Chapter 7
Rhizobium Biofertilizers: Mass Production Process and Cost-Benefit Ratio Analysis

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Abstract In the era of demanding food supplies for the increasing populations worldwide, the increasing use of nitrogen fertilizers resulted in adverse effects on human health and the environment. To overcome the rising threat of chemical fertilizers, researchers have sought to find an eco-friendly alternative. Nitrogen-fixing rhizobia are a very important microbial group used to supply nitrogen to leguminous crops, which act as host crops for bacteria. Rhizobial bioformulations have been used as biofertilizers for nearly a century. Currently, various types of rhizobial biofertilizers are commercially available in the market, which may be in solid or liquid form. This chapter deals with mass production and solid carrier-based bioformulations of *Rhizobium* species. Additionally, the calculation of the cost-benefit ratio is also described, aiming to develop the small-scale biofertilizer industry and attract entrepreneurs.

Keywords Nitrogen-fixing · Biofertilizer · Bioformulations · *Rhizobium* · Entrepreneurs

7.1 Introduction

Integrated plant supply systems (IPNS) consist of a judicious combination of organic matter, chemical fertilizer, and biofertilizer, which promises an optimal nutrient supply to crops with simultaneous conservation of soil productivity and ecological health. However, the high cost and environmental and human health issues associated with chemical fertilizers have necessitated the development of eco-friendly and cost-effective alternatives. In this context, biofertilizers have become efficient alternatives to chemical fertilizers. Biofertilizers are living microbial inoculants in the form of bacteria, fungi, or algae that increase plant growth. *Rhizobium* is specifically applied to leguminous crops, and its application leads to a 10–30% enhancement in crop yields through an estimated fixation of 40–250 kg/ha/year nitrogen fixation.

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The amount of nitrogen fixed depends on the efficacy of *Rhizobium* strain. *Rhizobium* as a bioinoculant was first commercialized in the USA by private enterprises in the 1930s (Smith 1992).

7.1.1 Rhizobium *Species*

Rhizobium is a Gram-negative root colonizer that generally colonizes leguminous roots. *Rhizobium* species work symbiotically with plants and drive atmospheric nitrogen in plants. The process starts when bacteria attach to the root hair, and the plant protein "lectin" binds the bacteria to the surface. Bacteria then penetrate root hairs, and the infected root cells divide to form nodules that provide an anaerobic environment that is necessary for the nitrogen fixation process (Gomare et al. 2013). It is recommended for crops such as groundnut, soybean, red gram, green gram, black gram, Bengal gram, lentil, fodder legumes, etc. However, colonization by *Rhizobium* strains is very specific to the host plant. Therefore, the industry that opted for *Rhizobium* biofertilizer mass production should produce multiple species of *Rhizobium* for application in various crops as per the farmer's demand and definitely for establishing reliable industry and earnings. The costs and technical details of different species are more or less the same. Some *Rhizobium* species and their host crops are listed in Table 7.1.

7.1.2 Mass Production

Mass production of *Rhizobium* biofertilizer is divided into the following stages:

(a) *Procurement of Standard Strain*
Pure cultures of various strains of *Rhizobium* species maintained in test tubes or vials can be purchased from Agricultural Universities, Indian Agriculture Research Institute, national/regional centers of organic farming, etc. After receiving pure cultures, it should be further subcultured and must be maintained purely for mass production using standard microbiological media and techniques by trained microbiologists.
(b) *Mother Culture Preparation and Maintenance*

Table 7.1 Some of the *Rhizobium* species and its host crop

Rhizobium species	Host crop
R. leguminosarum	Green pea and lentil
R. japonicum	Soybean
R. lupine ornithopus	Lupinus
R. meliloti	Melilotus
R. phaseoli	Phaseoli
R. trifoli	Trifolium

Table 7.2 Selective media for *Rhizobium* culturing

Components	Quantity (g/L)
Mannitol	10.0
K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2
NaCl	0.1
Yeast extract	0.5
Distilled water	1.0 L
pH	7.0
Agar	15

The selective medium for the mass *Rhizobium* strain is yeast extract mannitol (YEM) agar medium. The compositions are presented in Table 7.2. All ingredients should be mixed well and sterilized by autoclaving at 121 °C for 20 min at 15 lbs pressure. After making the slants, a single colony from the mother culture slant is transferred and incubated at 30 °C for 24 h.

(c) *Starter Culture Preparation*

Starter cultures for mass production can be prepared using the same YEM, excluding agar. The broth is dispersed into conical flasks and autoclave at 15 lbs pressure for 15 min at 121 °C, cool, inoculate with *Rhizobium* colonies, and incubate for 24–48 h at 30 °C in an incubator shaker. Inoculate the flask of 500 mL capacity with this starter culture and incubate it on shaker for 48 h and use this culture as an inoculum for the fermenter.

(d) *Mass Multiplication*

Prepare YEM medium as described and pour into a fermenter, sterilize (15 lbs pressure at 121 °C for 15 min), and cool to normal temperature. Add inoculum from the inoculation point of the fermenter (20 L capacity) at a rate of 5% and allow it to grow under optimum conditions for 4–5 days. Regulate the air flow to 3–10 L of air/hour/lit of the medium. Sterile air provides aeration and agitation for bacterial growth. Periodically, draw samples from the sampling point and analyze for growth and contamination by plating on YEM agar medium. Harvest the broth from the culture outlet for formulation once bacterial growth reaches 10^9 CFUs/mL.

The important considerations that should be kept in mind during the fermentation process are: a) it is not advisable to store the culture after optimum growth is reached, b) there should not be any fungal or other bacterial contamination during and/or after harvesting the culture. The final cost of the product is affected by the medium used for multiplication and the duration of the fermentation process. Therefore, it is essential to choose an appropriate medium and optimize parameters such as pH, temperature, and aeration to reduce the incubation time for different *Rhizobium* species. The ideal pH and temperature for *Rhizobium species* are 7.0 and 30 °C, respectively.

(e) Rhizobium *Bioformulations*

The fermented culture should be harvested in batch culture mode and mixed with the appropriate carrier material at a ratio of 1:5 (v/w). For instance, mix 1 L of enriched culture with 5 kg of carrier material to obtain effective bioformulations of 10^8–10^9CFUs/mL.

(f) *Processing of Carrier Material*

The use of an ideal carrier material is essential for biofertilizer formulations to maintain good quality until application in the field. The selection of an ideal carrier material should be based on: (a) cheaper cost, (b) nontoxicity, (c) high organic matter content, (d) more than 50% water holding capacity, (e) ease of processability, and, most importantly, (f) local availability. Generally, charcoal, press mud, peat soil, lignite, vermiculite, farmyard manure, and soil mixtures can be used as carrier materials for biofertilizer formulations. Neutralized peat soil or lignite is ideal for *Rhizobium* strain formulation. However, more recently, liquid formulations have attracted attention of entrepreneurs because of their low cost, easy storage, low space requirement, and easy maintenance.

(g) *Packaging*

Before packaging, the inoculum-mixed carrier material should be shade dried for 2–3 days at room temperature. Curing should be done by spreading the mixture on polyethylene sheets and keeping it in shallow trays with polyethylene covering. After 2–3 days of drying, the formulations could be packed in the desired quantities (200 g, 500 g, and 1.0 kg) depending on the market demand. Generally, polyethylene bags are used for the packaging of biofertilizers. However, it is important to consider that the bag should be of low-density grade, and the approximate thickness should be 50–75 μm (Sethi et al. 2018). Each packet should contain the product's name, manufacturer's name and address, details about *Rhizobium* strain used, the name of crops for which it can be used, batch number, date of manufacture, expiry date, storage instruction, mode of application, CFUs/g, etc. The population of inoculants in packed products should be checked at monthly intervals for a year to ascertain the quality in terms of CFUs/g count until use (Motsara et al. 1995).

(h) *Quality Control*

The quality of a formulated product is an important factor influencing its success or failure in the market. The correct type of organism in the desired number must be present in the formulations. Microbial processes are sensitive to contamination; therefore, quality should be checked for contamination at every stage of production, including mother culture subculturing, starter culture preparation, fermentation, carrier selection, and mixing with broth during packaging and storage. Quality control must include a serious consultation for microbiologists at each stage of the product. Finally, the product should meet some standards specified by the Indian government, i.e., the formulation must contain at least 10^7cells/g of carrier before the expiry date marked on the packet.

7.1.3 *Calculation of Pilot-Scale Production of* Rhizobium *for Small-Scale Industry*

Various facilities are required for the successful establishment of a biofertilizer unit for the production of *Rhizobium* strains. The infrastructure and laboratory facilities described here are not only applicable for *Rhizobium* production, but the same facilities can also be applied for the production of other nitrogen-fixing biofertilizers such as *Azotobacter* and *Gluconacetobacter*. Most private firms and governmental institutes produce various bacterial biofertilizers using common facilities to ensure the economic viability of project installation. The stepwise calculation for the small-scale industrial production of *Rhizobium* strain is divided into two parts: A) non-recurring cost described in Table 7.3, which includes fixed costs of capital investment in equipment; and B) recurring cost, which includes variable costs for raw materials used for production to packaging, manpower wages, marketing expenses, water and power utilities, and other miscellaneous expenses per year as described in Tables 7.4, 7.5, 7.6, and 7.7.

For small-scale production of *Rhizobium* strains, two 20 L capacity fermenters are sufficient for batch-wise harvesting. 1.0 L of culture is sufficient for mixing with 5.0 kg of carrier material if broth is enriched for 4–5 days under optimal conditions. Thus, 100 kg of *Rhizobium* biofertilizer can be produced from 20 L broth. Therefore, for a one-time harvest in a week from two fermenters, 200 kg of finished product will be formulated. Thus, the monthly production will be 800 kg, and the annual production will be 9600 kg. Thus, the approximate production with minimum

Table 7.3 Non-recurring cost calculation for initial capital investment

Sr. No.	Particular	Total cost in INR (in lakhs)
1.	*Equipment and machinery*	
	Vertical autoclave (600 × 350 mm) × 1	0.8
	Refrigerator (300 L) × 1	0.35
	Laminar air flow (3′ × 2′) × 1	1.0
	BOD incubator × 1	0.6
	Rotary shaker × 1	0.25
	Compound microscope (binocular) × 1	0.60
	Weight balance	0.15
	pH meter × 1	0.3
	Colony counter × 1	0.05
	Stainless steel seed fermenters (20 L cap.) × 2	3.0
	Polyethylene sealer × 1	0.15
	Total for capital investment for equipment	7.25
2.	*Miscellaneous fixed assets (computer, printer, fax, stationary items)*	1.5
3.	*Grand total* (1 + 2)	8.75

Table 7.4 Calculation of cost for production media

Components	Quantity (g/L)	Quantity (g/2000 L)	Approximate Indian rate	Cost for ultimate quantity required to produce 2000 L
Mannitol	10.0	20,000	550/kg	11,000
K_2HPO_4	0.5	1000	1500/kg	1500
$MgSO_4.7H_2O$	0.2	400	340/500 g	340
NaCl	0.1	200	230/500 g	230
Yeast extract	0.5	1000	2200/500 g	4400
Total cost				17,470
Total cost in lakhs (INR)				0.18

Table 7.5 Other miscellaneous raw material cost

Material	Quantity	Approximate rate	Total cost in lakhs (INR)
Carrier material	10,000 kg	4200/ton	0.42
Polyethylene bag and labels	Depending upon market demand for packaging (200 g, 500 g, 1 kg)	Approx.	0.5
Variable cost per annum for consumables (flasks, pipettes, test tubes, measuring cylinders, beakers, loops, gas cylinders, etc.)	Variable	Approx.	0.25
Total cost			1.17

Table 7.6 Man-power wages per annum

Category	Nos	Salary per head (Rs. in lakhs/month) (INR)	Total cost (Rs. in lakhs/month) (INR)	Total cost (Rs. in lakhs/annum) (INR)
Microbiologist	1	0.3	0.3	3.6
Assistant production officer	1	0.15	0.15	1.8
Administrative officer	1	0.15	0.15	1.8
Sales officers	2	0.15	0.3	3.6
Skilled and unskilled labors	3	0.045	0.135	1.62
Total cost			1.035	12.42

instruments and manpower will be approximately 10,000 kg. Therefore, the present model for the cost-benefit ratio is 10,000 kg/year *Rhizobium* biofertilizer production.

(a) Non-recurring cost
(b) Recurring (variable) cost per year

Table 7.7 Total recurring expenses per annum for 10,000 kg production

Particulars/annum	Cost (Rs. in lakhs/annum (INR)
Media cost (cost/10,000 kg/annum)	0.18
Other miscellaneous raw material cost	1.17
Man-power cost	12.42
Building rent/annum	3.6
Utilities—Power (2000 units @ Rs. 5/unit)	1.2
Contingencies	
Marketing and selling expenses	1.0
Repair and maintenance	1.0
Total cost	20.57

Table 7.8 Cost-benefit ratio calculation

Particulars/annum	Cost (Rs. in lakhs/annum (INR)
Expenditure/annum/10,000 kg production	20.57
Loss due to contamination	0.5
Depreciation cost of fixed assets at 5%	0.44
Total expenditure	21.51
Income/annum/10,000 kg selling	35.0
Net benefit	13.49
Profitability (%profit of sale)	38.54%

Raw Material Used for the Production of Rhizobium Species

Production of 2000 L of culture broth is required for 10,000 kg of the finished product of *Rhizobium* biofertilizer per annum. If one considers standard company ingredients then the cost for media (YEM) (2000 L) is given in Table 7.4.

Income per 10,000 kg of Selling

The average cost of solid *Rhizobium* solid formulations in the current market ranges 250–450 INR/kg. Government institutions and organizations may cost less than this, and on the other hand, some private industries may cost double. If we consider an average 350 Rs./kg the income will be 35.0 lakhs/annum (INR). Based on this selling and income, the cost-benefit ratio is calculated, as shown in Table 7.8.

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