Chapter 19
Principles and Application of Confocal Microscopy to Understand Symbiotic Fungi

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Abstract *Piriformospora indica* is an endophytic fungus of the Sebacinaceae family that colonizes the roots of a variety of plant species. As a result of this plant fungus association, plant benefits with respect to nutrient acquisition, resistance against biotic and tolerance to abiotic stress. The fungal hyphae form chlamydospores after entering into the root cortex. Confocal microscope can be used for the detailed analysis of the fungal chlamydospores, its ultrastructure, morphology, sporulation, germination. Confocal microscopy captures the high resolution image of living as well as dead cells. This instrument helps to take three dimensional image of the objects as it eliminates out of focus glare by filtering the laser light along with confocal pinhole and excitation pinhole in front of detector, whereas in other microscopy techniques the entire sample is illuminated, including the area adjoining the area of interest which interferes with the analysis. The basic features which make confocal microscopy better than other microscopy techniques are removal of out of focus glare, shallow depth of field, optional sectioning, volume analysis, live cell imaging and lambda scanning. *P. indica* is grown and also the culture is maintained on Hill and Kaefer media at pH 6.5 and 30 °C. Batch as well as continuous fermentation can be used for the production of fungal biomass and spores.

19.1 Introduction

Fast and reliable *in situ* imaging of biological species remains a long-standing goal in photonics to understand dynamic procedures including complex multicellular organisms. The fluorescence imaging delivers point-by-point construction of images in a volumetric space and has been broadly used to monitor complex cellular events (Prabhat et al. 2004; Siddhanta et al. 2017). Additionally, recent advances in super-resolution microscopy have revealed a new opportunities to track

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molecular dynamics in unique detail (Fernandez-Suarez and Ting 2008). The recent advancement of confocal microscopy and its application to study the physiological and metabolic changes in microbes–microbes, plant–pathogen interactions have revolutionized research into the character of selected biomolecules and cell machineries in pathogen infection strategies, identifying special molecular mechanisms (gene expression) and plant defense responses. Confocal microscopy allows high-resolution visualization of a variety of fluorescent and fluorescently tagged molecules in both fixed and living cells, not only in single cells but also in intact tissues. Confocal microscopes greatly improve image quality by reducing interference by out-of-focus light and can capture high-resolution optical images through samples in the z-axis. In combination with a range of computational image analysis techniques, confocal microscopy provides a potent tool by which molecules, earlier detection and characterization of disease, molecular interactions, and cell components can be localized and studied (Massoud and Gambhir 2003; Hardham 2012).

Piriformospora indica is an endophytic fungus with a wide range of higher plants and provides multifaceted amenities such as nutrient uptake, disease resistance, abiotic and biotic stress tolerance and growth promotional including value additions (Varma et al. 2012; Prasad et al. 2008, 2013; Gill et al. 2016). The hyphae of the fungus can enter into the root cortex and form chlamydospores. After culturing of fungus on synthetic media, we achieved confocal microscopy to analyze the form and structure of the hyphae and chlamydospores (Siddhanta et al. 2017). The hyphae are straight and hyaline, and the surface of the hyphal walls is smooth. The chlamydospores are pear shaped and have smooth walls. *P. indica* biotrophic colonization pattern can be accompanied by abroad-spectrum suppression of root innate immunity (Qiang et al. 2011). In the support of the large host range of *P. indica*, molecular and genetic analyses revealed that plant roots, similar to leaves, are equipped with an effective innate immune system where immune suppression by *P. indica* was considered as a prerequisite for successful root colonization (Qiang et al. 2011; Gill et al. 2016).

19.2 Principle and Application of Confocal Microscopy

Microscopy is a very important tool to study microorganisms, the revolution came when Anton van Leeuwenhoek (1675) observed microbes using a handcrafted microscope. Since, then enormous modifications have been made in this technique ranging from simple light microscope to highly advanced confocal and super resolution microscopy. The first ever microscope was developed by Zacharias and Hans Janssen in 1590. Later in the year, 1667 Robert Hooke made many changes in the compound microscope and published the famous “Micrographia”. In the year, 1675 Anton von Leeuwenhoek developed a simple microscope to observe bacteria and protozoans. Although he was not the first person to develop a microscope, but his was the best of the period. In the twentieth century, the resolution limit of visible light was overcome by UV light microscope. In 1931,

Knoll and Ruska developed the first transmission electron microscope. Later, TEM was improved by Ruska to form first Scanning Electron microscope. In 1988, Marvin Minsky, developed the first confocal scanning microscope. Later, first commercialized confocal scanning microscope was developed by Czechoslovak Mojmir Petran of Charles University in Plzen developed the Tandem Scanning Microscope, which was the first ever commercialized confocal microscope. Time to time many changes were made in the concept of confocal microscope according to the need of the experiment.

The technique has made imaging of cellular interactions, ultrastructure and morphology of cells possible which can help the scientists for doing better research leading to a variety of agricultural and biotechnological advancements (Ahmad and Khan 2012).

Imaging is a very powerful tool to view filamentous microbes such as fungi where morphology is a very important aspect from industrial point of view as directly or indirectly it is related to fermentation, be it's hyphal structures or spores, microscope plays a very important role in fungal research (Czymmek et al. 1994). *P. indica* is a plant growth promoting mycorrhiza-like fungus which helps in alleviating stress conditions in plants. It has shown to combat various abiotic and biotic stress in different plants hence the study of this fungi at microscopic level is essential (Varma et al. 2012). With the help of various staining methods fungal structures can be observed in roots which can help in interpretation of results. Roots of plants/fungus can directly be observed under bright field microscope to get a general overview and this method still remains the standard for root colonization studies. In depth analysis will require sophisticated and advance microscopy techniques. Confocal laser microscopy is highly efficient technique for researchers interested in better imaging and analysis of cell structure and function. With the help of reliable data accurate interpretations can be done. The present book chapter aims to give a brief description about confocal microscope (Fig. 19.1a).

Marvin Minsky in 1988 desired to image neural networks in living brain which drove him to invent confocal microscope. The technique was patented in 1957. Three dimensional images of biological and non-biological samples is possible by this instrument as it eliminates out of focus glare by filtering the laser light along with confocal pinhole and excitation pinhole in front of detector. Advanced optics along with transverse resolution results in high quality images. The laser intensity is reduced due to the presence of pinhole, to overcome this lasers are coupled with optical fibers so that increase in number of excitation wavelengths can give a bright, clear and haze free image. A 3D image can be produced with the help of powerful softwares (Singh et al. 1998). Removal of out of focus glare, shallow depth of field, optical sectioning, volume analysis, fluorescence recovery after photobleaching, förster resonance energy transfer, live cell imaging, lambda scanning etc., are some of the basic features which make this above all the others conventional microscopic techniques. Cells weather live or dead need to be stained with specific fluorescent probes. In conventional microscopes the samples when hit with a light of specific wavelength emits fluorescence, apart from the region of interest other areas also gets illuminated which interferes with the resolution of the specimen. Frequent problem occurs with samples

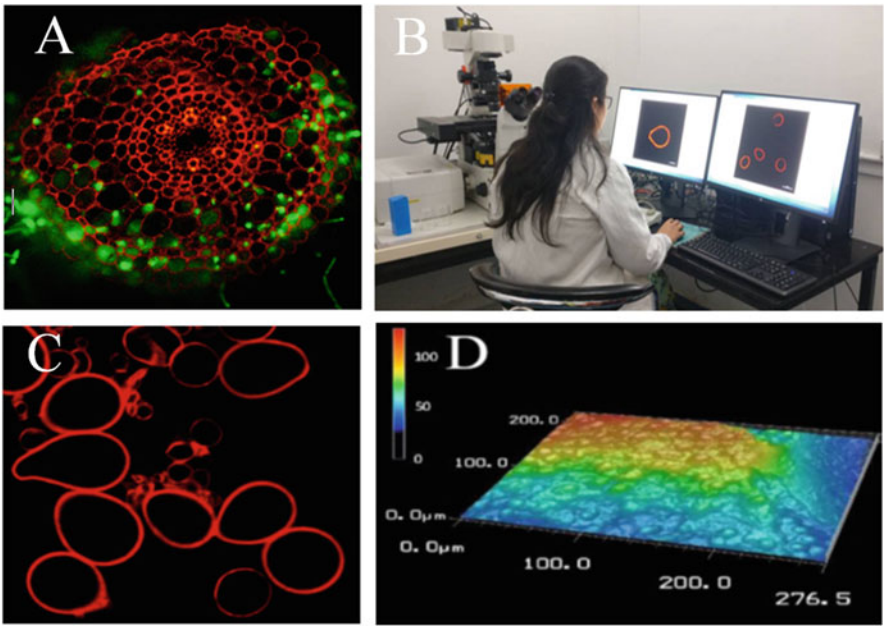

Fig. 19.1 (**a**) A confocal view of mycorrhized root, (**b**) Nikon Confocal A1, (**c**) *Piriformospora indica* spores viewed in Nikon Confocal A1 microscope, (**d**) CLSM image exhibiting the uneven and complex surface topology of a section of the fungal culture [reprinted with permission from reference Siddhanta et al. (2017), Copyright 2017 John Wiley & Sons]

having thickness >2 μm, secondary fluorescence emitted by the sample interferes with the resolution. Confocal laser scanning microscopes can solve this problem as the instrument has the ability to eliminate out of focus light and scanning done through lasers provides axial and lateral resolution thus giving a clear picture of the specimen. Talking about resolution and not mentioning electron microscope will be injustice to the most advanced microscopic techniques. In electron microscope the accelerated beam of electrons is used for acquiring image. Wavelength of electrons is much shorter than photons that's why the resolution is too high as compared to confocal and other conventional microscopy techniques. There is one major limitation with electron microscopy we can not observe live samples as we can do in confocal. This limits our studies only to dead samples whereas in confocal we can view the live samples for hours, even days with the help of time lapse and perfect focus system. We can say that confocal microscope has bridged the gap between the basic and advanced microscopy techniques (Fig 19.1b).

The question comes into mind how does confocal capture image which is sharp and haze free? In conventional microscope the whole sample is illuminated by light coming from a light source preferably a xenon or mercury lamp, this allows other areas adjacent to the region of interest to brighten up which interferes with the imaging of the interested point, we can directly see the image onto the eyepiece. However, in confocal microscopes the technique to image a sample is different. Here the laser source falls on the sample for illumination, it scans the specimen and

optical sections are produced. It is a non-invasive technique with which the focused light is used to section the specimen and instrument collect images in the form of optical sections. As the light source is laser we cannot see the specimen directly through eye piece, the signal produced by the sample is multiplied by photo multiplier tubes and the image can be viewed in computer (Amos and White 2003).

Now a days laser scanning confocal microscope is widely used in research, with modifications in the fluorescence microscope anyone can attain the benefits of this technique. It's simple design and user friendly approach has made it accessible to all. Sample preparation especially for confocal is not a problem as the protocol for both fluorescence and confocal is same. The difference lies that the instrument has laser as the light source which is coupled with the photomultiplier detectors, it multiplies the signal and a computer is attached to control all the scanning devices so that acquisition of the image can take place properly. Presence of pinhole is also a very important as it eliminates out of focus light. We can say that the presence of laser light source, pinhole and various dichoric mirrors, photomultiplier detectors along with the objective lenses are responsible for acquisition of a clear optical sectioned image which can produce a 3d view of the region of interest. All these features are not present in fluorescence microscope. After the acquisition of image it can be stored in computer and various types of analysis can be done with the help of various softwares.

Now going into functioning illumination as well as detection are restricted to a single diffraction limited point of the sample. Objective lenses which are available from 10× to 100×, depending on what magnification the image is to be acquired brings the point of illumination to focus in the sample. This is scanned by the scanner which is attached to the computer where the acquired image can be seen. The signals of sequence of the scanned image known as optical sections are detected by photo multiplier tubes through a pinhole. PMT's multiply the signals coming through pinhole and the output is displayed in the computer. The specimens are labeled with the fluorescent probes or dyes, when laser light falls on the labeled samples the photons get excited move to higher energy shells, while coming back to their ground state they emit fluorescence. This signal is captured to produce images (Shinya 2006).

Confocal microscopy is an advanced technique which is constantly used in mycological research. Ultrastructures, morphology, sporulation, germination and host pathogen interaction studies plays a very important role to give an insight to a whole new level of advanced research which can help in achieving great discoveries (Fig. 19.1c) (Singhal et al. 2017).

19.3 A Case Study: Confocal Microscope Used for Fungal Studies

Lagopodi et al. (2002) used confocal laser scanning microscopy (CLSM) to study the behavior of *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato root colonization (Fig. 19.2). *F. oxysporum* f. sp. *radicis-lycopersici* causes tomato

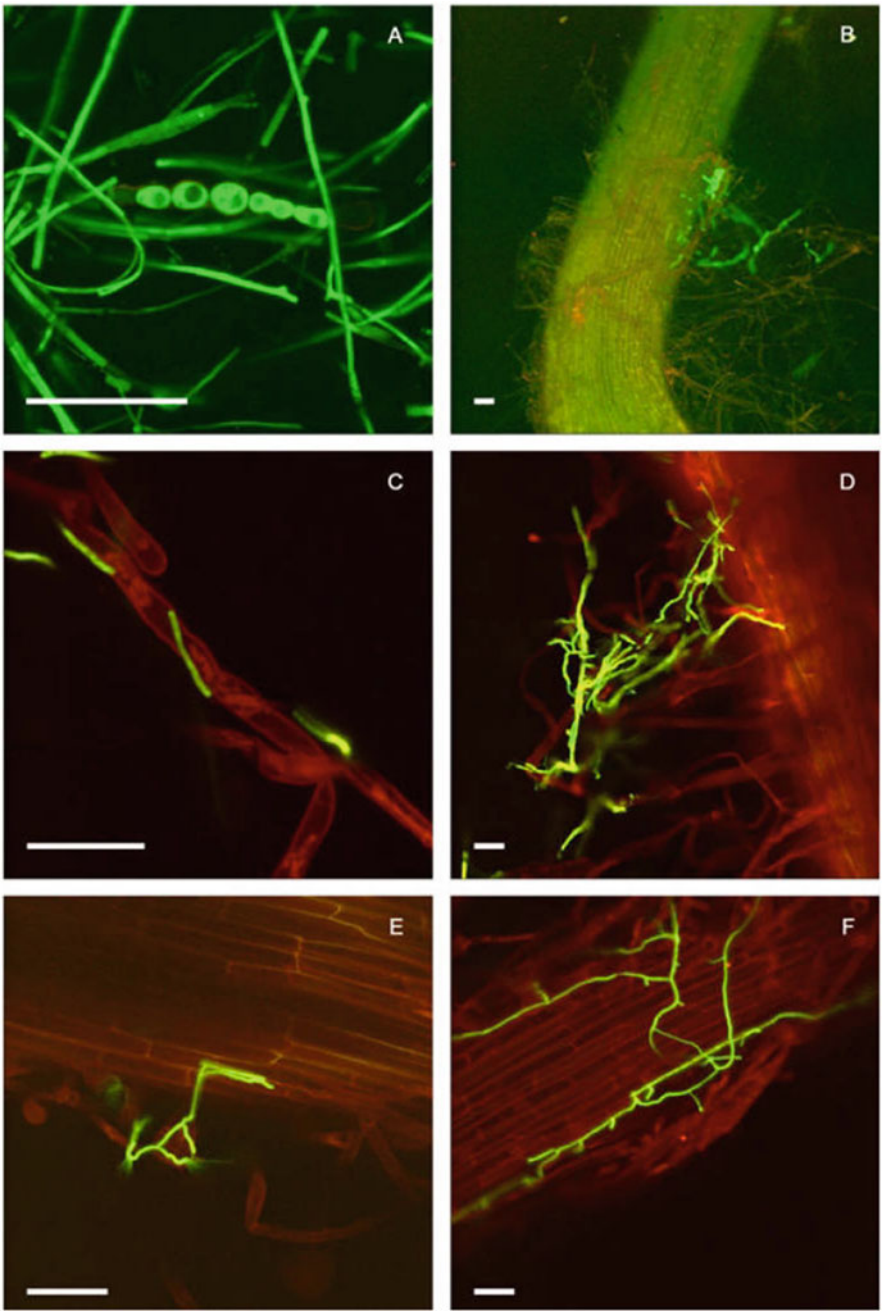

Fig. 19.2 Early stages of tomato root colonization by *Fusarium oxysporum f.* sp. *radicis-lycopersici* marked with gfp. Confocal scanning laser microscopy analyses of tomato roots grown after planting 2-day-old germinated sterile seedlings in sand containing spores *of*

foot and root rot disease. *F. oxysporum* is a soil fungus and is difficult to control. Green fluorescent protein from the jellyfish *Aequorea victoria* was used to label *F. oxysporum* f. sp. *radicis-lycopersici* in order to observe its presence and developmental stages in tomato. GFP's fluorescence is stable and does not depend on species. It also does not requires any substrate or cofactors for its reactions.

19.4 Plant and Microbe Interaction with Reference to *P. indica*

P. indica is a mycorrhiza like axenically cultivable plant growth-promoting root endophyte. It represents the order Sebacinales which is the elementary basidiomyceteous order projecting mycorrhizal capabilities (Matheny et al. 2007; Weiss et al. 2004, 2011). Further, *P. indica* which was formerly isolated from Thar Desert (Verma et al. 1998) is a biotroph and a model organism for investigational studies. *P. indica* is placed as a member of the Basidiomycetes order Sebacinales by the molecular phylogenetic analysis (Hibbett et al. 2007; Qiang et al. 2012; Weiss et al. 2004). The partial 18S rDNA sequence analysis placed *P. indica* in Basidiomycota close to the *Rhizoctonia solani* group (Varma et al. 2013a, b). A maximum likelihood analysis of 18S rDNA sequence confirmed these postulations. Further according to their similarities to Zygomycetes, *P. indica* is termed as an AM-like fungus (Franken et al. 2000). Leading further to the morphological traits of the fungus, *P. indica* has white to almost hyaline hyphae. The hyphae are thin walled and have a diametric range of 0.7–3.5 μm, irregularly septate and often exhibit anastomosis. The highly interwoven hyphae appear as intermingled cords and branch irregularly. External deposits, polysaccharides or hydrophobic proteins can be noticed on hyphal walls at regular intervals. The irregular septation of hyphae accounts for the presence of more than one nuclei in a single compartment. The distinct chlamydospores appear singly or in clusters. Initially the chlamydospores are thin walled and hyaline while they become thick walled towards maturity. Further no sexual structures or clamp connections were observed (Varma et al. 2001). The mycelium has a sub-surfaced and concentric growth on agar medium. When grown on solid culture media very few aerial hyphae were formed. Occasionally the mycelium fabricates periodic rings on agar medium, whereas the

Fig. 19.2 (continued) F. oxysporum f. sp. *radicis-lycopersici*. (**a**) Uniform expression of gfp in hyphae and chlamydospores of the transformed fungus grown on potato dextrose agar. (**b**) Fungal hyphae in contact with tomato root hairs, 2 days after inoculation. (**c**) Attachment of fungal hyphae to tomato root hairs, 2 days after inoculation. (**d**) Intermingling of hyphae with root hairs at the crown region, 3 days after inoculation. (**e**) Attachment of hyphae to the root surface and settling in the grooves between epidermal cells, 3 days after inoculation. (**f**) Colonization of the root surface by hyphae that are growing at the junctions of the epidermal cells, 3 days after inoculation. (**a–f**) *Scale bar* 50 μm (c.f. Lagopodi et al. 2002)

structure of the mycelium was homogenous. The morphological characters of the mycelium greatly differ with variations in conditions of cultivation or nutrient compositions of the culture medium. Readers are advised to see the article published by Weiß et al. (2016) have proposed the name Serendipitaceae for the family Sebacina and within it the genus Serendipita and have placed *Piriformospora indica* and *P. williamsii* (Weiß et al. 2016).

P. indica displays an endophytic lifestyle and have known to show symbiotic associations with majority of the terrestrial plants. It expansively colonizes the root hair zones inter and intracellularly while excluding the elongation and meristematic zones (Deshmukh et al. 2006). This pattern of colonization demarcates *P. indica* from ecto as well as arbuscular mycorrhizal fungi (AMF), which exclusively grow intercellularly or principally colonize the deeper cortex layers of younger roots (Smith and Read 2008). Symbiotic relationship of *P. indica* with various taxonomically unrelated hosts increases plant growth and biomass (Peškan-Berghöfer et al. 2004; Waller et al. 2005; Shahollari et al. 2007; Sherameti et al. 2008; Camehl et al. 2010, 2011; Hilbert et al. 2012; Nongbri et al. 2012; Lahrmann et al. 2013; Venus and Oelmüller 2013), higher seed yield, early flowering, and biotic and abiotic stress tolerance responses (Baltruschat et al. 2008; Singh et al. 2011). It has been reported that mutualistic associations of this fungus stimulates increased allocation of nutrients like phosphate to the plant roots (Yadav et al. 2010).

Since *P. indica* can be easily maintained and axenically cultured, it positions as an ideal models for beneficial fungus–plant interactions studies and has a promising perspective for application in sustainable horticulture and agriculture (Waller et al. 2005; Godoy et al. 2000; Kong et al. 2001; Ruan et al. 2011; Trivedi et al. 2012). Exploiting these plant benefitting properties, a formulation of *P. indica* with magnesium sulphite was prepared where magnesium sulphite acts as a carrier. For this, 2% (w/w) of fungal biomass served as effective and stable formulation. On an average the colony forming unit (CFU) count was maintained as 10^9 and moisture content was 20%. Application of this formulation on plants presented enhanced overall growth and resistance to biotic and abiotic stress.

19.4.1 Application of Piriformospora indica on Isabgol: A Case Study

Isabgol (*Plantago ovata*) are annual plant species that majorly grow in the arid and semi-arid regions and are extensively used in conventional and modern pharmacology (Patel et al. 1996). The seeds of blond psyllium are mainly valued for mucilaginous rosy white husk. The mucilage comprises of reserve carbohydrates mainly pantosans. The husk is commonly used for getting relief from constipation as per being a dietary fiber supplement acting as a bulk-forming laxative. It releases constipation through mechanically stimulating the intestinal peristalsis.

The seeds Isabgol (*Plantago ovata*) were treated with formulation of the AM fungi *P. indica* to study the effect on the growth and development of plant species. Nursery trails were conducted based on the season in the month of November. On application of *P. indica*, it was observed that the overall growth of the plant was promoted. The mean yield in Isabgol seed and husk respectively, increased to 57 and 33% g in *P. indica* treated seeds. There also was observed an early flowering in case of *P. indica* treated seeds.

19.5 Cultivation of *P. indica*

19.5.1 Culture Maintenance and Inoculum Preparation of P. indica

The culture of *P. indica* was maintained on Hill and Kaefer medium plates supplemented with 15 g/L agar. Plates were incubated at 30 ± 1 °C for 10 days and then stored at 4 °C (Prasad et al. 2005). For the preparation of inoculum, *P. indica* was initially grown on Kaefer medium in a petri dish and then transferred to the seed culture medium by punching out 8 mm of the agar plate culture with a sterilized cork-borer. The seed culture was grown in a 500 mL Erlenmeyer flask containing 100 mL potato dextrose broth at 30 ± 1 °C on a rotary shaker at 200 rpm for 4 days.

19.5.2 Cultivation of P. indica in Batch Culture

Batch culture is closed bacterial culture system with specific nutrient, temperature, pressure, aeration and other environmental conditions to optimise growth (Wilson 1995). Because nutrients are neither added, nor waste products removed during incubation, batch cultures can only complete a limited number of life cycles before nutrients are consumed and growth stops. In other words batch culture is a technique for large scale production of microbes or microbial products in which, at a given time, the fermenter is stopped and the culture is worked up. Cells, or products that the organisms have made, can then be harvested from the culture.

P. indica can be cultivated on Hill and Kaefer media under the optimized cultural conditions (inoculum size: 5%; agitation speed: 200 rpm; working volume: 50%; initial pH: 6.5; temperature: 30 °C) in 500 mL flask. The 500 mL flask containing 250 mL media was inoculated with 5% inoculum which was grown in a 500 mL flask containing 100 mL potato dextrose broth at 30 ± 1 °C on a rotary shaker at 200 rpm for 4 days. Now, keep this flask on rotary shaker at 200 rpm for 10 days (Fig. 19.3). In this method of cultivation maximum dry cell weight is

Fig. 19.3 (**a**) Typical view of a fermenter; (**b**) *P. indica* culture in fermenter

obtained after 5 days, the sporulation starts after 6 days of growth, and maximum spore yield is obtained after 8 days.

19.5.3 Cultivation of P. indica in Bioreactor

Continuous fermentation is a technique for production of microbes or microbial products in which nutrients are continuously supplied to the fermenter. The continuous culture of micro-organisms is a technique of increasing importance in microbiology. The essential feature of this technique is that microbial growth in a continuous culture takes place under steady-state conditions; that is, growth occurs at a constant rate and in a constant environment. Such factors as pH value, concentrations of nutrients, metabolic products and oxygen, which inevitably change during the 'growth cycle' of a batch culture, are all maintained constant in a continuous culture; moreover, they may be independently controlled by the experimenter. These features of the continuous culture technique make it a valuable research tool, while it offers many advantages, in the form of more economical production techniques, to the industrial microbiologist.

P. indica was cultivated in cylindrical stirred tank bioreactor of different capacity i.e. 3, 7 and 10 L (Fig. 19.3a) with a working volume of 50%. An agitator shaft with three Rushton flat blade turbine impellers was used for stirring. Each impeller consisted of six blades and the distance between two impellers was 8 cm (Khan et al. 2011). The orifice sparger was used for sparging air into fermentation broth. For all the bioreactor Bagde et al. (2010) experiments an active inoculum of 2% grown on potato dextrose broth for 4 days was used (Fig 19.3b).

19.5.3.1 Fermenter Sterilization

The fermenter vessel was filled with production media. Then the head plate consisting of the various probes and agitator module was fixed. The opening

pores like the sampling port and other opening pipes of the acid, alkali and antifoam supply was covered with cotton plugs. Finally the whole fermenter vessel along with the media inside was placed inside an autoclave and sterilized. The acid and the alkali were also autoclaved.

Spore count of inoculum used in above methods: The spore suspension was used as inoculum for fermentation (Kawaide 2006). The spore suspension was prepared by adding 10 mL of sterilized distilled water to the culture plates and then gently scraping the spores with the inoculation needle. Aqueous spore suspension consisting about 6×10^7 spores/mL was used (spores counted on Haemocytometer). The spore suspension was then transferred to another sterile empty test tube. Generally, 25 mL of the spore suspension was then inoculated into the fermenter vessel containing 3000 mL of the culture media.

However, when *P. indica* was grown in a 14-l bioreactor (Chemap AG, Switzerland) using Hill–Käfer medium maximum, dry cell weight was obtained after 42 h of growth, the fungus-initiated sporulation after 48 h, and a spore yield of 9.25×10^7 spores/mL was achieved after 60 h of growth. The early sporulation in this case may be due to rapid consumption of glucose. Owing to more efficient mixing and homogenized fungal suspension, the growth of fungus was faster in the bioreactor and resulted in early depletion of the carbon source and thereby in the early sporulation compared with the batch culture in shake flasks.

19.6 Conclusion

Fermentation techniques are employed for increased production of *P. indica*, which can be commercially used for the enhancement of nutrient uptake by different plants or help them to sustain through different stress conditions. To establish different experiments for confirmed results for these conclusions, confocal microscopy has helped a lot along with other techniques. This microscopy technique helps us to have a detailed knowledge about the ultrastructure of the fungal hyphae, its spores, sporulation, it's germination and host pathogen interaction studies. It removes the background image disturbances which gives a clear image of the area of interest.

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