International Journal of Pharmacy and Biological Sciences-IJPBS™ (2019) 9 (2): 144-155
Online ISSN: 2230-7605, Print ISSN: 2321-3272
Research Article | Biological Sciences | Open Access | MCI Approved
UGC Approved Journal

Food Industry Waste Material as A Growth Medium for Rhizobium

Ashok Kumar Singh, Gauri Singh and Gaurav Yadav

Department of Microbiology, Dolphin (PG) Institute of Biomedical and Natural Sciences, Manduwala, Dehradun (Uttarakhand) India.

Received: 12 Jan 2019 / Accepted: 12 Mar 2019 / Published online: 1 Apr 2019
Corresponding Author Email: gaurisingh2007@gmail.com

Abstract

Ever increasing industrialization have considerably increased the rate of water pollution. the management of waste is a major challenge for administrators and planners. To overcome those problems, there is an alternative process for using these wastes, as a source for cultivation media of micro-organisms. The present work was aimed to designing a food industry waste water based medium that included mushroom and dill, as a substitute for costly commercial media for growing rhizobium. Growth and optimization parameters of two strains of rhizobium namely R1 and R2 at different concentrations of food waste was observed. Growth of both the strains were highest in 90% waste. Growth effects of strains were observed at 90% food waste along with different synthetic media and was found to be superior to standard media (TY and YEM) used for Rhizobium. Media was optimized using 90% food industry waste along with different concentrations of yeast extract (1-7 g/l) and mannitol (1-7 g/l) in terms of OD at different intervals of time i.e. 24, 48 and 72 hours. The maximum growth was observed in 4g/l in yeast extract and mannitol in both the strains. Bacteria were grown on the waste water medium which produced effective results and Competed with standard media like YEM and TY. Hence, this study led to a new and efficient medium for bacterial cultures as well as decrease in the waste disposal expenses and efforts.

Keywords

Food industry waste, mannitol, Rhizobium, standard media, yeast extract.

*****

INTRODUCTION

Food processing wastes are those end products of various food processing industries that have not been recycled or used for other purposes. They are the non-product flows of raw materials whose economic values are less than the cost of collection and recovery for reuse; and therefore discarded as wastes.

A growth medium is a liquid or gel designed to support the growth of microorganisms. The commercially available media are very costly. The search for alternative, cheap media for use in laboratory agents for routine microbiological experiments is going on. Food and industrial wastes like saw dust, sugar cane, pulp, barley, spent grains, fish wastes, cheese whey, waste water sludge and

sugar waste [1-7] have also been reported to support the growth of microorganism.

Industrial food waste contains vast amounts of nutrients, which can be harnessed for growth of microorganisms and subsequent production of useful primary and secondary products. Fruit wastes rich in carbohydrate content and other basic nutrients could support microbial growth. The use of legume seeds as alternative nutrient media for bacteria and fungi has been reported [8].

Rhizobium is a gram negative soil bacterium that can fix atmospheric nitrogen. *Rhizobium* forms symbiotic relationship with roots of legumes. *Rhizobium* forms specialized organs, called nodules, on roots or stems of their hosts. Rhizobia inside nodule could reduce atmospheric nitrogen and make it available to the plant. Symbiotic nitrogen fixation is an important source of nitrogen, and various legume crops could fix as much as 200 to 300 kg of nitrogen per hectare or about 70 million metric tons of nitrogen per year [9-10]. The first step in the production of legume inoculants is massive growth of a selected Rhizobial strain in liquid medium [11]. The suitability of culture media for large-scale production of *Rhizobium* depends upon the utilization of carbon source and multiplication rate of bacteria.

For industrial production *Anethum graveolens* L. 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. The YMB medium (Yeast Mannitol Broth) has been mostly used for a laboratory-scale production [12]; however, its industrial use is limited due to high cost [13]. As in case of many other industrial fermentations, the economy of such a process is largely governed by the price of media utilized. In view of the growing demand of rhizobial inoculants it is imperative to search cheap and readily available substances to these costly ingredients.

The present work was aimed to designing a food industry waste water based medium (mushroom and dill) and compare it with a commercial medium for growing rhizobium.

Mushroom are fleshly fungi, spore-bearing bodies of typically produce above ground on soil or on their food sources which constitute a major group of lower plant kingdom. They do not contain chlorophyll like green plants the use of mushroom as food is very old. People have harvested mushroom from the wild thousands of years for food and medicine.

Anethum graveolens L. (dill) has been used in ayurvedic medicines since ancient times and it is a popular herb widely used as yields essential oil. It is an aromatic and annual herb of apiaceae family. *Anethum* seeds are used as a spice and its fresh and dried leaves called dill weed are used as condiment and tea. The aromatic herb is commonly used for flavouring and seasoning of various foods such as pickles, salads, sauces and soups. Fresh or dried leaves are used for boiled or fried meats and fish, in sandwiches and fish sauces. Dill oil is extracted from seeds, leaves and stems, which contains an essential oil used as flavouring in food industry. It is used in perfumery to aromatize detergents and soaps and as a substitute for caraway oil.

METHODS

Isolation of bacteria from root nodules of *Trifolium alexandrium* (Barseem):

Root nodulating bacterial Strains were isolated from the root nodules of *Trifolium alexandrinum* according to Vincent (1970) [14], collected from different agroclimatic regions of Dehradun. Plants were uprooted carefully and nodules were collected from the roots, washed with sterile water followed by surface treatment with 95% alcohal and again with sterile water. The washed nodules were surface sterilized with 0.1% mercuric chloride for 2-3 minutes and again washed for at least 10 times with sterile water as to remove the traces of mercuric chloride. The nodules were transferred in culture tube half filled with sterile water and crushed with a sterile glass rod to obtain a milky bacterial suspension. After serial dilution suspension was streaked on Yeast extract mannitol (YEM) agar plates and incubated for 2-3 days at 28°C one single colony was taken from each nodule extract directly or after purification through subsequent streaking.

Slants of YEM medium were routinely used for maintenance of the parent culture of rhizobia. The purity of the bacteria was checked by repeat streaking as well as microscopic examination with Gram staining.

Biochemical tests for differentiation of *Rhizobium*

Congo red Test: Rhizobia appear as white, translucent, glistening elevated and comparatively small colonies with entire margins.

Hoffer's alkaline broth Test [15]: *Rhizobium* grows in normal pH level. A medium i.e. Hoffer's alkaline having high pH of 11.0 was used to screen isolated nodulated bacteria.

Lactose agar Test: Rhizobia cannot utilize lactose. Culture of nodulated bacteria were streaked on lactose agar plate and incubated for 4-10 days.

Catalase test [16]: The 48 hours culture of the isolated strains on YEMA was flooded with hydrogen peroxide and observed for the bubble formation.

Oxidase test [17]: Few drops of p-amino dimethylaniline oxalate was added on the surface of 48 hours cultures of the rhizobial strains on YEMA and observed for the production of color.

Food waste as a substrate

The food waste (mushroom and dill) was collected from flex food industry, which is located at laal tappad, haridwar road, Dehradun.

Starter Culture

50 ml of culture was prepared by inoculating *Rhizobium* strains into Yeast Extract Mannintol Broth (YEM) and incubated at 28±2oC for 24 hours.

Media Optimization

Optimization of industrial food waste: Mushroom waste and dill waste were used for the current study.1% of starter culture was inoculated into different concentration of food waste i.e. 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% and growth was monitored by recording optical density (OD) at regular time intervals.

Comparison of food waste media with different Lab Media: Effect of different media i.e. 90% food waste, TY and YEM on *Rhizobium* was monitored in terms of absorbance at 650nm after every 24hrs,48hrs and 72hrs by inoculating bacteria with these media.

Optimization of food waste material with components of standard media: Yeast extract and Mannitol are two main components of the standard media which are very helpful for the growth of *Rhizobium*. So the growth of bacteria was also observed in 90% waste water along with different concentrations of Yeast Extract (1gm/l, 2gm/l, 3gm/l up to 7gm/l) and similarly 90% waste water along with different concentrations of mannitol (1gm/l, 2gm/l, 3gm/l up to 7gm/l).

RESULTS

Isolation of Rhizobium sp. From root nodules

Bacterial isolates were recovered from root nodules of *Trifolium alexandrinum (*Barseem plant) that were collected from fields of Manduwala, Dehradun. The two isolates that showed positive morphological and biochemical characteristics of Rhizobia were selected for further study. These isolates were designated as R1and R2.

Cultural characteristics for Rhizobium sp.

Rhizobial Colonies were obtained on YEMA agar medium after incubation at 28°C.The colonies had sticky appearance showing production of mucous through lower level. Pure colonies were also obtained on Rhizobium medium that appeared as pinkish white concave small colonies. Data analysed in table 1.0 clearly states that the isolates obtained from root nodules were of pure Rhizobial sp.

Table 1: Morphological and Biochemical characteristics of isolates

S.No.	characters	ISOLATES	
		R1	R2
1.	Gram staining	**Gram** -ve rods	**Gram** -ve rods
2.	CRYEMA Test	+ve	+ve
3.	Lactose agar test	+ve	+ve
4.	Hoffer's alkaline broth test	+ve	+ve
5.	Catalase	+ve	+ve
6.	Oxidase	+ve	+ve

Effects of Concentration of waste water on Growth of *Rhizobium* strains: Growth of R1 and R2 at different concentrations of waste water (10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%) was monitored by recording Optical density (OD) at 650nm after 24hrs,48hrs and 72hrs.

Growth of R1 and R2 on different concentration of mushroom waste water

In mushroom waste, maximum OD values obtained in 90% for R1i.e 0.482(Fig1) and R2 i.e. 0.437(Fig2) at 48-hour incubation period.

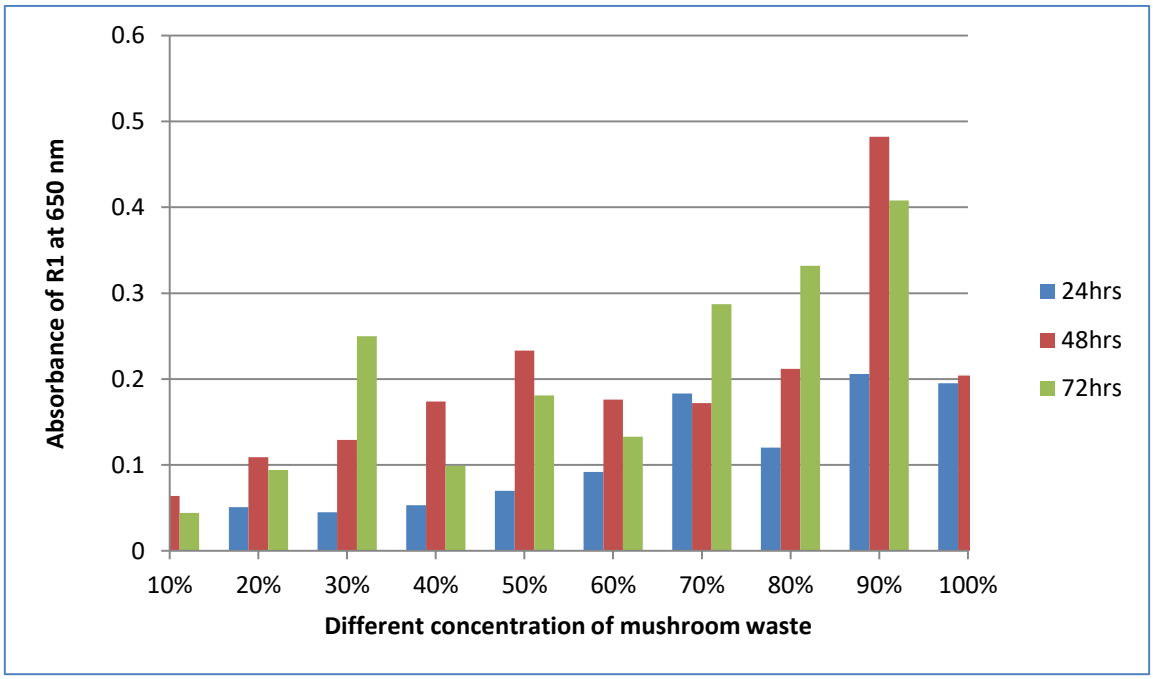

Fig.1: Growth of R1 on different concentration of mushroom waste

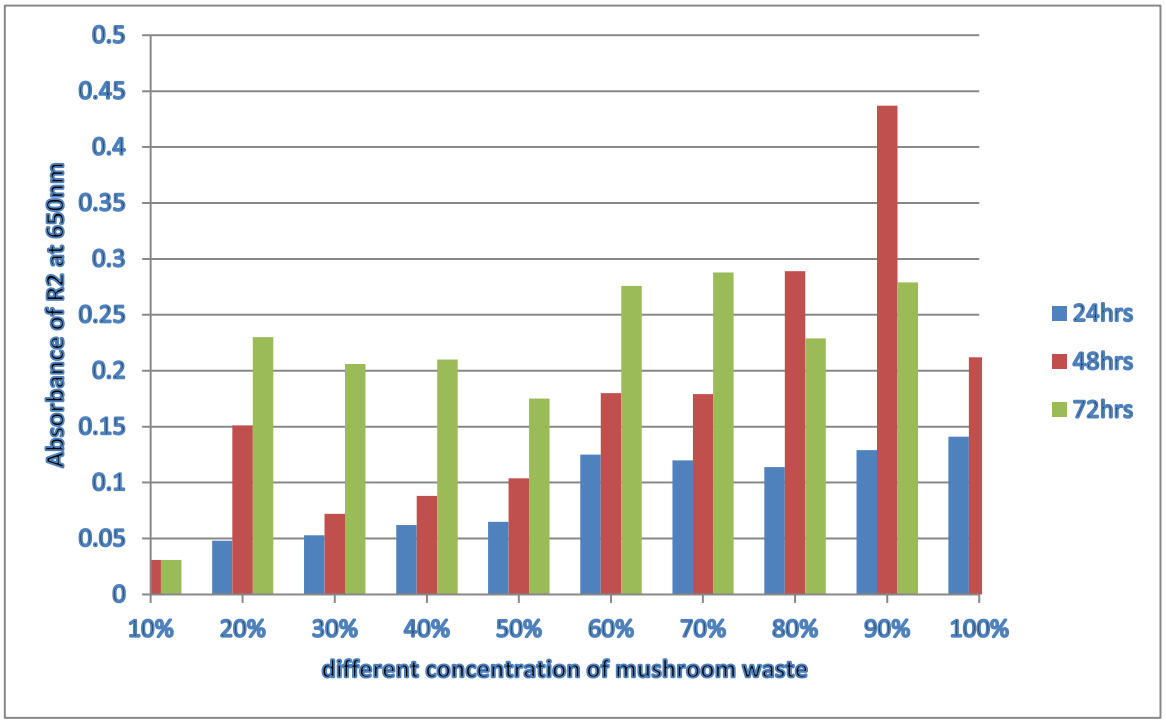

Fig.2: Growth of R2 on different concentration of mushroom waste

Growth of R1 and R2 on different concentration of dill waste water

In dill waste, maximum value obtained in 90% for R1 is 0.445(Fig 3) and R2 is 0.565 (Fig 4) at 48-hour incubation period.

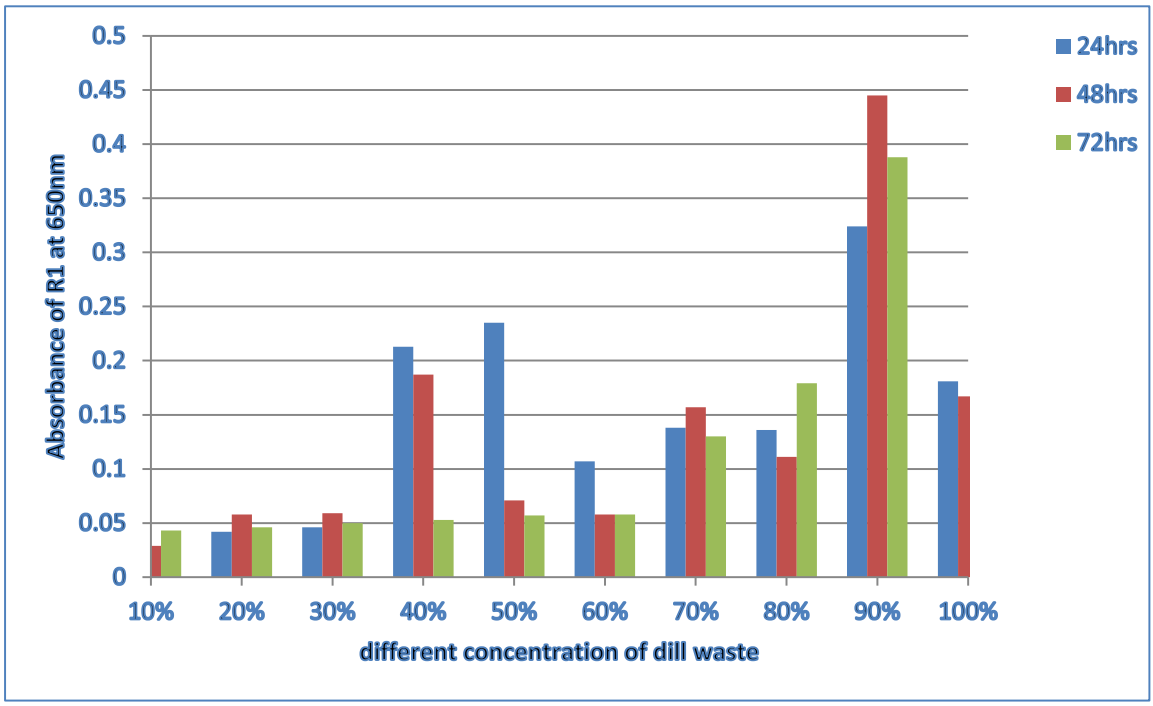

Fig.3: Growth of R1 on different concentration of dill waste water

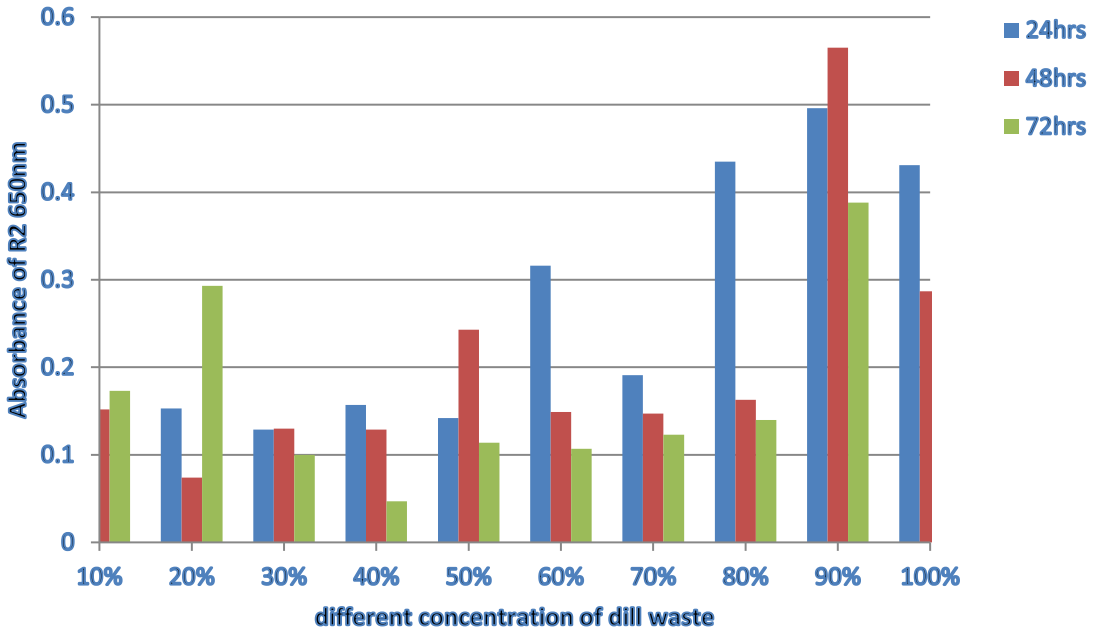

Fig.4: Growth of R2 on different concentration of dill waste water

Comparison of synthetic medium i.e. (YEM and TY) and waste water based medium (mushroom water, Dill water) for R1 and R2: Growth of R1 and R2 was observed in synthetic medium i.e. (YEM and TY Medium) and waste water (mushroom water, Dill water) separately within same conditions by recording Optical Density (OD) at 650nm after 24hrs.,48hrs and 72hrs.Maximum growth of R1 was found in mushroom waste ie.0.482 and minimum in YEM 0.384 at 48-hour incubation period(fig5). Maximum growth of R2 was observed in dill waste ie.0.565(fig6).

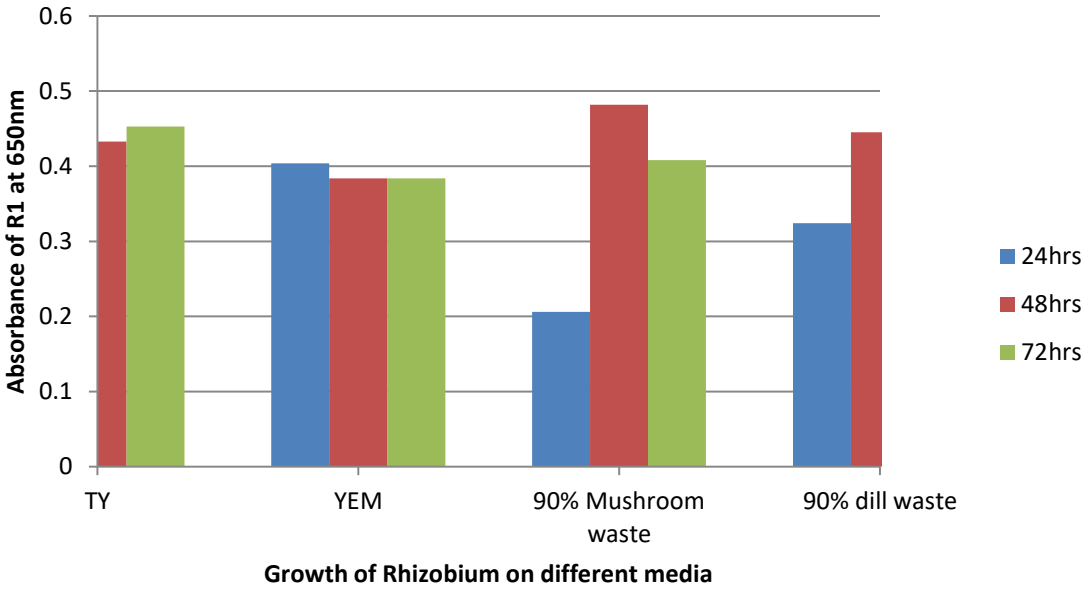

Fig.5: Growth of R1 on different media

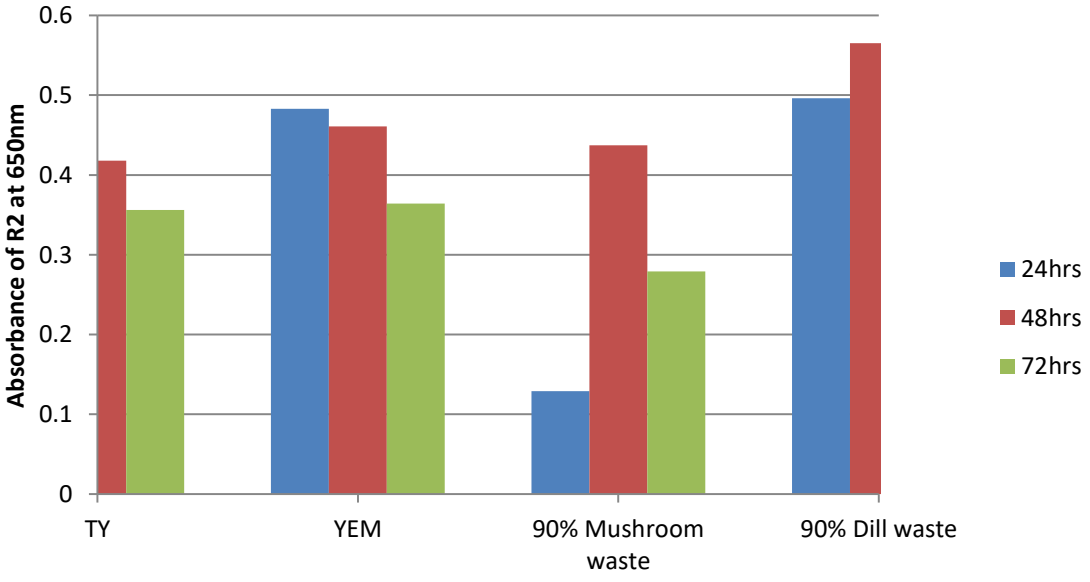

Fig.6: Growth of R2 on different media

Growth of Rhizobium on 90% waste water (mushroom and dill) along with different concentration of mannitol: Growth of *Rhizobium* strains were monitored at 90% waste water along with different concentration of mannitol (1-7g/l) in terms of optical density at different time intervals i.e. 24, 48 and 72 hours. In mushroom waste water, maximum growth of R1 was 0.754 (Fig7) and R2 was 0.728(Fig8) at 4g/l of mannitol. In dill waste, maximum growth was observed in R1, 0.818(Fig9) and R2, 0.389 (Fig10) at 4g.

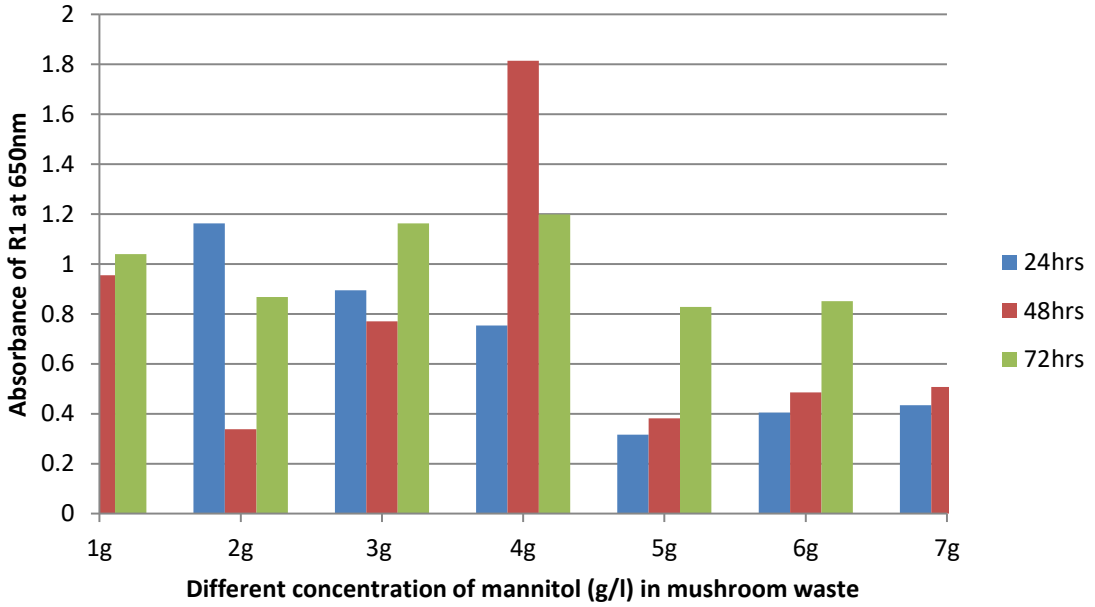

Fig.7: Growth of R1 on 90% mushroom waste with various concentration of mannitol at different incubation period

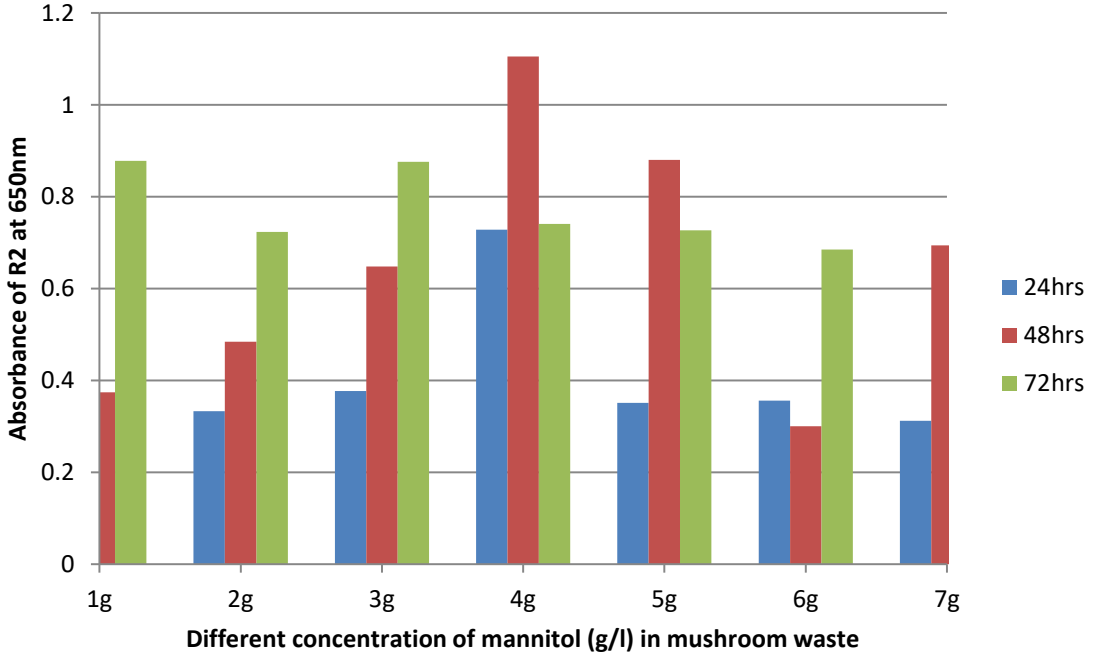

Fig.8: Growth of R2 on 90% mushroom waste with various concentration of mannitol at different incubation period

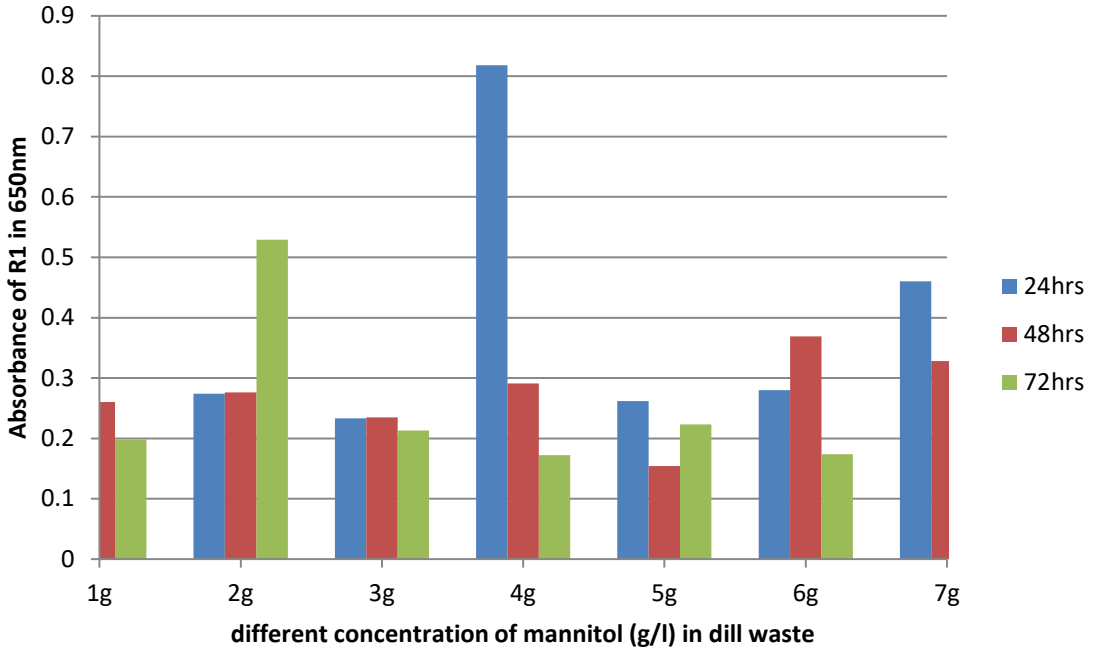

Fig.9: Growth of R1 on 90% dill waste at various concentration of mannitol at different incubation period

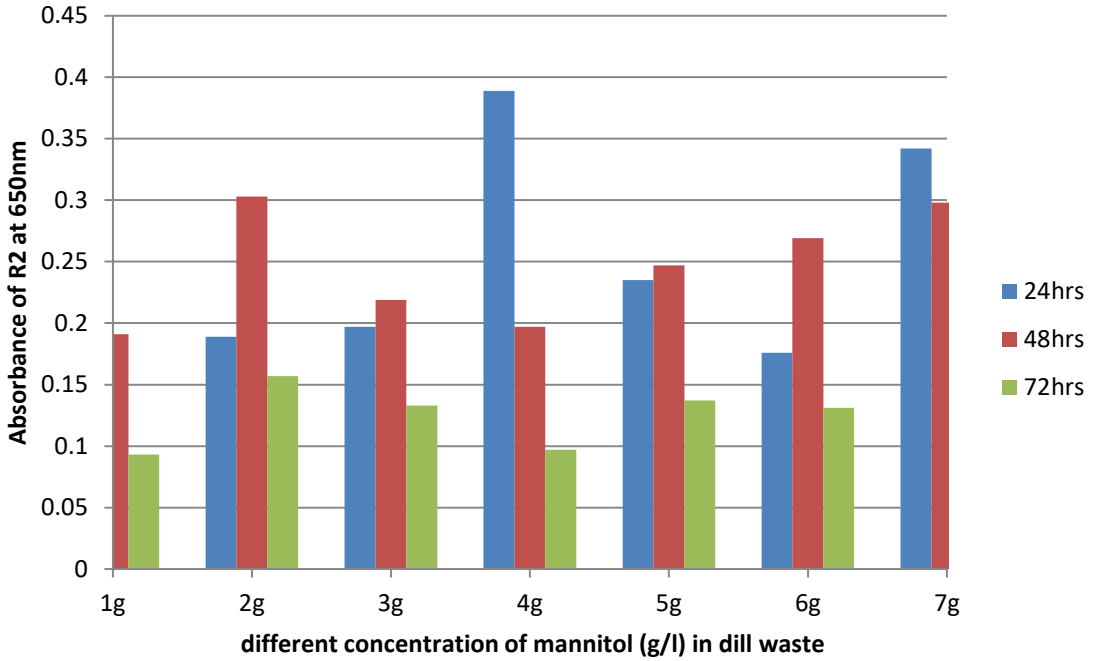

Fig.10: Growth of R2 on 90% dill waste with various concentration of mannitol at different incubation period

Growth of Rhizobium on 90% waste water (mushroom and dill) along with different concentration of yeast extract: Growth of *Rhizobium* strains were monitored at 90% waste water concentration along with different conc. of *Rhizobium* strains were exposed to various

concentrations of yeast extract (1-7g/l) in terms of optical density at different time intervals i.e. 24, 48 and 72 hours. In mushroom waste, maximum growth was observed in R1, 2.07 (Fig11) and R2, 1.959(Fig12) at 4g. In dill waste water, maximum growth was observed in R1, 1.558 (Fig13) and R2, 2.102 (Fig14) at 4g.

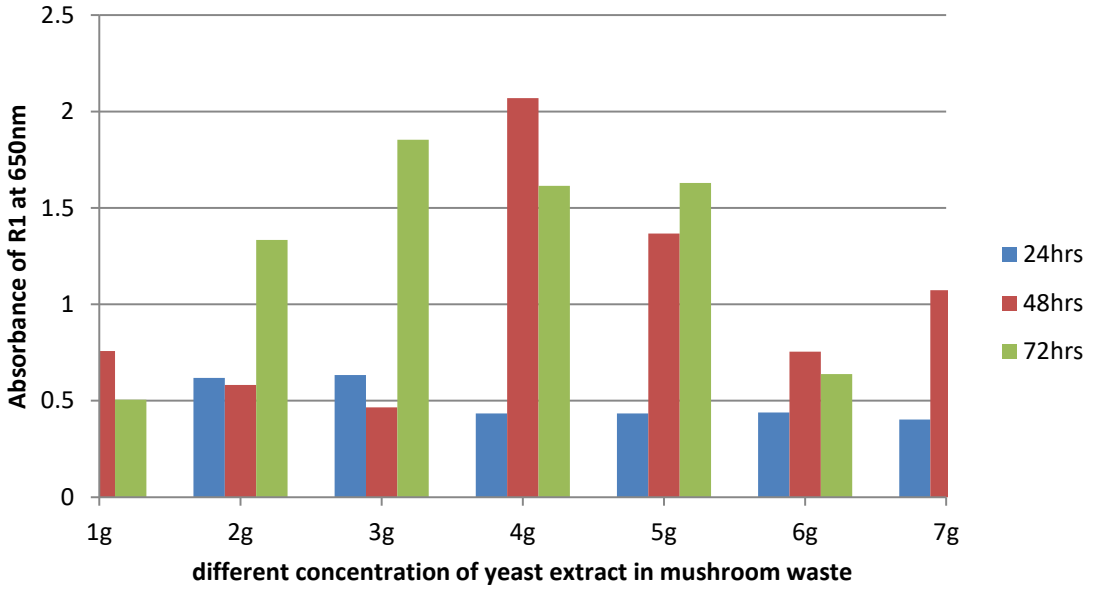

Fig.11: Growth of R1 on 90% mushroom waste with various concentration of yeast extract at different incubation period

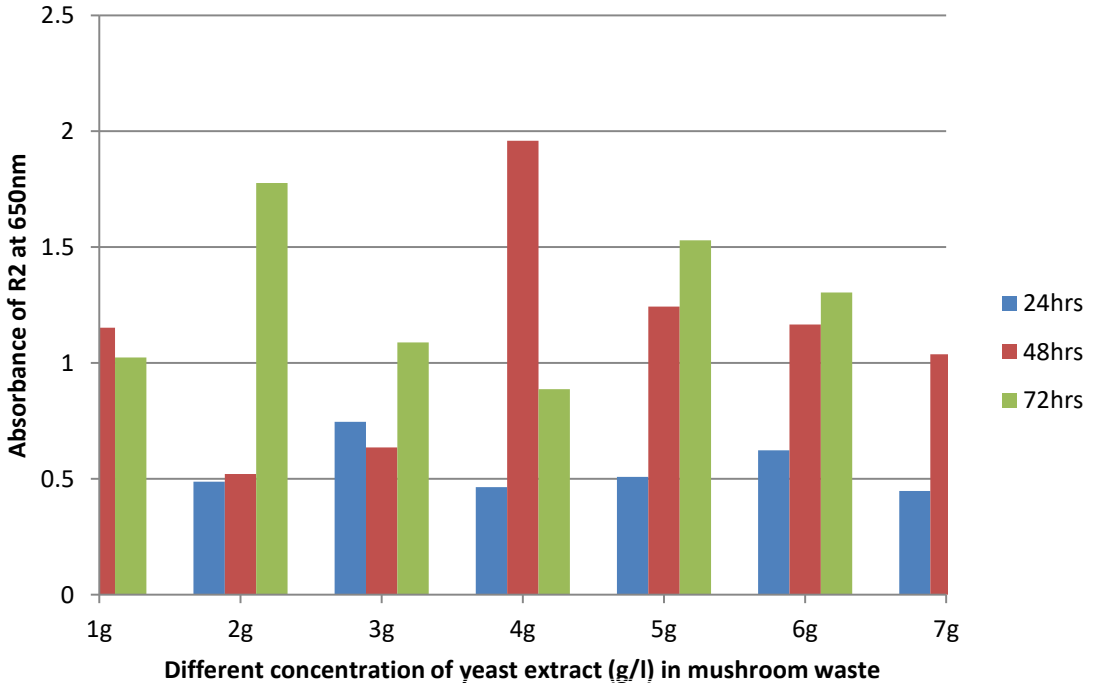

Fig.12: Growth of R2 on 90% mushroom waste with various concentration of yeast extract at different incubation period

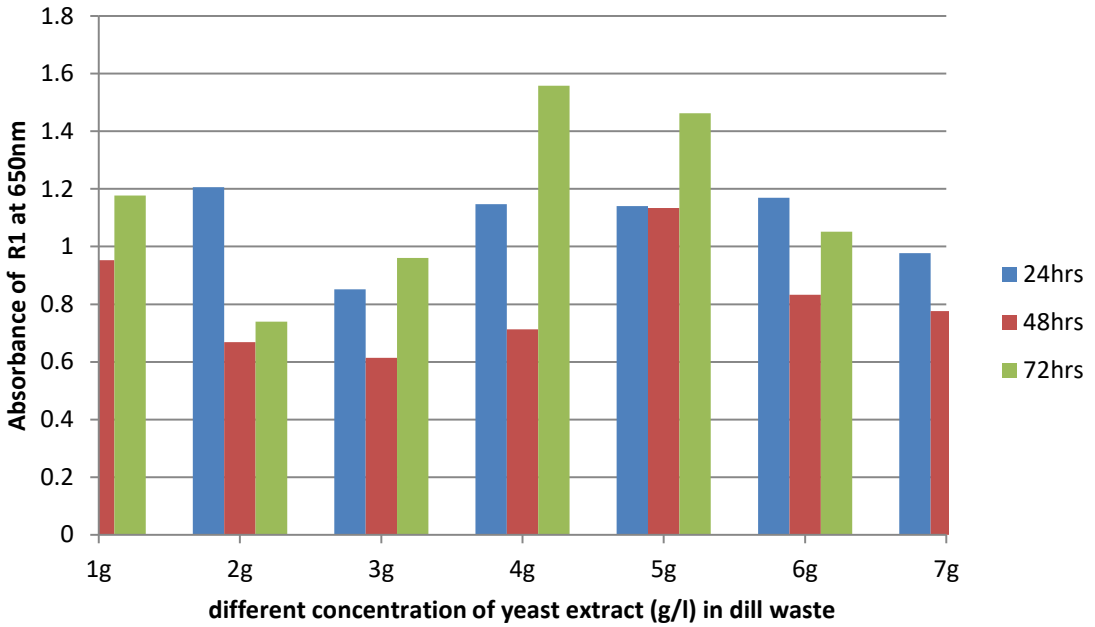

Fig.13: Growth of R1 on 90% dill waste water with various concentration of yeast extract at different incubation period

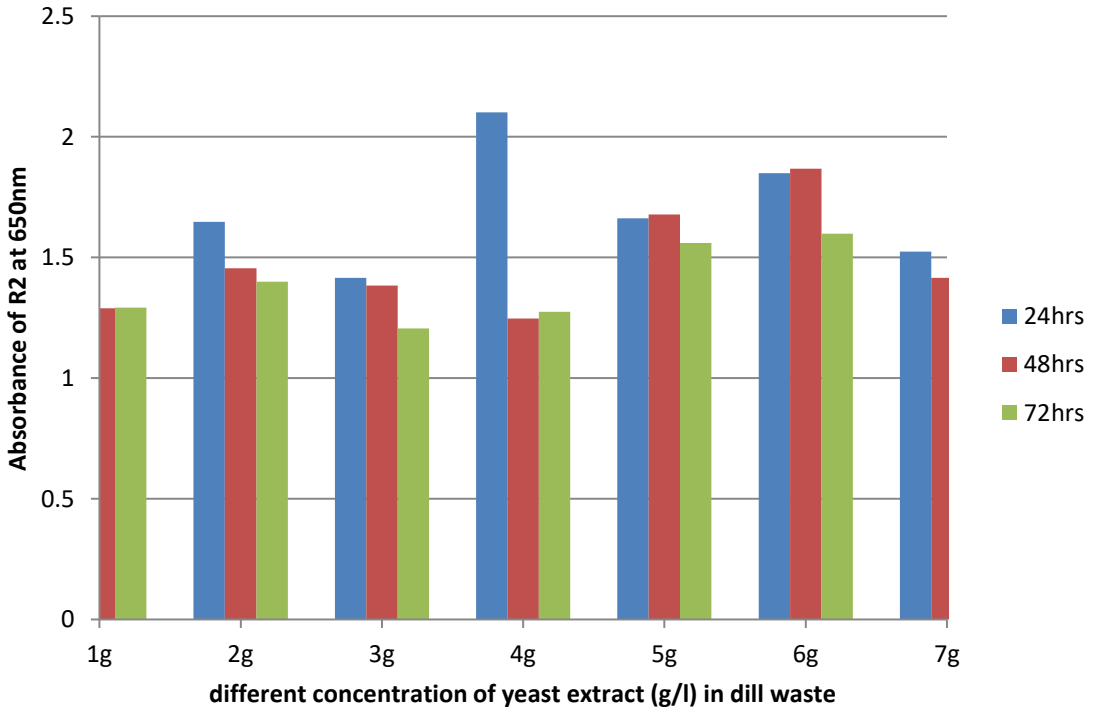

Fig.14: Growth of R2 on 90% dill waste water with various concentration of yeast extract at different incubation period

DISCUSSION

The Present study resulted in the isolation of root nodulating bacterial isolates from *Trifolium alexandrium* (Barseem plant) that were collected from Manduwala, Dehradun. Two microbial strains were recovered from root nodule of *Trifolium*

alexandrium. The general microscopic characteristics of the selected isolates showed rod shaped and gram-negative in nature and also similar to those reported earlier [18]. In the current study waste water (mushroom waste and dill waste) obtained from Flex food industry which is situated in laal tappad, haridwar road, Dehradun (U.K.) is used as a growth media for fast growing rhizobia. Growth of R1and R2 was observed at different concentrations (10%, 20%, 30%, 40%. 50%, 60%, 70%, and 80%, 90% and 100%) by recording Optical density (OD) at 650nm. Maximum growth of R1 & R2 was observed at 90% concentration. Singh et al., (2011) [19] optimized different concentrations of sugar waste and found maximum growth at 10%s ugar waste. Singh et al., (2013) [20] optimized different concentration of dairy sludge for fast growing rhizobia and observed maximum growth at 60% dairy sludge.

In the present work after optimizing the concentration of food industry waste water i.e. 90% growth of strains were observed at 90% waste water along with different synthetic media by recording optical density (OD) at 650 nm. The results of this study indicates that on medium containing only 90% waste water, cells of fast growing rhizobia (R1 and R2*)* grow rapidly and growth was superior to that of the control (standard media). These results demonstrate that 90% waste water can be satisfactorily used as a growth substrate of rhizobia.

Sellami et al., (2015) [21] studied the growth of fast-growing Rhizobiumlegumunosarium in industrial wastewater. They found that all wastewater samples sustained the bacterial growth. Almost a similar study was done by Mimb et al., (2014) [22] using two selected LNB strains (VUID1 and AHYP21), 16 local media (C and N sources) and groundnuts. The LNB biomass obtained from the local media was greater than the one obtained from the standard YEM media. Singh et al., 2011 [7] reported maximum absorbance at the growth of *R. meliloti* was found maximum in 10% sugar waste concentration along with other synthetic media like RMM and YEM, at 48 hrs.

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 [23]. Yeast extract and Mannitol are two main components of the standard media for the growth of *Rhizobium* therefore 90% waste water along with different concentrations of yeast extract (1gm/l, 2gm/l,3gm/l and up to7gm/l) and 90% waste water with different concentrations of mannitol (1gm/l, 2gm/l, 3gm/l and up to7gm/l) were further optimized. Growth in both conditions was studied at different time intervals i.e. 24, 48 and 72 hours. Our results demonstrated that maximum growth was observed in 4g/l of yeast extract and 4g/l of mannitol in both strains. **A**ccording to the findings of Kumar et al., (2016) [24] maximum growth of rhizobium was 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 along with spent wash. Tuzzimura and Muguro (1968) [25] reported that many types of sugars could be used to substitute mannitol as carbon and energy source for *Rizobium japonicum*. The effects of additional sources of nutrients on the growth of *S. meliloti* were studied in secondary sludge from the Quebec City wastewater plant [26]. Spent wash supplemented with different concentrations of yeast extract and glycerol increased the maximum cell counts. The highest increase was approximately six fold and was obtained with the addition of 4 g/L yeast extract and 7.5 g/L glycerol.

CONCLUSION

It is concluded from the current study that food industry waste water retains growth of rhizobia with higher than standard medium and different components used in YEM medium. It uses as an alternative media in commercial preparations of legume inoculants will considerably reduce the production cost of bio fertilizers.

REFERENCES

1. Green J H, Kramer A. Food waste processing waste management, 2 nd Edition. AVI Publishing Company Inc, New York. USA.1979; 82-88.
2. Kristinsson HG, Rasco BA. Fish hydrolysates: production, biochemical and functional properties. Crit Rev Food Sci Nutri 2000;40: 43-81.
3. Poenomo A, Buckle KA. Crude peptones from cowtail ray (Trygon sephen) viscera as microbial growth media. W J Microbiol Biotechnol 2002; 18:333-340
4. Kurbanoglu EB, Kurbanoglu IN. A new process for utilization of peptone as ram horn waste. J Biosci Bioeng 2002; 94:202-206.
5. Estrella M J, Pieckenstain F L, Marina M D, Ruizo A. Cheese whey: an alternative growth and protective medium for Rhizobium loti cells. J Indust Microb Biotech2004; 31: 122-126.
6. Ben Rebah F, Prevost D, Yezza A,Tyagi R D. Agro industrial waste materials and waste water sludge for rhizobial inoculants production: A review. Bioresour Technol 2007;98: 3535-3546.
7. Singh A K, Singh G, Bhatt RP, Pant S, Naglot A, Singh L. Sugars waste, an alternative growth and complete

medium for fast growing *Rhizobium* Cells African J Microbiol Research 2011;5(20):3289-3295,
8. Ravathie A, Sevvel P, Nirmala R, Kularajany N. Alternative culture media for bacterial growth using different formulation of protein sources. J natural product plant resources 2012; 2:697-700.
9. Peoples M B, Herridge D F, Ladha J K.Biological nitrogen fixation: An efficient source of nitrogen for sustainable Agriculture production. Plant Soil 1995; 174: 3-28.
10. Brockwell P J. Bottomley Recent advances in inoculant technology and prospects for the future. Soil Biol Biochem 1995;27: 683–697
11. Thompson J A. Australian quality control and standards. In Report of the Expert Consultation on Legume Inoculant Production and Quality Control, J.A. Thompson (ed.), pp. 107-11, Food and Agri. Orga. of the United Nations, Rome .1991.
12. Verma J P, Yadav J, Tiwari KN. Application of *Rhizobium* sp. BHURC01 and Plant Growth Promoting Rhizobactria on Nodulation, Plant Biomass and Yields of Chickpea (*Cicerarietinum*L.). International J Agricul Research2010; 5:148-156.
13. Tyagi R D, Prévost D, Ben Rebah F, Surampalli RY. Wastewater sludge as a new medium for rhizobial growth. Water Qual. Resear. J Canada 2002; 37:353–370.
14. Vincent J M. A manual for the practical study of root-nodule bacteria. Blackwell, Oxford Int Biol Prog .1970 ;164.
15. Hofer A W. Methods for distinguishing between legume bacteria and their most common contaminants. J Am Soc Agron 1935; 27: 228-230.
16. Graham P H, Parker C A. Diagnostic features in the characterization of root nodule bacteria of legumes. Plant Soil 1964 ;20: 383-396
17. Kovaks N. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature 1956 ;178: 703.
18. Gauri, Singh A K, Bhatt RP, Pant S, Bedi M K, Naglot A. Characterization of Rhizobium isolated from root nodules of *Trifolium alexandrinum*. J Agric Technol 2011;7(6): 1705-1723.
19. Singh A K, Gauri, Bhat R P, Pant S. Optimization and comparative study of the sugar waste for the growth of rhizobium cells along with traditional laboratory media. Res J Microbiol. 2011;6(9): 715-723.
20. Singh A K., Singh G, Gautam D, Bedi M K. Optimization of Dairy Sludge for the Growth of Rhizobium cells. Bio Med Research International 2013; Article ID 845264.
21. Sellami M, Oszako, Miled N, Ben Rebah B, Faouzi. Industrial wastewater as raw material for exopolysaccharide production by *Rhizobium leguminosarum. Brazalian* J Microbiol 2015 ;46 (2) :407-413.
22. Ngo M J A, Boyomo O, Laurette N, Hyppolite N N, Nwaga D. The use of local substrates in the production of legume nodulating bacteria inoculants for Sub-Saharan Africa: Preliminary results. J Appl Biosci 2014 ;(75):6183–6191.
23. Kivanc M, Kucuk C, Kinaci E. Characterization of Rhizobium species isolated from Bean. Turk J Biol 2006;(30): 127-132.
24. Kumar G, Singh A K, Singh G. Spent Wash as an Alternative Medium for Growth of Rhizobium. J Academia Industrial Research 2016;5(6) 81-84.
25. Tuzimura K, Meguro H. Respiration substrate of Rhizobium in the nodules. J Biochem 1968 ;47: 391
26. Ben Rebah F, Tyagi RD, Prévost D, Surampalli Y. Wastewater sludge as a new medium for rhizobial growth. Water Qual Resear J Canada 2002;(37): 353–370