

Culture Optimization for Mass Production of *Rhizobium* Using Bioreactor Made of Readily Available Materials and Agitated by Air Flow

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Abstract

Large quantities of *Rhizobium leguminosarum* by conventional technique are a time-consuming and labor-intensive method of production. The selection of different types of nutrient media for the production of large amounts of rhizobium at low cost, determination of environmental regulators of culture medium and the effect of different levels of air flow into nutrition media have been investigated. *Rhizobium* cultured in modified air lift bioreactor as improved conventional system using Yeast Extract Mannitol Broth by assessing aeration and agitation in their submerged growth. For selection of suitable nutrient media for *Rhizobium* before mass production, YMA (Yeast Mannitol Agar), YSA (Yeast Sucrose Agar), YMA + bromthymol blue (BTB), YMA + Congo red (CR) medium was tested. To optimize the growing conditions of different temperatures, pH levels and salt strengths were investigated. Three air flow speeds at the rate of 0.01, 0.05 and 0.1 VVM for oxygen supply were ensured. A thick off-white bacterial colony was observed on the YMA plate among different culture media. Room temperature (25°C - 30°C), neutral pH (7.0), low salinity (1%) was observed to be suitable among the environmental conditions for *Rhizobium* mass production. The maximum density of *Rhizobium* was observed using air supply of 0.1 VVM level in bioreactor system. The result seemed that the air supply and agitation was an important growth factor in submerged cultivation. This study provides useful information on large scale production of *Rhizobium* of interest employing low cost effective modified air lift bioreactor within less time.

Keywords

Rhizobium, Bioreactor, Agitation, Mass Propagation, Bacteria

1. Introduction

In terms of business the term, “large scale production” refers to the production of a commodity on a large scale with a large sized firm. It requires huge investments in plants and machinery. Large scale production can be carried out if the market size is large and expanding. In this research bioreactor for *Rhizobium* large scale production is usually described in a biochemical sterile environment that capitalizes on liquid nutrient or liquid/air flow and outflow systems, designed for intensive culture and control over micro environmental conditions (agitation, aeration, temperature, and pH).

Rhizobia are famous for their potency to embed a symbiosis with legumes. They inhabit root nodules, where they reduce atmospheric nitrogen and make it available to the plant. The *Rhizobium* bacterial strains in the starter cultures were needed to be grown on a large scale for which their mass production was urgently needed. In order to produce large quantities for a microorganism, the inoculums must be purified and liquid form would be cultured. Next, have to select the culture vessel used for liquid culture. In this case the size of the culture vessel is adjusted as required and productivity depends on vessel size [1]. Environmental requirements for microbial culture vessels have to be ensured.

Mass propagation at a higher rate after isolation of microbial biofertilizer is a challenging issue. This involves the culturing of large volumes of bacteria to obtain enough material to spectrally characterize an isolated compound. Currently, it is estimated that upwards of 90% of all culturing experiments in biotechnology are performed through shaken cultures [2]. The benefits of using shaken cultures are numerous and include the ability to culture in high density, a relatively low-cost barrier, and the use of easily accessible equipment. There are, however, a number of drawbacks to using shake flasks [3]. Primarily, shake flasks rely on surface aeration to transfer oxygen into the culture, resulting in low oxygen transfer compared to stirred bioreactors [4]. For natural product researchers, the application of shake flasks for the large-scale cultivation of microorganisms for natural product isolation has additional challenges. Although high-capacity shakers capable of handling numerous flasks at one time are available, they are expensive. This necessitates the use of smaller, cheaper orbital shakers that requires multiple rounds of culturing to generate enough material for compound isolation and characterization [3].

Conventional funnel or balloon shaped glass containers are taken for the purpose of cultivating more bacteria in a short period of time. It is not cost effective as low cost parts are assembled. Agitation of these types of bioreactors is accomplished by the flow of sterile air instead of mechanical stirring. Sometimes fermenters also used; the equipment, which provides the proper environment for the growth of a desired organism. It is generally a large vessel in which, the organism is kept at the required temperature, pH, dissolved oxygen concentration and substrate concentration. A sophisticated fermenter contains pH regulator, oxygen level regulator, anti-foam device, temperature controller, etc.

Multifarious field of application needs bulk amount of *Rhizobium* inoculums. Cultivation of these valuable bacteria in conventional methods is a costly, laborious and time consuming process. It is important to introduce *in vitro* mass bacterial cultivation methods by introducing any other simple and affordable method. Bioreactor system enabling aeration, agitation and maintaining OD from outside of the system during cultivation that hastens the submerged limitation. Modified air-lift bioreactor is completely offering for plenty production of *Rhizobium* which costs low and takes only couple of days. Bioreactor yielded liquid state entire biomass can be incorporated with carrier material Ramanujam *et al.* [5] to meet the ever increasing demand of world focused organic farming. The mass production of the bacterial strain to be utilized as a biofertilizer. Aimed at mass production in full *in vitro* condition to optimization of culture media, culture conditions including agitation for liquid form of *Rhizobium*, multiply the *Rhizobium* largely in modified air-lift photo bioreactor with less time and cost.

2. Materials and Methods

2.1. Optimization of Culture Conditions

For selection of suitable nutrient media for *Rhizobium* before mass production YMA (Yeast Mannitol Agar), YSA (Yeast Sucrose Agar), YMA + bromthymol blue (BTB), YMA + Congo red (CR) preparation were used to test the purity of culture. YEMA medium and 6.8 pH was selected as standard for this experiment to find out the optimum temperature for growth of *Rhizobium*. Difference in the range of growth temperature was investigated by incubation of bacterial cultures in YEM agar at 20°C, 25°C, 30°C and 35°C. To find out the optimum pH condition for growth 25°C temperature was selected as standard. The influence of medium pH on rhizobial growth was investigated at 5.0, 6.0, 7.0 and 8.0 by using HCl and NaOH in a pH meter after mixing of media ingredients. The salinity tolerance was measured by culturing the bacteria on YEMA medium containing different salt concentration 1%, 2%, 3%, 4% (w/v) NaCl.

2.2. Preparation of Liquid Media

Yeast Extract Mannitol Broth media (Vincent, 1970) [6] components were mixed properly and adjusted the pH to 6.8 ± 0.02 . All culture media were sterilized at 121°C for 20 minutes by autoclaving. All culture vessels, rubber pipes, rubber corks and spargers were also sterilized at 121°C for 20 minutes by autoclaving. Selected bacterial strains were grown in slants and transferred to liquid broth to detect the growth of *Rhizobium* under static and agitation conditions YEMB observed after 3 days of inoculation.

2.3. Establishment of *Rhizobium* Culture in Bioreactor System

Modified Air lift bioreactor was used as an automated culture 2 L vessel to propagate bulk amount of the *Rhizobium* inoculants. The modified vessel has

three outlets with pipes one for air supply into the medium and other two for let out the bubbles and gases to control produced gas pressure. One liter of yeast mannitol broth medium was prepared for each bioreactor system. Sterilized culture media poured into the vessel inside the laminar air flow hood. Then 48 days old *Rhizobium* cultured into the medium with sterilized pipette.

2.4. Culture Conditions for Bioreactor System

For appropriate growing condition of *Rhizobium leguminosarum* following parameters were applied in bioreactor system that used for mass production of *Rhizobium leguminosarum*. A set of three bioreactors were used in the preparation to evaluate the need of air supply into the culture medium of *Rhizobium* cultivation. An air pumper with regulator was entered into the bioreactor system. Three nose of air supplier connected with the outlet of respective bioreactor. Aeration convinced by three speeds of air as 0.01, 0.05, 0.1 VVM (Vessel volume per minute) through air passing by air compressor regulation from outside of system. Another bioreactor system had no air supply as control in the aeration experiment of oxygen necessary. After that sealed all outlet of system with parafilm after rubber band and incubated the system in growth chamber.

2.5. Estimation of Optical Density (OD)

Rhizobial growth in broth culture was become opaque in 2 - 3 days. Broth optical density was checked by using spectrophotometer (at 540 nm). Values of OD. correlate to number of cells. If OD. values will be high then cells number also will be high [7].

2.6. pH Test for Purity of *Rhizobium* Culture

Fast-growing *Rhizobium* produce a little acidic compound. After incubation, the pH was decreased. (Example, pH before growing = 7.0, after growing pH = 6.4 - 6.6). If broth pH of contaminants [8].

2.7. Determination of Colony Forming Unit (CFU)

After 24 - 48 hours of incubation, the plates with different colonies were selected for counting. Counting of colonies was normally done by visual observation. Then total CFU were determinate according to the following equation [7].

$$\text{CFU} = \frac{\text{Colonies count on an agar plate} \times \text{Amount plated}}{\text{Total dilution of tube (used to make plate for colony count)}}$$

3. Results and Discussions

3.1. Optimization of Culture Conditions

The organism was identified with nutrient medium dependence for selection of suitable nutrient medium of *Rhizobium* before mass production. *Rhizobium* usually do not absorb CR. BTB containing YMA plate show an alkaline reaction,

fast growing *Rhizobium* turn the medium yellow. Noted that growth and color reactions described here were dependent on the strain metabolism on the standard YMA media.

Growth of bacteria on Yeast Extract Mannitol Agar (YEMA) medium indicates their ability to fix atmospheric Nitrogen to the plants (Figure 1). The use of Yeast Extract Mannitol Agar (YEMA) medium for the isolation of Nitrogen fixing (Rhizobia) bacteria was earlier been reported by some workers [9] [10] [11]. After incubation of 2 - 5 days the bacteria was grown well on YEMA + Congo red medium, white thick colony color and medium color remained as pink. The colony grown on medium YEMA + bromthymol blue grows as well, the medium color of green turned yellowish after *Rhizobium* grows. This indicates that the isolate was a fast-grower and acid producer as reported by [12].

Highest growth of *Rhizobium* on YEMA plates were shown 2.3×10^9 CFU/ml in 25°C and 2×10^9 CFU/ml in 30°C (Figure 2). The culture which incubated at 20°C was not satisfactory. Culture incubated at 35°C was unfavorable for *Rhizobium* species. Allison and Minor [13] reported most species of Rhizobia were showed the optimum growth rate lies between 29°C and 31°C . Beijerinck [14] stated that room temperature was the most favorable for clover bacteria. Laurent [15] obtained the compatible growths at 22°C to 26°C ; at 30°C . The growth was no better than at the lower range.

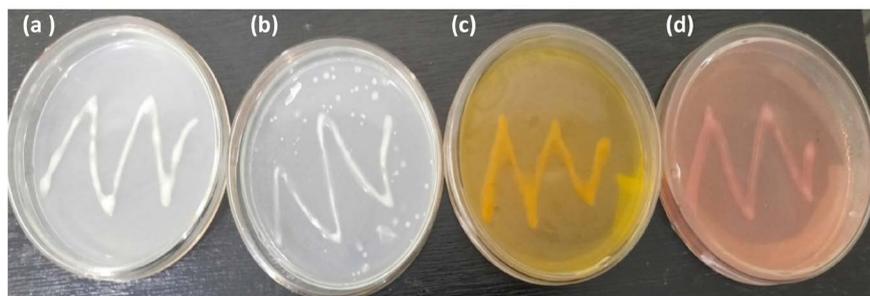


Figure 1. Growth performance of *Rhizobium* in different media, (a) = YMA, (b) = YSA, (c) = YMA + bromthymol blue (BTB), (d) = YMA + Congo Red (CR).

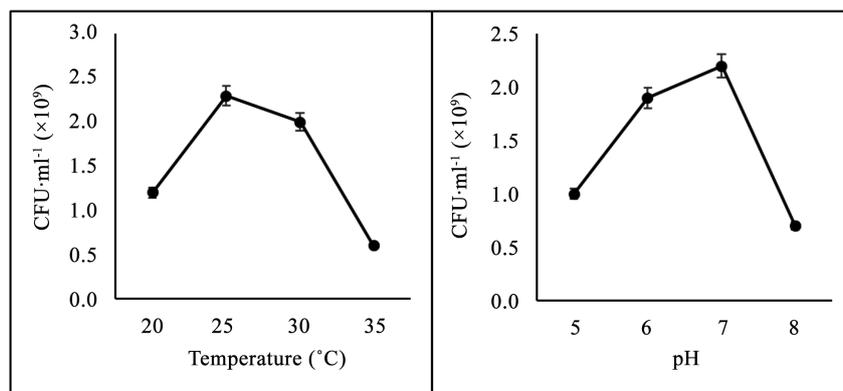


Figure 2. Colony forming unite of *Rhizobium* on YEMA at different temperature and pH level.

The response of *Rhizobium* growth rate to nutrient medium was observed between pH 5 - 8 (**Figure 2**). The maximum growth was found in 7 pH adjusted culture and the lowest CFU/ml found in 8 pH treating culture medium the CFU was 0.7×10^9 6 pH culture media was influence the *Rhizobium* to grow well after highest growth of 7 pH. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth [16] [17].

3.2. Salt Tolerance of *Rhizobium*

To determine tolerance in saline environments strains of rhizobia showed different growth rate at different concentration of NaCl, maximum growth rate was observed at 1% (w/v) NaCl and minimal at 4% (w/v) NaCl (**Table 1**). Jida and Assefa [18] also reported high tolerance to NaCl the *Rhizobium* could grow well with 1% NaCl. However, at higher concentrations, the percentage of tolerant isolate decreased with increasing salt concentration. The isolated bacteria were showed no growth on the YEMA with 2% NaCl, thus confirming the *Rhizobium* [19].

3.3. Response of Agitation for Large Scale Production

Environmental suitability is created by agitation for micro-organisms. The colour of culture medium was changed with agitated condition in drowned cultivation as off-white to brown (**Figure 3, Table 2**). Poor growth was observed in non-agitated condition in large vessel due to using only static medium conditions and it was denser than the medium of static condition. Highest optical density was observed in agitated vessel because of bacterial growth was improved due to agitation plays a role in dissolving oxygen through nutrition. Usually agitated or shaken culture did not allow the bacteria to be clumped on the surface and distributed properly into whole medium.

Table 1. Effect of NaCl concentration on Rhizobial growth.

Concentration of NaCl	Growth
1%	+++ve
2%	++ve
3%	+ve
4%	-ve

*Legend: +++ = excellent growth; ++ = less growth; + = poor growth; - = no growth.

Table 2. Influence of agitation on mass production of *Rhizobium* spp.

Culture conditions	Colour response	Growth performance	OD
Agitation	Brown	Good	1.969
Non-Agitation (Static)	Light yellow	Poor	0.984

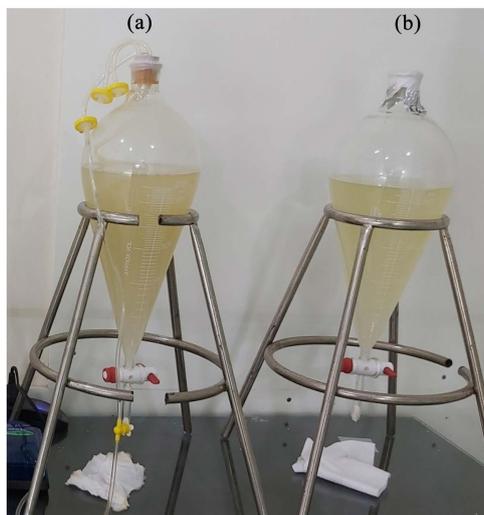


Figure 3. Influence of agitation on growth of *Rhizobium* (a) = agitation, (b) = without agitation.

3.4. Effect of Air Flow Rate on Mass Production of *Rhizobium*

Bioreactors are widely used for microbial mass production in laboratory conditions. *Rhizobium* are very sensitive to changes in the culture environmental conditions, such as aeration, agitation, nutrients, and pH. Influence of air flow on *Rhizobium* multiplications are shown in **Figure 4**. Minimal growth was observed in non-aerated condition in large vessel due to using only static medium conditions. In the absence of agitation through liquid nutrients, bacterial colonies usually form only on the surface of nutrients due to lack of oxygen. Maximum bacterial growth was found using 0.1 VVM air supply (**Figure 5**). The rate of growth of *Rhizobium* is similar to the rate of increase in air supply through liquid nutrition. Air circulation occurred at the rate of 0.01, 0.05, 0.1 VVM whereas 0.1 VVM air passing influence for highest cell growth of *Rhizobium* (1.769) when optical density at 540 nm after 72 hours. The medium speed of 0.05 VVM resulted 1.410 O.D. at 72 hours after.

The color of culture medium was changed with the passing of air speed in drowned cultivation as off-white to light brown (**Table 3**). The brown color of 0.10 VVM aerated bioreactor is relatively darker than other bioreactors which indicates that 0.10 VVM air supply increases the production of *Rhizobium*. The supply of air causes bubbles in the culture media, causing mild agitation in the media, which causes all the media to be used for growth of *Rhizobium*. The non-aerated bioreactor was found light off-white color, due to absence of agitation.

3.5. Purity of Production Media

As fast-growing *Rhizobium* produce acids media pH tends to decrease with the increasing bacteria. Before inoculation we can see the media pH was 6.8 ± 0.02 (**Figure 6**). The number of bacteria increases and at the same time the media pH

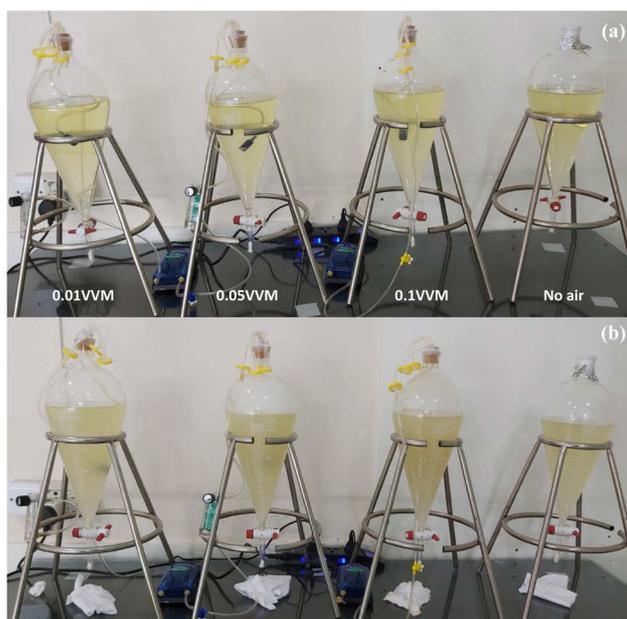


Figure 4. Impact of aeration on mass production of *Rhizobium* using bioreactor. (a) = 00 hours and (b) = 72 hours after inoculation.

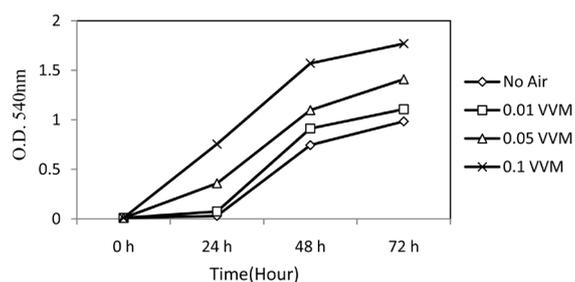


Figure 5. Influence of different air flow level on growth of *Rhizobium* spp.

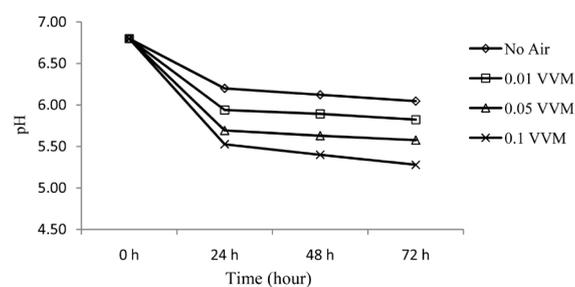


Figure 6. Observation of pH at different time for growth of *Rhizobium* spp.

Table 3. Influence of air supply on media color and growth status of *Rhizobium*.

Air supply (VVM)	Culture color	Growth
0.00 (No air)	Light yellow	Poor
0.01	Off white	Medium
0.05	Light brown	Good
0.10	Brown	Good++

decreases. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth [16] [17]. Rhizobia showed growth in 3 days and turned the yeast mannitol agar media containing bromothymol blue to yellow color showing that all were fast growers and acid producer [20].

4. Conclusion

This research was an effective process of producing large amounts of *Rhizobium* in a short time at a low cost. In this study, biofertilizers produced by *Rhizobium* was found to be potent since it could be active at 0.1 VVM air supply shows good results. Therefore, it was found that biofertilizers produced on lab scale might be beneficial tools for agriculture and environment. In the future, emphasis can be placed on the proper and economical utilization of abundantly present *Rhizobium*.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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