International Journal of Scientific Research in Engineering and Management (IJSREM)
Volume: 07 Issue: 03 | March - 2023 Impact Factor: 7.185 ISSN: 2582-3930

OPTIMISATION OF MEDIA FOR PRODUCTION OF COST-EFFECTIVE RHIZOBIUM [BIOFERTILIZER]

RAJESH JORGEWAD[#], ADITYA PILLAI[#], ABHISHEK MOHITE[#], ANIKET SAWANT[#], SAMARTH BAKARE[#]

[#] *Department of Biotechnology, KIT'S College of Engineering*

Abstract - Rhizobium is a soil habitat, gram negative bacterium associated symbiotically with the roots of leguminous plants. Rhizobium helps in the process of nitrogen fixation. Screening and selecting the most effective strain play a key role in the process of biological nitrogen fixation. The present work was undertaken to bring into light different characteristics, growth, isolation, and secretion of rhizobial strains (bradyrhizobium). These are found in the root region of the leguminous plants where it forms nodules and fix nitrogen. The study revealed that the species used was non-spore forming, rod-shaped, motile, aerobic, gram- negative soil bacterium. These rod-shaped bacteria are 0.8um in diameter and 2um in length, and often have flagellae. These species are non-pathogenic to humans. The rhizobium bacteria were grown on three different types of medias namely – nutrient broth, CRYEMA(Congo Red Yeast Extract Mannitol Agar), and modified media containing jaggery. The common ingredients of the culture media included Peptone, Meat Extract, Mineral Salts, Carbohydrates and Water. Growth analysis, substrate estimation and biomass estimation was done and yield coefficient was calculated. Rhizobium was then applied as a biofertilizer for good improvement in plant growth and yield.

***Key Words*:** Biofertilizers, Biological Nitrogen Fixation, Bradyrhizobium, Inoculants, Leguminous plants, Optimized Media.

INTRODUCTION

Legumes can establish ecologically important symbiosis which can lead to the development of new plant organ in response to nitrogen fixing bacteria. The leguminous plants are a kind of cash crops. Nearly one-third of human dietary protein is obtained from the grain legumes[1]. Along with being a rich source of protein, legumes are important because they have the ability to produce good amounts of organic nitrogen through the help of symbiotic biological nitrogen fixation[2]. Biological Nitrogen fixation is a process by which atmospheric nitrogen (N2) is converted into ammonia and subsequently available for plants. Legumes such as beans, clover, soybean and pea help to feed the meat producing animals as well as humans. The family Rhizobiaceae currently involves six general Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium, which are collectively referred to as Rhizobia. It has been estimated that 1g of soil may contain a community of 10^9 microorganisms with Rhizobia representing around 0.1% of soil microbes or 10^6 g^{-1} soil. Nitrogen is important for maintaining and improving crop yield[3]. The long-term use of chemical fertilizers in agriculture has created several environmental impacts including degradation of soil fertility, deterioration ofthe organic matter of soil, and decreased water and nutrient holding capacity[4]. Due to these ill effects, the use of biofertilizers is increasing day by day.Beneficial free-living soil bacteria are commonly referred to as a Plant Growth Promoting Rhizobacteria (PGPR). Soil temperature, acidity and rainfall areimportant conditions required by Rhizobia for its growth and development[5]. Biological fertilizers could potentially play key roles in the productivity and sustainability of soil. Biofertilizers are cost-effective and eco-friendly. The use of

biofertilizers improves soil fertility by fixing atmospheric nitrogen, solubilizing insoluble phosphates, producing plant growth-promoting substances in the soil, and promoting nodulation ability, which increases yield by 16–60%[6]. Biofertilizers are known as microbial inoculants, which are artificially multiplied cultures of certain soil microbes that can improve soil fertility and crop productivity[7]. They are organic products containing living cells of different types of microorganisms, which have the ability to convert nutritionally important elements from unavailable to available form through biological process [8]. Keeping in view the importance of Rhizobium in legume plants, the present study was undertaken to characterize and study different medias usedfor Rhizobium production.

As per the guidelines issued in the biofertilizer manual by the Japan Atomic Industrial Forum (JAIF) there are certain parameters that are followed in the production of biofertilizer[9].

METHODOLOGY: -

ISOLATION: -

The Rhizobium isolates were obtained from the root nodules of the leguminous plant. Nodules located on the roots were spherical (2-4 mm) and pink in color. Root nodules were sterilized in 95% (v/v) ethanol for 10 seconds and then washed 7 times with sterile distilled water. Individual nodules were crushed using sterile glass rod and streaked on Yeast Extract Mannitol agar containing 0.0025% (w/v) Congo red. After incubation for 2-3 days at 30ºC, single colonies were selected and transferred on Yeast Extract Mannitol Agar (YEMA) agar for pure culture.[10]

Figure 1

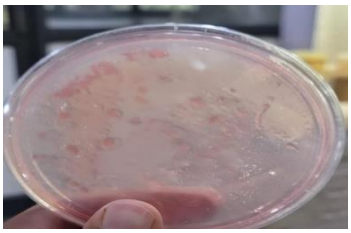


Figure 2

Figure 3

Rhizobium colonies cultured on different medias. Fig 1 indicates the growth of rhizobium on Congo Red Yeast Extract Mannitol Agar (CRYEMA) media. Fig 2 indicates the growth of rhizobium on nutrient broth and Fig 3 shows the growth of rhizobium on modified media.

PREPARATION OF MICROBIAL CULTURE: -

Freshly prepared 30 ml Cryema media was sterilized and then it is used as a enrichment liquid media. Inoculation was done with 5 loops of bacterial growth on preserved spread plate into 30 ml media. Cell count was taken after 24 hours. Average 4.165 ×10^7cells/ml were counted and 1 ml of mother inoculum was dispersed into each flask containing different medias.

SHAKE FLASK FERMENTATION: -

Shake flask method is different from the method of surface culture. Since the microorganisms are suspended in the culture broth, the oxygen concentrations are low. Shake Flask Fermentation method was used for the production of biofertilizer from Rhizobium bacteria.

GROWTH ANALYSIS: -

The bacteria (rhizobium) show exponential growth. The cells show 2nmultiplication rate. (Where n is the number of cells).

The bacteria (rhizobium) grow exponentially in CRYEMA media and all four phases were observed, namely: - lag phase, log phase, stationary phase, and death phase on 24 hrs. basis.

SUBSTRATE ESTIMATION: -

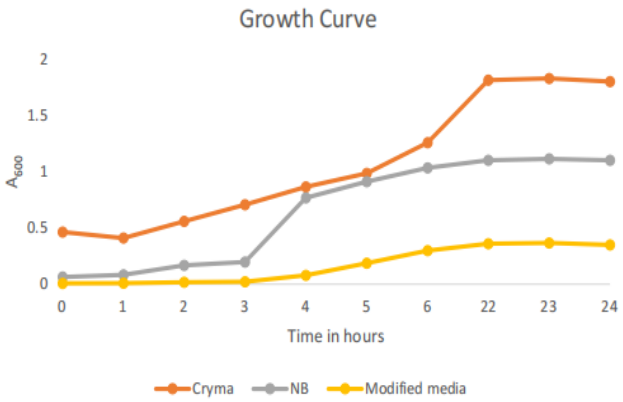
Substrate estimation is done in order to estimate the amount of substrate consumed at a particular interval of time, and with the help of this the yield is calculated, and from this the best media can be selected for the growth of the desired bacteria.

The method used for substrate estimation in this project is Phenol Sulphuric (Phenol H_2SO_4) method.[11]

The basic principle of this method is that carbohydrates, when dehydrated by reaction with concentrated sulfuric acid, produce furfural derivatives. Further reaction between furfural derivatives and phenol develops detectible color.

OBSERVATIONS: -

Graph 1. Growth Curve of Rhizobium on the 3 medias.



For Growth curve of Rhizobium on three different medias

Lag Phase :- Between 0 – 3 hrs.

Log Phase:- Between 3 – 6 hrs.

Stationary Phase:- Between 6 – 24hrs.

Graph 2. Standard Curve for substrate estimation

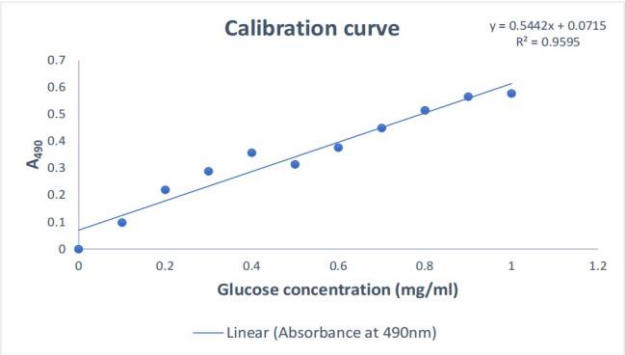
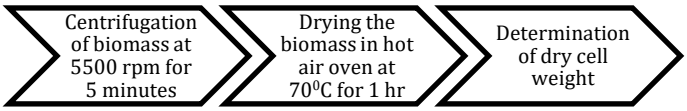


Table 1 Comparison of readings at different intervals of time for substrate estimation.

Concentration	Absorbance at 0hr	Absorbance after 24hrs	Absorbance after 48hrs	Absorbance after 72hrs
Sample 1	0.301	0.245	0.216	0.074
Sample 2	0.216	0.179	0.135	0.121
Sample 3	0.236	0.234	0.195	0.136

BIOMASS ESTIMATION: -



- Biomass Estimation was done using centrifugation method.[12]
- Weigh the empty centrifuge tube.
- Centrifuge the slurry (with biomass) at suitable rpm for required time.
- Decant the supernatant after centrifugation.
- Weigh the centrifuge tube along with biomass. Subtract the weight of emptybiomass tube from it, this will give you the wet weight of the biomass.

- Alternatively, the biomass can be dried in hot air oven at specific conditionsof temperature and time and then the weight of biomass.

Table 2 Comparison of observation for biomass estimation.

Time	After 24hrs.	After 48 hrs.	After 72hrs.
Sample 1 biomass (For 300 ml)	3.08g	4.62g	4.86g
Sample 2 biomass (For 300 ml)	2.05g	3.01g	3.12g
Sample 3 biomass (For 300 ml)	1.03g	2.02g	3.01g

YIELD COEFFICIENTS: -

The yield coefficient is also known as the substrate to biomass ratio. It is used to calculate the growth rate of a microorganism within a productand also to calculate the mass of the product with the increase in the cell growth.

$$Yxs = \frac{X_{final} - X_{initial}}{S_{final} - S_{initial}}$$

Table 3 Comparison between estimated readings of biomass and substrate for 3 medias.

(X =biomass and S=substrate)

Time	X(g) Cryema	S (mg) Cryema	X(g) Nutrient Broth	S (mg) Nutrient Broth	X(g) Modified Media	S (mg) Modified Media
0hr	0	5.4033	0	3.8548	0	4.2185
24hr	3.08	4.386	2.05	3.064	1.03	4.179
48hr	4.62	3.85	3.01	2.356	2.02	3.461
72hr	4.86	1.247	3.12	2.106	3.01	2.374

Table 4 Calculated Yield based upon Table 3.

Time (hrs.)	Cryema (g/l)	NB (g/l)	Modified Media (g/l)
24	0.7022	0.64	0.246
48	1.2	1.277	0.5836
72	3.897	1.42	1.2699

RESULTS: -

Table 5 Results of Samples for substrate estimation.

Conc. of Sample 1 at 0^{th} hour	5.4033 mg/ml
Conc. of Sample 2 at 0^{th} hour	3.8566 mg/ml
Conc. of Sample 3 at 0^{th} hour	4.2185 mg/ml

Conc. of Sample 1 after 24 hours	4.389 mg/ml
Conc. of Sample 2 after 24 hours	3.164 mg/ml
Conc. of Sample 3 after 24 hours	4.179 mg/ml

Conc. of Sample 1 after 48 hours	3.85 mg/ml
Conc. of Sample 2 after 48 hours	2.356 mg/ml
Conc. of Sample 3 after 48 hours	3.461 mg/ml

Conc. of Sample 1 after 72 hours	1.247 mg/ml
Conc. of Sample 2 after 72 hours	2.106 mg/ml
Conc. of Sample 3 after 72 hours	2.374 mg/ml

The concentrations of the samples of the three medias at different intervals of time were estimated using the Phenol Sulphuric acid to determine the sugar content consumption at different intervals of time. The bacteria use sugar for growth and thus the consumption determines the growth of microbes in the sample. This also helps to determine the yields on different methods.

CONCLUSION: -

It was observed that when the medias were formulated for 300ml, CRYEMA – the selective media of Rhizobium culture showed high yield i.e., 3.897g, than the other medias NB 1.42g and modified media 1.26g.

COSTING: -

Table 6 Cost of 1L biofertilizer.

Sr. No.	Chemical required	Quantity (1L)	Total Cost (Rs) (1L)
1.	Mannitol	10g	15.19
2.	Yeast Extract	0.4g	5.06
3.	NaCl	0.1g	10.13
4.	$MgSo_4.7H_2O$	0.2g	10.13
5.	K_2HPO_4	0.5g	4.05
6.	Sucrose	8g	0.4
Total	-	-	45

The above given cost is applicable for the production of 1 liter of biofertilizer.

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