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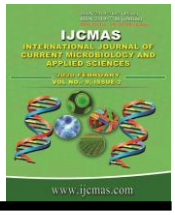

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Formulation of Alternative and Cost Effective Growth Medium for Beneficial Microorganisms using Post Biomethanated Distillery Spentwash

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A B S T R A C T

The increasing cost of microbial growth media has necessitated continuous search for more readily available culture media at affordable prices. The agricultural based industries are generate significant quantities of organic wastes and it is necessary to convert them to useful end products. It is now realized that these waste could be utilized as cheap raw materials for some industries or used as cheap substrates for microbiological processes. Distillery is one of the promising industries in India and in recent years its growth is phenomenal which besides alcohol production, generates enormous quantity of wastewater. Distillery wastes are rich sources of organic matter and nutrients especially potassium, calcium, magnesium and sodium. There is an increasing interest in distillery spentwash due the possibility to use it as a cheap carbon and nutrient source and the presence of several other compounds. These include minerals, organic compounds and vitamins, and this makes the modified spentwash medium as an inexpensive and economic alternative media for Bio inoculants growth. Now an attempt has been made to modify the distillery spentwash to support the beneficial microorganism growth. Comparative studies of Azospirillum sp growth in modified spentwash medium and N-free Malic acid broth were recorded with 24hrs time between 0 to 168 hrs. Maximum population of Azospirillum in N- free Malic acid and modified Spentwash medium was 2.0 x 107 of CFU ml-1 and 2.5 x 107 CFU ml -1 at 120 hrs interval respectively. The Azospirillum sp. colonies present in modified spentwash were showed positive growth confirmation with various sources of carbon and methyl red biochemical test but negative in the voges test. Azospirillum grown in modified spentwash medium produced the maximum amount of IAA (5.6 mg L-1).

Introduction

Microbiological studies depend on the ability to cultivate and maintain microorganisms under laboratory conditions by providing suitable culture media that offers favorable conditions. A nutrient media prepared for the growth of microorganisms in a laboratory is called culture media. Microorganisms can obtain energy directly from sunlight while carbon can be made available in organic forms such as carbohydrates or inorganic forms such as carbon dioxide and water. Nutrient agar medium is commonly used as general purpose medium for the cultivation of broad range of bacteria (Jadhav *et al.*, 2018).

It is a basic medium composed of peptic digest of animal tissue, beef extract and yeast extract, sodium chloride and agar. Commercially available media such Nutrient Agar, Cetrimide Agar, MacConkey Agar are used for the growth of microorganisms but these are very expensive (Jamel *et al.,* 2008). Higher cost of cultivation media is a matter of concern. Therefore, various alternative media are formulated and alternatives for agar are tested, so as to reduce the cost involved.

Distillery is one of the promising industries in India and in recent years its growth is phenomenal which besides alcohol production, generates enormous quantity of spentwash annually. The treated distillery spentwash is a nutrient rich liquid organic waste generated as a byproduct after the distillation of molasses. It is also a very good source of readily available major and micronutrients (Palaniswami *et al.,* 2011). Recently, the presence of appreciable quantity of plant growth promoters viz., gibberellic acid and Indole Acetic Acid (IAA) have also been detected which further enhance the nutrient value of spentwash (Hukkeri *et al.,* 2013). Distillery wastewater are rich in sugar and nutrients are easily assimilated by microorganisms; this makes the wastes suitable materials for growth of microorganisms. Inability to salvage and reuse such materials economically results in the unnecessary waste and depletion of natural resources (Malarvizhi and Subramanian, 2016).

Distillery Spentwash is a by-product of the distillery industry, there is an increasing interest in spentwash due the possibility to use it as a cheap carbon and nutrient source and the presence of several other compounds besides sucrose. These include minerals, organic compounds and vitamins, and this makes the modified spentwash medium as an inexpensive and economic alternative media for Bio inoculants growth. With this background, a study was undertaken to aim at Modification of post biomethanated distillery spentwash as an alternative medium for Bio inoculants growth study and evaluates the performance of *Azospirillum* grown in modified spentwash medium.

Materials and Methods

Collection and characterization of Post biomethanated Distillery Spentwash

The spentwash sample was collected from the M/s Sakthi Sugars Ltd. (Distillery Division) at Appakudal, Erode District, Tamil Nadu. The biomethanated spentwash was collected in polycarboryl containers, properly sealed and stored at 4^{o}C for further analysis. The distillery spentwash was analyzed for physical, chemical and biological parameters as per the following standard procedures (Table 1).

Collection and Purification of Microbial culture

The microbial cultures *Azospirillum* was collected from the culture collection bank maintained in the Department of Agricultural microbiology, Tamil Nadu Agricultural University, Coimbatore. The purification of *Azospirillum* was done by Streak plate technique (Cain *et al.,* 2013). The selective media, N-free Malic acid medium *for Azospirillum* was prepared, sterilized in an autoclave for 15 minutes at 20 lbs pressure (Dobereiner and Day,1976). The Petri plates were cleaned with ethanol and sterilized in hot air oven at 160^{o} C for 2 hours. The sterilized media and petri plates were transferred to laminar airflow chamber to maintain a sterile condition. The media was melted in the microwave oven and poured into Petri plates and allowed for solidification. A loopful of pure microbial culture was

streaked in a zigzag manner on the respective media (Christine, 2002). The plates were then incubated for 3 days in an incubator at 37^0 C for colony formation. The colonies were sub cultured in the respective slants and incubated at 37^0 C for 3 days in the incubator for maximum growth and preserved in the refrigerator at 4^0 C for further use.

Formulation of Modified Spentwash medium

Modification of distillery spentwash study was conducted in the Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore. 10% concentration spentwash was selected as the base material for developing the alternative media and the 10 % concentrated spentwash was analysed for relevant physico-chemical parameters according to internationally accepted procedures and standard methods (Table 1). In 250 ml Erlenmeyer flask 100 ml of 10% spentwash (10ml spentwash + 90 ml of water) was taken and carbon Source *viz.*, Malic acid for *Azospirillum* was added at the rate of 0.5 g per 100 ml of 10% spentwash and sterilized in an autoclave for 121 °C for around 15–20 minutes and broths were allow to cool. A loopful of the pure culture of *Azospirillum* was inoculated on to the respective flask in the laminar airflow chamber. The flasks were incubated in the incubator at 37^0 C for the growth of maximum population of the microorganisms. The growth of microbes in modified spentwash was further confirmed by most probable number method and biochemical test (Roszak and Colwell, 1987).

Estimation of *Azospirillum* population using most probable number (MPN)

In most cases, the numbers of N- fixing bacteria (free living, associative and endophytic) to allow are estimated by the most probable number method using McCardy's probability tables (Okon *et al.*, 1977). Counts are made on N- free semi solid medium by inoculating 0.1 ml of each dilution in three tubes containing the medium specific for the target organism, placing the inoculum from the bottom to the centre of the tube, as these bacteria are sensitive to O_2. These tubes are then incubated at 30-34^{O}C without shaking from 5 to 7 days, to allow pellicles to form. The method used to quantify the MPN is based on the presence or absence of pellicles in the semi solid medium and approximate numbers computed using McCardy's MPN.

Biochemical test for Bio inoculants grown in modified spentwash

Methyl red –Voges test

In 250 ml conical flask 0.5 g of glucose, 1.5 g peptone and K_2HPO_4 were mixed in 100ml distilled water sterilized in an autoclave for 121 °C for around 15–20 minutes. One ml culture were inoculated on to the respective flask and incubated for 3 days. Then few drops of methyl red indicator were added to the culture tube. A positive reaction is indicated, if the colour of the medium changes to red within a few minutes (Acharya, 2013)

Carbohydrate fermentation test

Azospirillum was confirmed by Carbohydrate fermentation test (Table 2). 100 ml broth was prepared with addition of different carbon source *viz.*, glucose, sucrose, lactose, Mannitol (0.5 g of peptone+ 1 g of carbon source is added in 100 ml of distilled water). Then 0.5 ml of methyl red was added and dispensed into test tube. Inverted Durham's tubes were introduced into the each test tubes and autoclave for 121 °C for around 15–20 minutes. Inoculated the culture and incubated

for 24hrs. Appearance of gas bubble in the Durhams tube was indicates the Positive result (Reddik, 1975).

Estimation of IAA production

Bio inoculants were grown for 48 hrs on their respective media at 36±2 °C. Fully grown cultures were centrifuged 10000 rpm for 20 minutes at Department of Environmental Sciences, Tamil Nadu Agricultural University, Coimbatore. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M FeCl3 solution). Development of pink colour indicates IAA production. A blank containing Salkowski reagent and water (3+2 ml) was used for calibration in UV Spectrophotometer. The test samples were read at 530 nm for absorbance. OD values were compared with a standard graph made from authentic IAA to calculate the actual quantity of IAA and was expressed as µg/ml (Jayaprakashvel *et al.,* 2014).

Results and Discussion

Studies on the Physico Chemical characteristics of post biomethanated distillery spentwash

The distillery spentwash was dark brown in colour with an odour of burnt sugars. The brown colour could be ascribed to the presence of melanoidins, which are the products of the maillard reaction between sugar and amino acid produced upon heating (Sharma and Mittal, 2014). The specific gravity of spentwash was 1.06 g cc^{-1}. The spentwash was found to be neutral in reaction with pH of 7.75. For creating a favourable pH for the growth of methenogens, the acidic pH of the distillery spentwash was shifted to neutral thereby the PMDSW possessed a neutral. Being originated from the plant sources, the spentwash contained considerable amount of plant nutrients and organic matter. Among the plant nutrients, nitrogen recorded to a level of 1,700 mg L^{-1} followed by phosphorus was 450 mg L^{-1}. Potassium was found higher amount in the spentwash with the value of 10,820 mg L^{-1}. The organic carbon content of the spentwash was 26,110 mg L^{-1}. The cations *viz*., calcium, magnesium and sodium were recorded at 4600 mg L^{-1}, 1,752 mg L^{-1} and 845 mg L^{-1}, respectively. Though the spentwash does not contain any toxic metals or other toxic constituents, it appears to have relatively small amounts of micronutrients which followed: Fe >Mn> Zn > Cu. Organic compounds present in post biomethanated distillery spentwash are of humic in nature (Naik *et al.,* 2008), similar to those in soil, except that fulvic acid predominated over humic acid. Therefore, it may be considered as a nutrient rich organic liquid fertilizer, which can support plant growth (Mahimairaja and Bolan, 2004).

Studies on the growth of *Azospirillum* in modified spentwash medium in comparision with N-Free Malic acid broth

Comparative studies of *Azospirillum sp* growth in modified spentwash medium and N-free Malic acid broth were recorded with 24hrs time between 0 to 168 hrs. The data showed that the *Azospirillum population* was higher in modified spentwash medium at all the growth stages tested. These results are presented Fig 1 and 2. Maximum *Azospirillum* population (2.5 x 10^7) was noticed in modified spentwash medium during 120 hrs and after that gradual decline was observed. In *Azospirillum* population in N- free Malic acid broth was comparatively lesser and it was recorded the population of 2.0 x 10^7 at 120 hrs intervals. The better growth of the organisms in modified spentwash may be attributed to the richness of

the medium with all essential nutrients including nitrogen. The increase in microbial biomass might be due to soluble carbon along with additional nitrogen and phosphorus content present in the Spentwash. These results corroborate with the findings of Sellami *et al.,* (2015). Jintaridth *et al.,* (2002) who also reported that the spentwash was rich in nutrients and organic material, particularly easily oxidizable and soluble organic carbon with all essential nutrients and this might have favoured the proliferation of microbial population. Industry effluent having easily degradable organic matter might have increased the microbial activity. Similar result was also reported by Tharmila *et al.,* (2011).

Table.1 Analytical methods followed for the analysis of distillery spentwash and leachate

S.No	Properties	Methodology	Reference
1.	Colour	Assessed by visual comparison with distilled water	APHA (1989)
2.	Density	Weight/Volume	A.O.A.C (1962)
3.	pH	pH meter	Jackson (1973)
4.	EC	Conductivity meter	Jackson (1973)
5.	Organic Carbon	Chromic acid - Wet Digestion method	Walkley and Black (1934)
6.	Nitrogen	Bremner method	Jackson (1973)
7.	Phosphorus	Vanadomolybdate Colorimetric Method	APHA (1989)
8.	Potassium	Flame photometric method	Jackson (1973)
9.	Calcium	Versenate titration method	Jackson (1973)
10.	Magnesium	Versenate titration method	Jackson (1973)
11.	Chloride	Mohr's method	Jackson (1973)
12.	Sodium	Flame photometric method	Jackson (1973)

Table.2 Carbohydrate fermentation test

Carbohydrates	*Azospirillum*	*Azotobacter*
Glucose	AG	AG
Sucrose	AG	AG
Lactose	A	AG
Mannitol	A	AG
Galactose	AG	AG
***A-Acid production; G- Gas formation**		

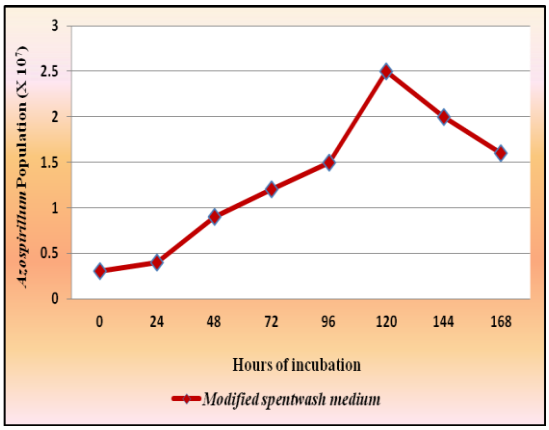
Table.3 Biochemical test for *Azospirillum* grown in modified spentwash medium

S.No	Microbes	Catalase test	Methyl red test	Voges test
1.	*Azospirillum*	+ ve	+ ve	- ve
2.	*Azotobacter*	+ ve	-ve	+ ve
+ve - positive result; -ve – negative result				

Table.4 Carbohydrate fermentation test

S.No	Carbohydrate	*Azospirillum*	
		Acid production	Gas formation
1	Glucose	+ve	+ve
2	Sucrose	+ve	+ve
3	Lactose	+ve	-ve
4	Mannitol	+ve	- ve
5	Galactose	+ve	+ve
+ve - positive result; -ve – negative result			

Fig.1 Studies on the growth of *Azospirillum* in modified spentwash medium comparison with N-Free Malic acid broth



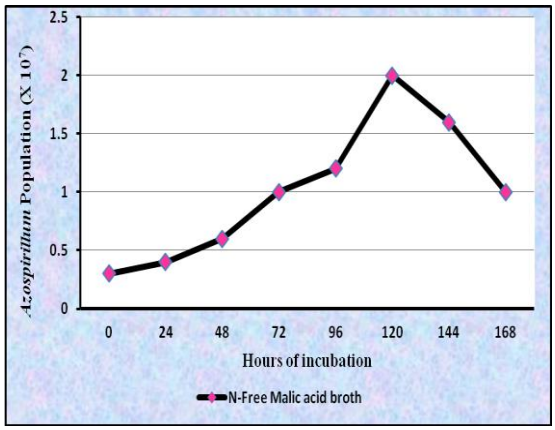


Fig.2 Studies on the growth of *Azospirillum* in modified spentwash medium comparison with N-Free Malic acid broth

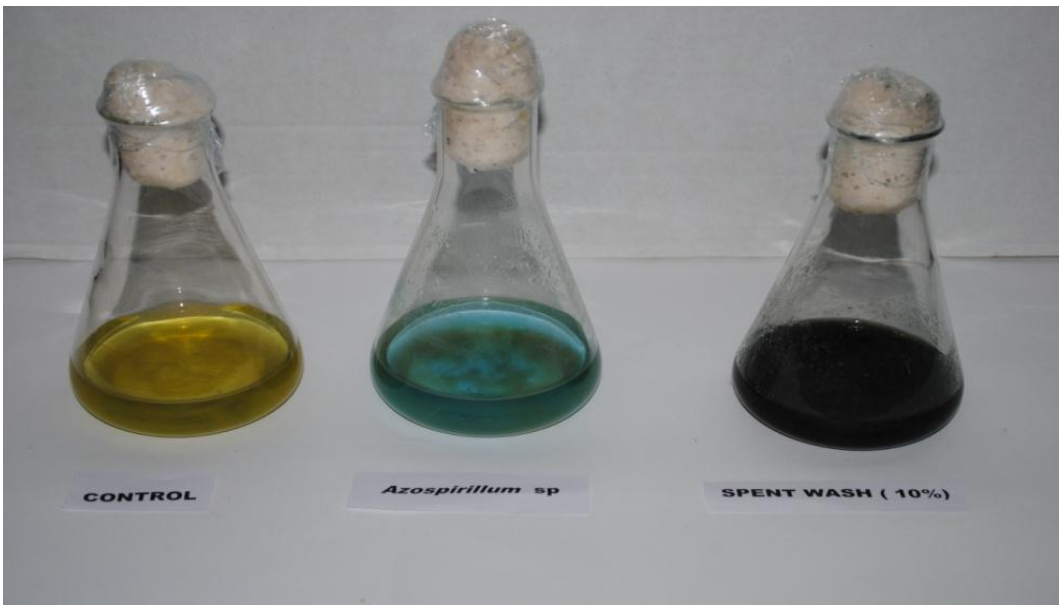
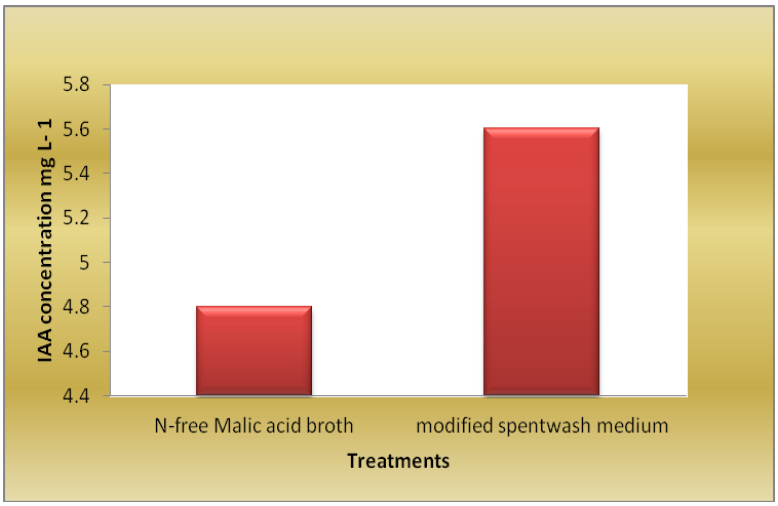


Fig.3 Studies on the IAA production of *Azospirillum* grown in modified spentwash medium comparison with conventional broth



Biochemical test for Bio inoculants grown in modified spentwash medium

Bio inoculants grown in modified spentwash medium were subjected to various biochemical tests based on Bergy's manual of determinative bacteriology. The *Azospirillum sp.* colonies present in modified spentwash were showed positive growth confirmation with various sources of carbon and methyl red biochemical test but negative in the Voges test (Table 3 and 4). These results proved the *Azospirillum* was grown very well in the modified spentwash. MacFaddin (2000) who also reported that the positive results indicated that the *Azospirillum* was able to ferment sugars due to have specific enzyme that was responsible for sugar fermentation and production of acid and gas.

Studies on the IAA production of Bio inoculants grown in modified spentwash medium in comparison with conventional broth

Indole acetic acid (IAA) production is a major property of rhizosphere bacteria that stimulate and facilitate plant growth. In this study bio inoculants was used for the quantitative estimation of IAA grown in modified spentwash medium and the IAA production was compared with bio inoculants grown in conventional microbial broth(Fig. 3). *Azospirillum* grown in modified spentwash medium produced the maximum amount of IAA (5.6 mg L^{-1}). Indole acetic acid and Indole lactic acid were produced by *A. brasilense* which gave positive results on salkowski reagent. Same trend of result was reported by Hormens *et al.,* 1985.The maximum amount of IAA production was noticed in the *Azospirillum grown in* modified spentwash medium. Sridevi and Mallaiah, (2007) reported that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability.

In conclusion from the results of this study, post biomethanated distillery spentwash contain minerals and nutrients that can meet the nutritional requirements of microorganism. Therefore, they can be utilized as alternative materials in the formulation of culture media for teaching and research purposes. An important advantage of alternative media was produced from post

biomethanated distillery spentwash for the cultivation of Bio inoculants, which was found to be cost effective in the present scenario of getting conventional medium. It solving the problem of the shortage of culture media for laboratory practical, the result of this research will go a long way in ameliorating this problem. Further investigation is recommended in the application of modern tools and methods in the study of microbial physiology as this will assist in manipulation of other readily available local products into suitable media to further check the exorbitant cost of conventional media.

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