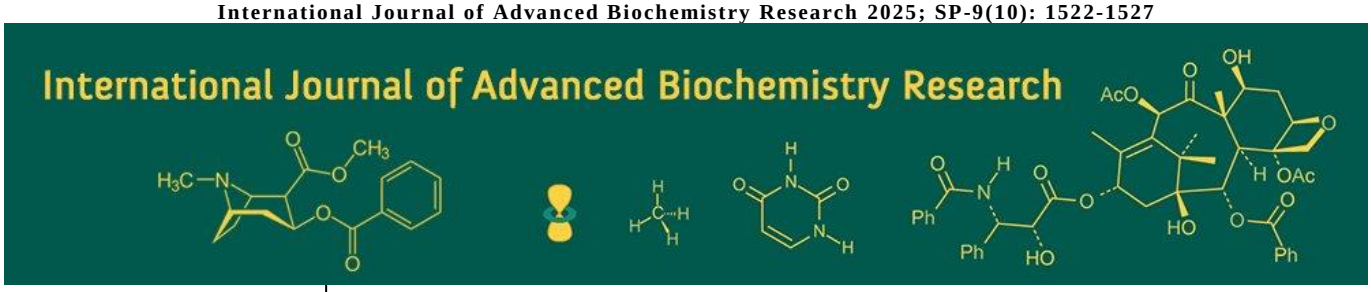

ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; SP-9(10): 1522-1527
www.biochemjournal.com
Received: 02-07-2025
Accepted: 05-08-2025

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A review on advanced techniques in mass production and bankable enterprise development for biocontrol agents

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DOI: https://www.doi.org/10.33545/26174693.2025.v9.i10Ss.6049

Abstract
Chemical pesticides usage lead to degraded soils, groundwater pollution, and food safety concerns. To address the environmental and health concerns of chemical pesticides. Need for effective, less harmful pest management strategies. So, Biopesticides are an eco-friendly alternative. Biopesticides represented 3% of the overall pesticide market in India and are expected to increase. This growth is attributable to enhanced awareness, policy support, and improvements in mass production and formulation technologies. Solid state fermentation (SSF) remains widely used for fungal agents like *Trichoderma viridae* and *Metarhizium anisopliae*, where substrates such as wheat bran or sorghum are used to enhance sporulation. However, liquid fermentation (LF) is increasingly gaining traction for bacterial agents like *Bacillus thuringiensis* and fungal formulations due to its higher yield potential, automation, and ease of downstream processing. LF can yield over 1.5-2 × 10^9 CFU/ml of viable spores compared to 10^7-10^8 CFU/g in SSF. Different designs and operating conditions of SSC bioreactors might encourage the development and production of conidia from particular microbes. Compared to solid substrate fermentation techniques, submerged liquid fermentation is less labor-intensive, produces a higher output, and has a longer shelf life. It occurs in deep tank bioreactors that are automated and readily scalable to thousands of liters. Successful production management requires a variety of tools and rearing supplies, including cages, storage racks, glassware, plastic ware, and chemicals. The types of biocontrol agents generated will determine the equipment selection. Innovative production techniques, multi-tiered policy support, and interdisciplinary research aiming at improving the effectiveness, shelf-life, and cost-efficiency of bioagents are key to the future of biocontrol in India.

Keywords: Biopesticides, liquid fermentation, yield, shelf life and policy support

Introduction
The public concern is growing over chemical inputs in agriculture. Chemical pesticides usage lead to degraded soils, groundwater pollution, and food safety concerns. To address chemical pesticides' negative effects on the environment and human health. Need for effective, less harmful pest management strategies. So, Biopesticides are an eco-friendly alternative (Gupta & Dikshit, 2010) [12]. Biopesticides are "Certain types of pesticides derived from such natural materials as animals, plants, bacteria, and certain minerals, Less threat to environment and human health".
Microorganisms (bacteria, viruses, fungi, nematodes) or naturally occurring substances (plant extracts, semiochemicals). The benefits include being less hazardous, causing less environmental stress, being target-specific, being effective in small amounts, breaking down rapidly, and being appropriate for Integrated Pest Management (IPM).

Types of Biopesticides
1. **Microbial Pesticides:** Contain a microorganism (bacterium, fungus, virus, protozoan, alga) as the active ingredient. Specific to target pests (e.g., *Bacillus thuringiensis* (Bt) for insects).
2. **Plant-Incorporated Protectants (PIPs):** Plants produce Pesticidal substancesfrom added genetic material (e.g., plants engineered to produce Bt protein).

3. **Biochemical Pesticides:** Naturally occurring substances (plant extracts, fatty acids, pheromones) that control pests by non-toxic mechanisms (e.g., growth regulators, repellents/attractants) (Varsha Bharti and Shahida Ibrahim. 2020) [26].

As per estimates by the Central Insecticides Board and Registration Committee (CIBRC), India had over 121 registered biopesticide products (Biofungicides and Bioinsecticides). The area under biopesticide application expanded from approximately 0.2 million hectares in 2007 to over 1.2 million hectares by 2014 (Sithanantham, 2014) [25]. Biopesticides represented 3% of the overall pesticide market in India and are expected to increase. This growth is attributable to enhanced awareness, policy support, and improvements in mass production and formulation technologies.

Mass Production Technology - There are two types of production methods

1. **Solid State Fermentation:** Organism grown on the surface of a solid medium. (e.g., nutrient agar, potato dextrose agar)
2. **Liquid State Fermentation:** Organism grown beneath the surface of a liquid medium. (e.g., nutrient broth, potato dextrose broth).

Solid-state fermentation (SSF) remains widely used for fungal agents like *Trichoderma viridae* and *Metarhizium anisopliae*, where substrates such as wheat bran or sorghum are used to enhance sporulation. However, liquid fermentation (LF) is increasingly gaining traction for bacterial agents like *Bacillus thuringiensis* and fungal formulations due to its higher yield potential, automation, and ease of downstream processing (Kumar *et al*., 2010) [16]. For example, LF can yield over 1.5-2 × 10^9 CFU/ml of viable spores compared to 10^7-10^8 CFU/g in SSF (Rangaswamy *et al*., 2014) [23].

Solid-State Culture for the Production of Entomopathogenic Fungus (EF) Conidia: An aerobic or anaerobic procedure known as "solid-state culture" (SSC) involves growing the microbe on a solid matrix, such as a substrate or support. The carbon supply, nutrients, and water needed for the microorganism's culture are all present in the solid matrix (Manan and Webb, 2017) [17]. The grains of rice, wheat, sorghum, and millet are the most often utilized substrates. However, inert supports enhanced with a culture media and agro-industrial by-products (bagasse, bran, husk, fibers, and fruit peels) can be used. Since the solid matrix is the means of transporting nutrients, the amount of water present is crucial for microbial development in SSC. The growth of significant EF from the genera Metarhizium, Beauveria, and Paecilomyces is favored by substrates with a water activity of 0.98. The bed must feature interparticle gaps that facilitate mycelium growth, conidia generation, metabolic heat removal, and gas exchange in addition to having the chemicals required for microbial growth (Méndez-González *et al*., 2022) [19]. Conidia production and quality are influenced by the gaseous atmosphere's temperature and composition. The majority of EF found globally thrive in temperatures between 20 and 32°C. Temperatures outside of this range typically have a major impact on conidia quality, production, and microbial proliferation. Additionally, the quantity and quality of conidia are significantly influenced by the amount of O_2, CO_2, and volatile chemicals present in the gaseous atmosphere. O_2's low availability restricts microbial development since aerobic bacteria (like EF) need it to get energy from the substrate (Finger *et al*., 1976). However, high CO_2 atmospheres (~5% v/v) have been shown to have a negative impact on conidia production (in Beauveria bassiana) (Garza-López *et al*., 2011) [10], and volatile chemicals like 1-Octen-32-ol reduce the quality of conidia (Muñiz-Paredes *et al*., 2017) [22]. Consequently, procedures for gas exchange and temperature maintenance must be taken into account while designing SSC systems. The core component of the SSC system is the bioreactor. The conditions for microbial growth are provided by bioreactors. Static bed and mixed bed are the two layouts available for SSC bioreactors. According to their capacity, these bioreactors can be divided into four categories: bench (2 to <5 L), pilot (5 to <50 L), laboratory (a few milliliters to <2 L), and industrial size (>50 L) (Méndez-González *et al*., 2022) [19]. They can also be categorized as high-tech or traditional based on the differences in monitoring and control equipment. Different designs and operating conditions of SSC bioreactors might encourage the development and production of conidia from particular microbes.

i. Traditional Bioreactors

The two main forms of bioreactors used in traditional processes, which were established in the middle of the previous century, are plastic bags and compact, rigid containers made of glass, metal, wood, or plastic. *Tolypocladium cylindrosporum*, *Metarhizium anisopliae*, *Beauveria bassiana*, *Lecanicillium lecanii*, and *Culicinomyces clavispurum* are among the conidia that are produced in low-capacity covered containers (CC), such as wooden baskets and covered pal bioreactors (Roberts and St. Leger, 2004). A thin layer of infected substrate, about 4-5 cm high, is spread out inside the container of this kind of bioreactor and covered with either porous or non-porous material. Diffusion across the membrane facilitates gas exchange when the covering material is porous. On the other hand, non-porous materials require that the bed surface not be entirely covered in order to provide aeration. The high-density polypropylene used in plastic bag bioreactors (PBB), which are used to produce EF conidia, typically has a capacity of 200-1000 g of substrate (Méndez-González *et al*., 2020) [20]. Micropores in the production material enable gas transmission through diffusion (Durand, 2003). Nevertheless, CO_2 buildup may result from the poor diffusive transport velocity across the membrane. In certain situations, bags are left partially open or plugs with more porosity than the bag material typically cotton are employed to enhance gas exchange. The usage of these porous plugs is necessary for certain PBBs composed of non-porous polymers (like polyethylene). Because CC and PBB have a low capacity (≤1 kg of wet mass (kg wm), it is required to process a high number of units every batch, which necessitates a lot of work and big incubation areas. Furthermore, it is difficult to apply monitoring and control tools because of the features of the bioreactor. Significant differences in conidia output and quality can result from a lack of process monitoring and control, which can then have an impact on marketing pricing and manufacturing costs.

Nonetheless, the culture procedures in CC and PBB enable the low-tech and low-capital creation of propagules of various EFs. With a capacity of 300 to 350 kg of conidia annually (enough to treat 7000 hectares), these bioreactors are therefore perfect for setting up small production units (Grzywacz *et al*., 2014) [11].

Mass production of *Trichoderma viride* in Solid state fermentation: The medium chosen for mass multiplication should be affordable, easily accessible, and have the right ratio of nutrients. Since solid-state fermentation (SSF) yields micropropagules with a greater conidia content, it is a successful technique for producing fungal biopesticides in large quantities. As substrates, a variety of inexpensive cereal grains are utilized, including ragi, rice, millets, and sorghum. To produce *Trichoderma viride* in sorghum in large quantities, place 100 grams of sorghum in a 250 milliliter flask and soak it for six hours in a 2% sucrose solution. After that, filter the water out of the flask and autoclave it to disinfect it. After cooling to room temperature, the grains were infected with 10 milliliters of Trichoderma *viride* suspension the next day. They were then incubated for 10 to 15 days to develop the dark green spore covering that *Trichoderma viride* produces on grains.

ii. High-Tech Bioreactors

High-tech bioreactors can be outfitted with devices to track and regulate culture conditions, addressing important factors like temperature, moisture content, and gaseous O_2 and CO_2 concentrations. Controlling these characteristics guarantees a process that produces high-quality goods, is repeatable, and has high production yields. Static bed systems like TB and PBCB, as well as those with mechanical mixing like Rotating Drums (RRB) and Internal Stirred Bioreactors (ISB), are examples of bioreactors in this category.

i. **Tray Bioreactors (TB):** Tray bioreactors (TB) are one of the most popular high-tech SSC bioreactors in the business. The basic design of TBs consists of a tray with a rectangular surface on which a thin layer of substrate is spread. Metal, plastic, and wood are some of the materials that can be used to make trays. Trays may or may not have perforations, depending on the needs for gas exchange and heat removal. Natural convection and diffusion carry CO2 and O2 between the headspace and bed in the traditional kind (non-perforated trays). Evaporation and conduction are the methods used to remove heat (Diniz-da Silva *et al*., 2022) [4].

Conversely, forced aeration through the bed is made possible by perforated trays, which enhances the supply of oxygen and eliminates heat, CO_2, and volatile substances. As bed height increases, heat and mass transport in TBs are impeded. Research conducted on *Beauveria bassiana* and *Metarhizium anisopliae* (da Cunha *et al*., 2019) [2] shows that raising the bed height from 2 to 8 cm causes heat buildup, which lowers the formation of conidia (between 63 and 76%). Thus, it is essential to keep the bed height between 2 and 4 cm in order to avoid anaerobic zones and overheating.

Production of Conidia from *Beauveria bassiana* in Tray Bioreactors: Conidia from *B. bassiana* have been produced at pilot scale in tray bioreactors with chambers of 60 x 60 x 200 cm. With a bed height of 4 cm and a porous bottom (35-60 mesh), these chambers hold 25 parallel trays that may hold 2-3 kg dm of the substrate (rice grains). Production yields range from 1.8 to $2.7X10^9$ conidia/g dm with a viability above 90%. The conidia production process takes around 7 days, with an incubation temperature near 25^OC and HR values above 90%. Techniques like aeration and water spraying keep the temperature and moisture levels necessary for the fungus's growth and conidiation stable. Some businesses have employed this method to produce *B. bassiana* conidia on a big scale, with a yield of $3.72X10^9$ conidia/g dm following 10 days of incubation (Fernando Méndez-González *et al*., 2018**)** [7].

ii. **Packed Bed Column Bioreactors:** The cylindrical systems known as packed bed column bioreactors (PBCB) are powered by airflow that flows through the bed. Both the top and bottom of the bioreactor can supply forced aeration, which encourages heat loss and gas exchange through convective mechanisms. Process condition monitoring and control are made easier by the design of PBCBs. As a result, huge quantities of conidia can be produced under control using these techniques. The complexity of PBCB scale-up, however, restricts its application in the sector. The accumulation of metabolic heat is one of the primary issues with the PBCB scale-up. Temperature control in laboratory-scale PBCBs is made possible by heat removal in both axial and radial directions. Heat transfer via the wall is minimal in bioreactors with larger capacities, but (Finkler *et al*., 2021). As a result, conduction, evaporation, and convection are the primary axially directed methods that remove heat. The bioreactor's maximal dimensions determine how well these devices maintain the bed temperature. By encouraging the opening of vacant spaces in the bed, texturizers enhance heat evacuation and air distribution (Méndez-González *et al*., 2022) [19]. When sugarcane bagasse was applied to the rice bed, saw a 40% increase in M. anisopliae conidia production.

Production of Conidia of *Metarhizium spp*. in Packed Column Bioreactors: Good growth and conidia output from 4 to $6X10^9$ conidia/g dm at 175 hours of culture were made possible by aeration rates ranging from 1 to 60 mL/min (in bioreactors with a capacity of 14 g dm, roughly). Conidia generated by using packing densities ranging from 0.270 to 0.357 g/cm3 were around $4.6X10^9$ conidia/g idm. However, research on the composition of culture media allowed for a rise in conidia output. In bioreactors with a capacity of 8.5 g dm and a forced air supply of 0.34 mL/h g idm, the use of growth media based on mixes of rice bran and rice hust (BH medium) produced conidia yields of $1.15x10^{10}$ conidia/g dm. Up to $8x10^9$ conidia/g idm was achieved using a hemp fermentation bed impregnated with culture media containing glucose (200 kg/m3), which is almost 57% more than with rice grains ($3.4x10^9$ conidia/g idm).

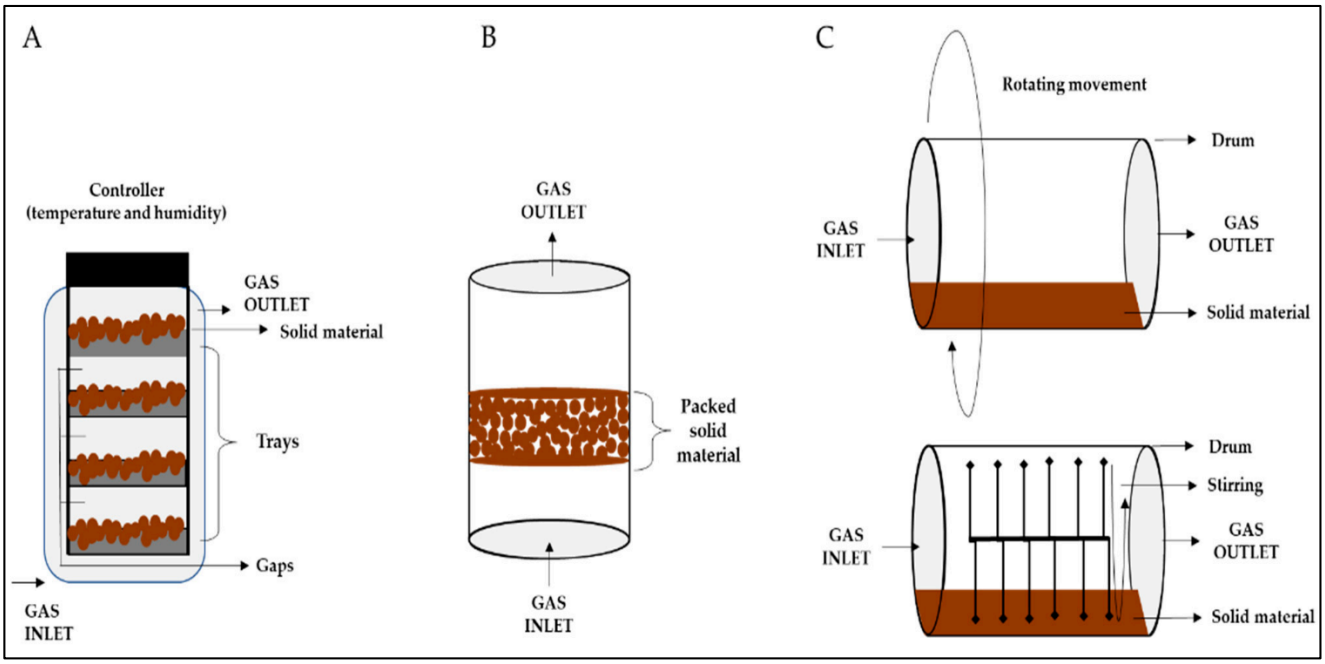

Fig 1: SSF bioreactors with occasional agitation and without forced aeration (**A**). Tray bioreactor); with occasional agitation and forced aeration (**B**). Packed-bed bioreactor); and with slow continuous agitation and without forced aeration (**C**). Two models of stirred drum

iii. **Agitated Bioreactors:** Agitated bioreactors (AB) present two configurations: rotating drum (RDB) and internal stirred (ISB). In these bioreactors, heat removal and gas exchange are facilitated by intermittent or continuous mixing. Similar to PBCB, AB can benefit from forced aeration, which enhances heat and mass transport in the bioreactor (Martínez-Ramírez *et al*., 2021) [18]. Enzymes and animal feed have been produced on an industrial scale (up to 25 t) using ABs (A-type bioreactors), which enable controlled procedures. RDBs (Rotating Drum Bioreactors) are cylinders that rotate horizontally. The mycelium sustains modest damage from the drum's smooth rotation, but substrate agglomerations cannot be broken up by it. ISBs (Inoculated Solid-state Bioreactors) have a system of blades for mixing the substrate and are cylindrical (horizontal and vertical). While stirring promotes bed uniformity, it also mechanically damages the mycelium and substrate.

2. **Submerged liquid fermentation (SLF):** In contrast to solid substrate fermentation (SSF) techniques, submerged liquid fermentation (SLF) occurs in automated deep tank bioreactors that are easily scalable to thousands of liters and require minimal personnel. It is simple to assess the right nutritional and environmental conditions for generating large quantities of active fungal propagules, such as submerged conidia, blastospores, mycelium, microsclerotia, and chlamydospores, by manipulating the nutrient composition and physical facts (i.e., carbon, nitrogen, minerals, vitamins, carbon-to-nitrogen ratio, aeration rate, temperature, rheology, osmotic pressure, and pH). Many templates for the food, bioenergy, and biotechnology sectors have been developed in liquid fermentation technology that can be modified to produce EPF.

Mass Production of Trichoderma viride in Liquid State Fermentation: Liquid state fermentation (LSF) is utilized for processes where microbial growth is facilitated by soluble ingredients in water. Typically, molasses-yeast medium, wheat bran, and potato dextrose broth are used to grow significant amounts of *Trichoderma* spp. by liquid fermentation technology. Black gram soak water, etc., are also used for mass production. Before inoculation, prepare 100 milliliters of potato dextrose broth, incubate it for 15 minutes at 121 degrees Celsius and 15 pounds of pressure, and then allow it to cool to room temperature. To stop germs from growing, add 0.1 grams of antibiotic to the soup. Incubate 1 milliliter of *Trichoderma viride* suspension in 100 milliliters of potato dextrose broth for five to six days at 25 to 30 degrees Celsius.

EPNs may be mass-cultured *in vitro* (using solid and liquid media, such as kidney extract, beef fat, and pig kidney, along with symbiotic bacteria for axenic culture, e.g., *Steinernema glaseri*) or *in vivo* (using a dead insect body function as a bioreactor in a symbiotic association with bacteria). Baculovirus biopesticides are produced using *Helicoverpa armigera virus/Helicoverpa zea* cell systems, initially containing $2x10^{12}$ OB/L. Using a liquidizer or a cool glass pestle and mortar, the cadavers are homogenized in sterile ice cold water at a magnitude relation of 1:2.5 (w/v). After being filtered through two layers of fabric, the material is continuously rinsed with water. To remove debris, the filtrate is centrifuged for 30 to 60 seconds at 500 rpm. Next, the supernatant is centrifuged at 5,000 rpm for 20 minutes. After that, the pellet containing the solid occlusion bodies (POB) is suspended in sterile water and centrifuged in water at a low rate, followed by activity at a high rate, for three rounds of washing.

Table 1: Comparative Yield and Shelf-Life of Mass Production Techniques for Key Biocontrol Agents (Dr Nanda Ram, 2017) [5]

Technique	Agent Type	Yield Potential (CFU/g or CFU/ml)	Shelf-life (Months)	Preferred Carrier
S olid-state fermentation (SSF)	*Trichoderma, Metarhizium*	10^7-10^8 CFU/g	4-6	Talc, lignite
L iquid fermentation (LF)	*Bt, Pseudomonas, Beauveria*	1.5-2 × 10^9 CFU/ml	6-12	Liquid suspensions
Encapsulation/gel beads	*Pseudomonas, Trichoderma*	10^9 CFU/ml (approx.)	8-12	Alginate, polymer beads

Microbial Strains Improvement for Better Biopesticide Production: "Strain improvement" refers to the application of metabolic engineering or biotechnological methods to consistently increase the output of biopesticides by manipulating microbial strains and using propagation strategies. Zhu *et al*. used the CRISPR/Cas9 technology to create a robust strain of *B. thuringiensis* that produced a lot of melanin. According to the researchers, this strain has 80% insecticidal efficacy against *Helicoverpa armigera*, the cotton bollworm, while the control sample only showed 20%. When entomopathogenic fungi are inserted into the cuticle layer, they release digestive enzymes into the hemolymph, which encourages the use of sugar. Trehalose, the main carbohydrate found in insect hemolymphs, can be broken down by the trehalase enzyme that *Metarhizium acridum* excretes, more especially by acidic trehalase metarhizium 1 (ATM1). The generation of a mutant of *M. acridum* that has the capacity to overexpress the ATM1 gene after successfully reaching the locust hemocoel has been made possible by the use of biotechnological methods in metabolic engineering. In comparison to the wild-type strain, this improvement reduced LC_{50} and promoted *M. acridum* development in the locust hemocoel.

Bankable enerprise development for biocontrol agents

The activities carried out in the production facility involve,

- Regulation of environment,
- Handling of different life stages of insects,
- Sanitation in the workplace,
- Preparation of feed materials for the insects, and
- Preparation or processing of biocontrol agents.

Thus, several types of equipment and rearing supplies, including cages, storage racks, glassware, plastic ware, and chemicals, are necessary for effective production management. The types of biocontrol agents generated will determine the equipment selection.

Scope: Even if there are currently 140 biopesticide production facilities in the nation, less than 1% of the planted area may be supplied by them. The only way to close the gap is to establish an increasing number of facilities for the manufacturing of biopesticides. Given the yearly rise in demand, there is potential to improve the manufacturing and application of biological control agents in the days ahead. This calls for significant financial outlays as well as private involvement.

Location: Facilities for biopesticides must be established in regions with suitable climates in order to get the best outcomes. because it is less expensive to regulate the temperature in areas without harsh weather. In addition to the weather, the location's closeness to the market is crucial. However, in order to avoid the manufacturing facilities becoming contaminated by pesticides from the farming regions, care must be taken to ensure that they are situated at least a quarter of a mile away. Additionally, the production should be situated away from urban and industrial regions because air pollution might harm biopesticides.

Objectives

- Establishing the viability of mass production of diverse bioagents is the main goal of biopesticide programs.
- To provide entrepreneurs who might be interested in establishing biopesticide units with guidance and support.
- To encourage the establishment of more bio-control production facilities.
- To spread the technology widely.

Type of facilities: Nowadays, the majority of the facilities used to produce biocontrol agents are for commercial production and research & development. The former seeks to discover and classify different types of biocontrol agents, investigate their biology and efficacy against agricultural pests, develop economical production techniques, establish quality control standards, and show the agents' efficacy in real-world settings. The goal of the commercial manufacturing facility is to provide farmers with a big quantity of high-quality biocontrol chemicals so they may profit from them. In order to find solutions for pressing daily issues and to stay up to date with the evolving demands of producing biocontrol agents, a commercial facility may have an internal research and development section (Fernando Méndez-González *et al*., 2024**)** [8].

Design & space requirements: When designing a facility, the program's overarching goals and the associated processes are crucial. But no matter how advanced a facility is, manufacturing will not be consistent unless it is correctly built. Not every insect and its biocontrol agent needs the same kind of space, tools, and practices.

Design & space requirements: production complex may be divided into several sections viz., administration, quarantine, production, storage, wash and waste disposal.

Equipments & materials (glassware & chemicals)
Quarantine area: When new insect or biocontrol agent specimens are brought into the lab from the field or another lab, they should never be transferred to the production area without first being checked for biological contamination. The specimens must so be maintained in a quarantine environment.

Conclusion

As the country faces escalating concerns regarding environmental degradation, pesticide resistance, and food security, biocontrol offers a viable path forward. However, substantial efforts in capacity building, research funding, and farmer education are necessary to ensure the scalability and success of biocontrol technologies in India’s diverse agricultural landscape. The future of biocontrol in India lies in innovative production methods, multi-tiered policy

support, and interdisciplinary research aimed at enhancing the efficacy, shelf-life, and cost-effectiveness of these agents. By addressing the current barriers and seizing emerging opportunities, biocontrol agents can significantly contribute to the sustainability of Indian agriculture, ensuring a healthier environment and safer food for generations to come.

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