Chapter 5
Mass Multiplication of *Trichoderma* in Bioreactors

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Abstract Global statistics show that the biopesticides market is far behind as compared to their chemical counterparts. Therefore, in order to increase their share in the global market, low-cost mass production technologies need to be developed. The most exploited genus in the biopesticide industry is *Trichoderma* whose formulation is developed using various organic and inorganic carriers either through solid or liquid fermentation technologies. However, the major trouble faced by both farmers and manufacturers regarding the bioproduct is the instability of the developed product under different environmental conditions. Therefore, through this chapter, we have tried to summarize the various production technologies with necessary precautions to be taken for making a successful *Trichoderma* product for the end users.

Keywords *Trichoderma* · Propagules · Fermentation · Quality assurance · Shelf life

5.1 Introduction

Industrial production of effective biocontrol agents for commercial use requires well-defined criteria of selection and also thorough knowledge of the process for the development of microbial products for disease control in plants. This chapter discusses the selection of novel microbial control agents and their production process for commercial use. An important factor in the success of *Trichoderma* is the adoption of cost-effective means of production and development of a product that remains stable in the environment and multiplies and performs the desired functions as a biocontrol agent (Singh et al. 2013; Fraceto et al. 2018).

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In the process of mass manufacturing or production of *Trichoderma* spp., the factors to be considered are the types of production, i.e., submerged fermentation, solid-state fermentation, and formulation of the products, i.e., liquid, powder, and granular etc. The company or the manufacturer has to make a choice on the type of product the customer requires and accordingly the infrastructure of the plant has to be made.

5.2 *Trichoderma* spp. Commercially Important Propagules: Mycelia, Chlamydospores, and Spores

Trichoderma spp.is a fungus that produces different structures throughout their life cycle. The main structural organization and components are mycelia, spores, and chlamydospores. Each is considered as propagules which have capabilities of multiplying and growing into a new colony if suitable substrate and other favorable conditions are available (Singh et al. 2014b).

Solid-state fermentation (SSF) produces mostly aerial spores also known as conidia and the other kind of spores known as blastospores are produced under submerged fermentation (SmF). Spores produced by SSF, i.e., aerial spores have longer viability compared to submerged spores. Other advantages of SSF aerial spores are that they have more virulence resistance to stress conditions, higher UV resistance over SmF. Blastospores also have few advantages for commercial uses as its infection is much faster and also germination is faster than SSF spore.

5.2.1 Mass Production

There are various research going on across the country and throughout the world to identify, isolate, and characterize the most efficient *Trichoderma* as a biocontrol agent. But there is a huge gap when the efficient strain has to be mass multiplied and supplied as a product. A lot of emphasis is to be given to this area of research where such knowledge is very much required (Keswani et al. 2016). The mass production of the *Trichoderma* in a bioreactor is a very important step in the process of commercialization of the *Trichoderma* and having a potential strain does not serve the purpose unless and until the art of mass multiplication is known (Kumar et al. 2014; Prasad and Rangeshwaran 2000). Hence, as a futuristic approach, a lot of research has to be undertaken in generating as much knowledge as possible in this front. Any typical mass production unit of *Trichoderma* comprises of the following:

1. Production Lab—Production lab comprises basic infrastructure like laminar airflow, BOD incubators, orbital shaker, etc. The production lab is the starting point of the activities of commercialization. Activities done in production lab are as follows:
   (a) Culture maintenance at 4, −20, −80 °C as glycerol stock, agar slant, or Lyophilized form

(b) Seed culture preparation
(c) In-process sample checking
(d) Harvest sample analysis
(e) Finished good analysis

5.2.2 Production Area (Upstream and Downstream)

The production area is the next step in the mass production of *Trichoderma*. It comprises of fermenters depending upon the type of fermenters (SmF/SSF), utilities like boilers, compressors, water supply etc. The fermentation process of *Trichoderma* is conducted by the adoption of standard operating procedures and documentation of the process. The in-process samples during fermentations are drawn and analyzed in the production lab. The samples are analyzed for various parameters like pH, microscopy, spore count, detection of contaminants (i.e., other than the desired microbes) and substrate residual analysis. These parameters are very much required to be studied to assess the quality of the product (Al-Taweil et al. 2009).

The output of the fermentation is formulated into different formulations. The formulation can be of anything ranging from liquid, powder, granules, water dispersable granules, aqueous suspension, suspension concentrate, etc. The formulation is an important step in the product development. Intense research is required to optimize the formulation steps to ensure the spore survivability in different forms. If the formulation and the formulants are not compatible with *Trichoderma*, this may lead to a very poor shelf life of the product.

5.2.3 Quality Assurance Unit

In any manufacturing unit, the role of the quality assurance unit is of utmost importance as it plays a major role in ensuring product quality (Keswani et al. 2016). The activities of the quality assurance unit are as below:

1. Seed inoculum purity checking.
2. Pre-inoculum and inoculum purity checking.
3. Fermenter in-process sample checking. The samples are checked for growth, pH contamination, microbial count, and spore count.
4. Harvest samples checking. The quality assurance department checks the harvest samples, assesses the quality of the product, and approves the batch to the harvested.
5. Formulated sample checking. The samples after formulations are also checked by the quality assurance unit. The parameters checked are pH, moisture, CFU count, total viable count, and presence of contamination in any formulation should not exceed 10^4 per gram.

6. The formulated product should be tested negative for human pathogens like *Salmonella*, *Shigella*, and *Vibrio*. The quality assurance unit conducts the entire above tests to ensure the quality of the product.

5.2.4 Packing and Dispatch of the Final Product

Once the product is formulated, necessary quality checks are done. The product is either stored as semifinished goods or as packed units.

5.3 Product Stability Study

Any formulated product of *Trichoderma* should remain viable during the period of storage as claimed in the label. The quality assurance unit conducts periodical tests for viability of the *Trichoderma*, moisture, pH, and presence of any contaminants (Fig. 5.1) if any (Bhat et al. 2009). Selection of compatible polymers are again an

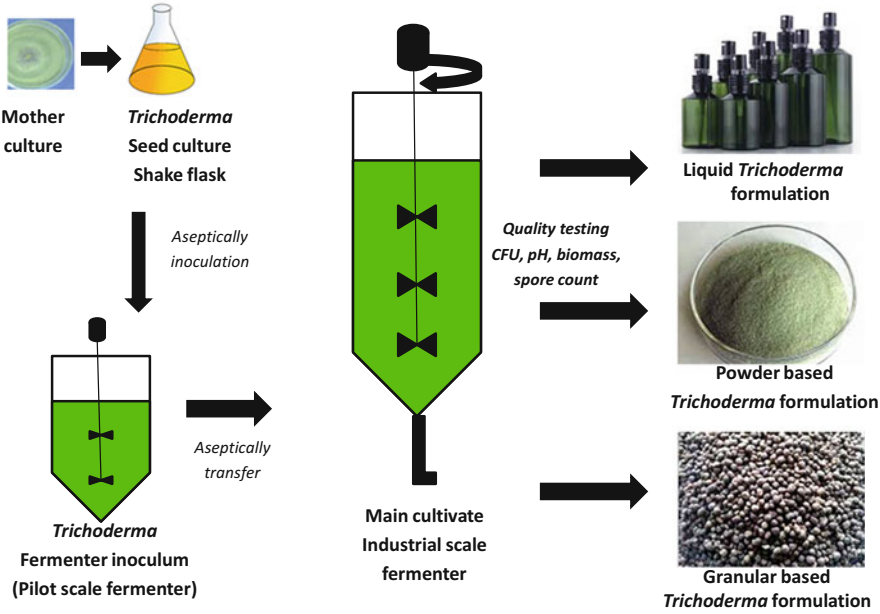

Fig. 5.1 Product development process flow in submerged fermentation

important factor for ideal product development. Different statistical approaches are employed for the optimization study.

Bioinoculants normally has to be produced at large scale with utmost care and good manufacturing practices. Good manufacturing practices play a major role in maintaining the quality of the bio inoculants. Mostly bio inoculants/bio fungicides like *Trichoderma* spp. are being manufactured across the country as small scale industry practices (Ramanujam et al. 2010).

5.4 Different Types of Fermentation and Bioreactor Designing

Trichoderma spp. as the commercial formulation is manufactured using fermentation technology which is through SmF and SSF procedures.

5.4.1 Submerged Fermentation

Industrial process using SmF can be carried out by three major types of fermentation:

1. Batch fermentation
2. Fed-batch fermentation
3. Continuous fermentation

Batch fermentation is also known as closed-system fermentation. In this approach, the fermentation medium is prepared, inoculated and the process runs till the harvest of the products. There is no intermediate addition or removal of broth or ingredient from the vessel (Fig. 5.2). This type of fermentation is mainly followed in the case of mass multiplication or whole-cell fermentation.

Fed-batch fermentation is also known as semi-closed system fermentation. In this process, the medium is prepared, inoculated and the process runs for a stipulated time. The new ingredients or substrate are added at regular intervals. This process is known as feeding strategy. There is no removal of cells or ingredients from the system/vessels (Fig. 5.3). This type of fermentation is generally followed in a case where secretory secondary metabolic substances are products (intercellular, intracellular, or extracellular).

Continuous fermentation is also known as open system fermentation where a sterile medium is added and inoculated and after a certain period of time, and cell and medium containing metabolites are removed and replenished with new medium (Fig. 5.4). The log phase is prolonged. This type of fermentation is usually followed in pharmaceutical industries where metabolites are products. This type of fermentation process runs for several weeks to months.

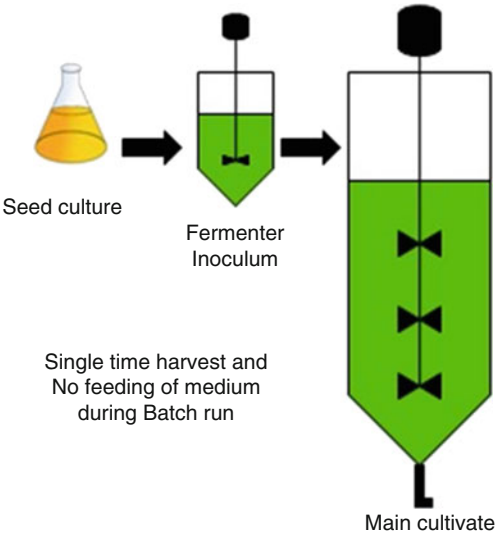

Fig. 5.2 Typical schematic of batch fermentation

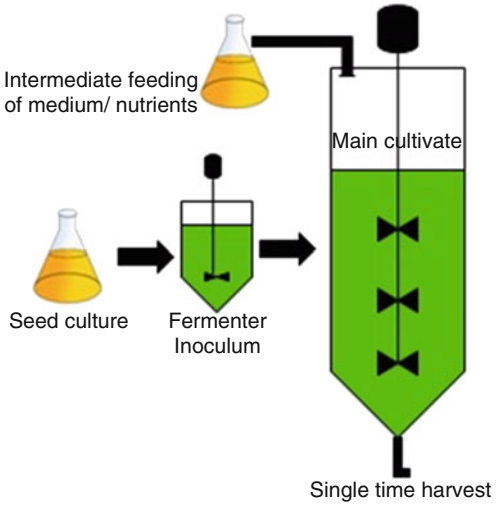

Fig. 5.3 Typical schematic of fed fermentation

For commercial production of *Trichoderma*, batch fermentation is more convenient and most commonly used throughout the industries worldwide (Lejeune and Baron 1995). The selection of the type of fermentation depends upon the final products.

5.4.2 Solid-State Fermentation

SSF is a type of fermentation process mainly used for microbial biomass production at large scale manufacturing (Fig. 5.5). SSF has very minimum free water or

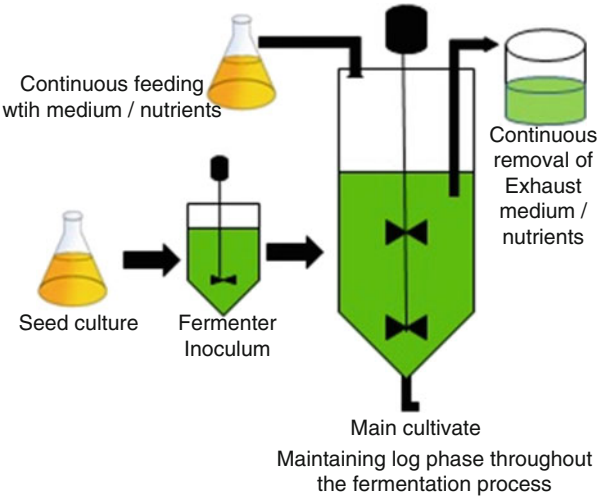

Fig. 5.4 Typical schematic of continuous fermentation

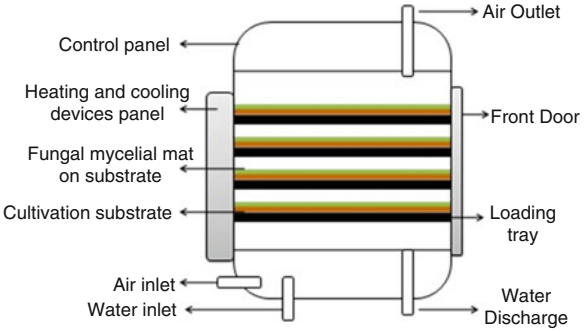

Fig. 5.5 Typical schematic of chamber type solid fermenter

sometimes completely absent. The use of natural sources of carbon substrate is the source of energy for the microbes to grow. This is a major difference with SmF where free water is the medium. In SSF the substrate which is used contains sufficient moisture to support the metabolism a growth of the microorganism. In the case of *Trichoderma* spp. commercial production as a biocontrol agent (BCA), both SSF and SmF are used for large-scale production. In SSF the *Trichoderma* spp. mycelia grows on the surface and on the minor and major cavities of the substrate (Smits et al. 1996). Water activities are a major factor which influences the spore production. Higher moisture content will decrease the amount of aerial spores resulting in a low count of product per gram of harvest material. There are several other factors that eventually influence the productivity when grown as SSF culture. Culture condition, substrate type like particle size, moisture, pH, etc. plays a major role. *Trichoderma* is cultivated in Petri dish, flask, polypropylene bags, column reactors, tray reactors, and packed bed reactors.

5.5 Factors Affecting the Growth of *Trichoderma* spp. in Bioreactors

For production of effective bioformulation of *Trichoderma* spp. there are several key factors that are considered. The production is directly or indirectly influenced by different factors. In both the cases, SSF and SmF there are few common parameters whereas other factors are unique with respect to each process (Vrabl et al. 2019). Key factors affecting the cultivation are summarized as below:

1. Strain/microorganism
2. Nutritional factors
3. Physical factors—temperature, pH, agitation, oxygen requirement, moisture, etc.
4. Inoculum quantity, strength and age
5. Mechanical and design of bioreactor
6. Process scale-up

5.5.1 Strains

Trichoderma strains have biocontrol activities against several fungal plant pathogens. *Trichoderma* exerts biological control by different approaches like antibiosis, mycoparasitism, and induced systemic resistance to plants and by competing for nutrition as *Trichoderma* is a fast-growing fungus. Likewise, *Trichoderma* directly or indirectly helps in protecting plants from many plant diseases when applied as a foliar spray, soil application, or as a seed treatment. But the selection of strains is of utmost importance as the strain to be used for commercial activities needs to fulfill all the criteria of a biocontrol agent. Isolation source plays a crucial role in adapting the isolates into different cultivable condition (Kumari et al. 2014). Screening of strains is performed at the research phase through dual cultural techniques against severe plant pathogens like *Fusarium, Pythium, Pythophthora, Sclerotinia, Alternaria, Cylindrocladium,* etc. Selected strains are then considered for the developmental phase (Singh et al. 2013).

5.5.2 Nutritional Requirements

Trichoderma or any other microbial strains require any major and minor nutrients for growth and metabolism for SSF or SmF. Carbon and nitrogen are the major nutrient sources for growth along with P, K, Mg, Ca, Mn, Fe, etc. Different sources of carbon and nitrogen play a major role in the growth and sporulation of the fungus.

There are different forms of organic and inorganic sources of carbon and nitrogen. Organic sources of carbon are jaggery, molasses which are cheap and readily

available, whereas glucose, maltose, sucrose are other types of carbon sources which are considered for production but are less used due to its expensive cost. Different sources of nitrogen are like yeast extract powder, meat extract, corn steep liquor, and inorganic sources like ammonium chloride, ammonium sulfate, etc. Other forms of trace elements are also added at a suitable combination to fasten upon the fungal growth. In SSF, there are other cheap sources of carbon and nitrogen sources which are commonly used like wheat bran, rice flour, and different pulses processed powder. SmF and SSF components for raw material varies as SmF requires more soluble particle for optimum growth whereas bigger size particle is required for SSF since *Trichoderma* utilizes the substrate and grows on the surfaces (Ming et al. 2019).

There are several forms of statistical approaches where the selection of ideal raw material for optimum growth is carried out. Factorial design and Plackett–Burman are the two most common tools for initial screening and optimization of raw materials. The value after initial screening is further statistically analyzed through response surface methodology (RSM). The statistical method of screening and optimization helps to understand the variables factors and its level and marginalizes the critical components essential for the growth of *Trichoderma*. The components of raw materials are sometimes selected on the basis of background and available research data which can save time which can otherwise be time-consuming studies.

5.5.3 *Physical Factors: Temperature, pH, Moisture, Oxygen Concentration*

As mentioned previously that raw material and nutrients play a crucial role in the production of *Trichoderma*, physical factors like temperature, pH, and oxygen concentration are the major influence for the optimum growth and ideal development of inoculants. Temperature plays a crucial role in growth where it influences the spore production along with other metabolic pathway determination (Mishra and Khan 2015; Singh et al. 2014a). The optimum pH range for the production of *Trichoderma* is 25–30 °C. pH on the other hand is equally important for the ideal manufacturing of *Trichoderma* formulations. It grows well at pH range between 4.5 and 7.0. The most important is oxygen concentration especially in the case of SmF where oxygen transfer and its uptake has a vital influence on the growth. Less or excessive oxygen concentration can also damage or delay the bioprocess. Each factor discussed above is the driving factors for the ideal production of *Trichoderma* formulation in SmF or SSF. Moisture is a limiting factor in SSF and moisture percentage in the range of 50–80% is ideally suited for proliferation and growth of *Trichoderma*. Optimum moisture of 75% is ideally suited for the spore production of *Trichoderma*. Moisture above 80% in solid-state fermentation is reported to decrease the sporulation. Oxygen is supplied to the fermenter vessel through specialized pipelines with a perforation at the bottom known as sparger in SmF. The dispersion

of oxygen is carried out by impellers where air or O_2 bubbles are broken into small size for immersion into the suspension. Oxygen is an important factor for the growth of *Trichoderma*. In SSF oxygen supply was a constraint so far but with modern equipments, the supply has become efficient where oxygen is suplied externally.

5.5.4 Inoculum and Seed Culture

The seed culture, i.e., from slant to plate and to liquid broth for preparing a first-generation liquid culture sets is the first step to the production process (Singh and Nautiyal 2012). The inoculum plays a crucial role where cell concentration, stage of inoculation decides the take of the bioprocess. Percentage inoculum is optimized at the research phase prior to production through various studies in shake flask fermentation.

5.5.5 Mechanical Factors

Mechanical factors are mainly considered for the bioreactors used for the cultivation of microbes. The bioreactors used for solid-state fermentation or submerged fermentation have significant effects or the final yield and productivity (Flodman and Noureddini 2013; Olaniyi and Oyesiji 2015). The yield in submerged fermentation depends on the design of the reactors. Ideally, a manufacturing unit should have similar types of symmetrical bioreactors for commercial production. This reduces the variability and ensures standardization of bioprocess. Microbial fermenter for production has a specific dimension ratio of height and diameter. A typical microbial submerged bioreactor has 2.5:1 to 3:1. It consists of a middle vertical shaft with an impeller. The impellers are rotatory blades that rotates at a fixed axis which helps in oxygen supply to microbes form growth. Microbial bioreactors have Ruston turbines impellers of four to six blades. The supply of oxygen is exerted through the bottom ring sparger. Spargers are hollow pipes connected inside the fermenter vessel with a perforation at the lower surface of the ring. Vessel design influences the scaling-up strategies of the bioprocess. On the other hand in SSF, the structure is less complicated but the raw material plays a crucial role. Oxygen transfer, temperature, pH, and water content are limitations in the case of SSF. There are different types of SSF reactors like tray bioreactor, rotating drum bioreactor, packed bed bioreactor, and other forms varies industry-wise. For the production of *Trichoderma* trays, rotator drums and poly bags reactors are most common across industries. And stirred tank bioreactor is the most used submerged fermenter for *Trichoderma* commercial production.

5.5.6 Scale-Up of Bioprocess

Any microbial process which needs to be commercialized for the manufacturing of products undergoes a series of events, popularly known as the scale-up process. The laboratory and pilot study are operational and research activities emphasize on the standardization of research and development phases (Ortiz et al. 2015; Hardy et al. 2017). A laboratory shake flask study is initiated for initial screening and evaluation of input v/s output. Studies for optimization of raw material, temperature, pH, O_2 concentration, inoculum percentage are conducted. The initial investigating parameters set forth the process for the developmental phase, i.e., for piloting the bioprocess. Shake flask fermentation is a miniature form of bioreaction that significantly lays down the prototype for bioprocess.

At the development phase, laboratory-scale bioreactors of varying sizes are used for scaling up of *Trichoderma* formulation from 1 to 100 L. The configuration of bench-scale and pilot-scale bioreactors are exactly similar to commercial-scale bioreactor where cultivation of *Trichoderma* is controlled effectively. *Trichoderma* production using SSF trays and bag are used for commercial production which is less complicated in scale-up process as compared to the submerged one.

For scale-up process of *Trichoderma*, maintenance of an optimum oxygen concentration is of utmost importance in phases of the life cycle (Schmidt 2005). As oxygen is poorly soluble in water, the mechanical factors play a crucial role in the supply of O_2 from air to cell through water as a medium. Oxygen transfer rate (OTR) and oxygen uptake rate (OUR) have to be proportional at the exponential phase of *Trichoderma* cultivation. The exponential phase reaches at 30–48 h from the start of the bioprocess. The cell count per gram or milliliter is 2×10^7 to 5×10^8 with a spore count of 5–8 crores/ml. The wet cell biomass at this stage is ideally from 80 to 200 g/l. All the values obtained indicate a proper optimization of a *Trichoderma* production process. The process of scale-up is controlled through a constant factor known as the volumetric mass transfer coefficient, also popularly known as KL*a* (Tribe et al. 1995). This KL*a* is determined at various phases of scale-up through various calculations with respect to vessel volume or dissolve oxygen concentration.

$$\mathrm{KL}a = \mathrm{OTR} - \mathrm{OUR}$$

where KL is the overall transfer coefficient, "*a*" is the area of bubble per unit of liquid volume (mm^2/ml).

In terms of oxygen flux = KL*a* ($C^* - C$) per unit area of bubble.

C^* = dissolved oxygen concentration which would be at equilibrium with the bubble and C = dissolved oxygen in the liquid phase.

The *Trichoderma* production requires high oxygen supply during the early sporulation stages. The agitation and aeration rate is controlled in such a way that the growth is optimum. High agitation must be avoided for maintaining an optimum dissolved oxygen (DO) concentration as this may cause shearing of mycelia that reduces the spore production. The DO can be maintained through the supply of pure oxygen in certain cases where the air is not sufficient enough. Too much production

of mycelia biomass of *Trichoderma* is also not recommended for commercial formulation. An ideal *Trichoderma* inoculant must contain plenty of spores/conidia as they are more stable and have longer shelf life during the storage period.

Modern bioreactors used for the production of *Trichoderma* are controlled with gas monitoring system (GMS) which is automatically regulated using PLC (programmable logic control). The data is managed and recorded through SCADA (a software and program for complete control and record of data for bioprocess). The airflow or oxygen flow is set as per the scale-up parameter as calculated in VVM (vessel volume per minute). Scale-up of *Trichoderma* production process required precision and zero deviation to optimize from pilot scale to commercial scale. The calculations throughout the scale-up study are important as it impacts the optimum output. The required agitation for oxygen supply is calculated throughout the geometric volume of bioreactors with respect to the tip speed of the impeller. This is dependent on the volume of the bioreactor and the diameter of the impeller. The value for tip speed can be calculated as:

$$3.14DN\ (D = \text{diameter of impeller}, N = \text{RPM}).$$

Also, power and volume *P*/*V* calculation is considered for minimizing the stress and shearing caused due to high agitation. This further ensures the *Trichoderma* produced is of high quality for commercial use. In case of SSF sclae up, quantity of substrate in tray or polybags need to be optimized, also water activity and oxygen supply are critical for ustilization of entire substrate and to produce high quantity of spores.

5.6 Factors Affecting Quality of *Trichoderma* Product

The quality of the bio inoculants is of utmost importance. Any potential strain is considered as ideal strain only if it is able to survive, multiply, and performs its desired function. In the process of manufacturing, many aspects are to be taken into consideration to achieve the above characters of ideal inoculants. Among the various factors which affect the quality of the *Trichoderma* when manufactured under large scale includes virulent potential of the strain, sporulation percentage, CFU count of the final product, moisture percentage, pH of the final formulated product, contaminations present in the final formulated product, kind of raw materials used for the production of *Trichoderma* in the fermenters, quality of carriers used for powder and granular product.

5.7 Conclusion

For better utilization of the potential of *Trichoderma*, research in future should focus on testing the suitability of commercially produced *Trichoderma* for management of both foliar and soilborne pathogens, development of better delivery system

preferably liquid/oil formulations with long shelf life and product with good resilience potential against harsh conditions. In addition, percolation and better penetration to end users can be achieved by the support of policymakers who by fastening the registration process can help industries in scaling up the process.

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