Title: A NOVEL BIOREACTOR FOR MASS PRODUCTION OF ARBUSCULAR MYCORRHALIZAL FUNGI

Abstract: The present invention provides a novel bioreactor to maximize aseptic mass production and up scaling of AM fungi by cultivating the fungal spores and roots in a transformed hairy root of plant. The bioreactor includes a steel or glass, misting forming device, a special type of inoculation assembly, culture holding mesh and mist controlling system wherein liquid nutrient medium steadily floods in a form of mist inside the vessel and then drains out and recycles at regular intervals. Present invention provides novel, improved systems/apparatus for the growth and cultivation of AM Fungi affording efficient oxygen, nutrient distribution capacity throughout the reactor vessel, and provides improved oxygen, nutrient supply at low shear to prevent damage of shear-sensitive tissues of transformed hairy root to produce AM fungal spores which are contamination free, simple, inexpensive and effective using the novel bioreactor.

Figure 11
SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.


Declarations under Rule 4.17:
- as to applicant’s entitlement to apply for and be granted a patent (Rule 4.17(ii))
- of inventorship (Rule 4.17(iv))

Published:
- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
A NOVEL BIOREACTION FOR MASS PRODUCTION OF ARBUSCULAR MYCORRHIZAL FUNGI

[0001] The present invention relates to a novel gas-phase (mist) bioreactor for the in vitro production of arbuscular mycorrhizal fungi (AMF’s), especially cultivating the fungal spores and roots using transformed plant roots in a limited aseptic space and the bioprocess using the said developed bioreactor where liquid nutrient medium steadily dispersed in a form of nutrient mist and drains out after condensation and precipitation and recycles at a regular intervals.

BACKGROUND OF THE INVENTION

[0002] Arbuscular mycorrhizal fungi (AMF) are beneficial symbiotic fungi in that they colonize the cells of roots of plants and stimulate absorption of essential plant nutrients. Mycorrhizal fungi is proved to be a kind of microbial fertilizers due to their stimulating actions on plant growth which is confirmed by many research reports.

[0003] Other than the essential nutrient uptake the actions include: (1) promoting resistance of infected plants to pathogens; (2) enhancing capability of drought resistance; (3) suppressing absorption of noxious elements; and (4) connecting with other plants via hyphae.

[0004] Mycorrhiza can be applicable to a wide range of plants from floricultural, horticultural, silvicultural, to agricultural species including legumes, tree and plant species, except a few like mustard (Brassica sp.) and Sugar beet (Beta vulgaris). Finished product has easy storage at ambient temperature and prolonged shelf life at room temperature.

[0005] However, despite the voluminous work carried out to date, the main difficulty in applying AMF’s is that, it is not known how to produce their propagules in large quantities in reactors or under fermentation for commercial application. It is believed that no one has ever succeeded in cultivating them in a sterile medium without the host plant despite the large amount of domestic and overseas researchers carried out to date.
The most difficult reason to apply AMF as one of microorganism fertilizers is no cheaper and more efficient method that is found now to mass-produce AMF inoculums of a high quality without any adulterated microbial contaminants for commercial applications. Therefore, the price is still high enough to affect the farmer willing of adopting the above-mentioned fertilizer. It will be very helpful for generalizing this excellent microbial fertilizer to know in advance how to lower the cost of using AMF inoculums.


[0007] 1. Potted cultivating method carried out with traditional potted plants having AMF inoculation which is easily understood from the previous description is first preparing some mycorrhizae or spores of AMF as inoculums to be inoculated into the radicle of a host plant cultivated in pots or a greenhouse and then cultivating AMF therein. However, the time required for cultivation of AMF is quite long and the product is unexpectable due to the possibility of introducing contaminants in the soil at the same time.

[0008] 2. Hydroponic cultivating method is to cultivate symbiotic host plants inoculated with AMF in a specialized hydroponic device with nutrient liquid in which the host plants and AMF inoculums therein are submerged to enhance the growth of mycorrhizae and sporulation of AMF. However, though efficiency of AMF production for this method is better than the method used for potted plants, frequently refreshing the nutrient liquid is needed to prevent from serious problem of adulterated microbial contaminants. And the soaked state of the host plants and AMF therein caused by aquatic environment in this method is not the normally and naturally growth condition for AMF which results in the quantity of AMF sporulation failing to increase.
3. Aeroponic cultivating method is to cultivating symbiotic host plants inoculated with AMF in a specialized aeroponic container where vaporized nutrient liquid is provided to the plants and AMF therein. Cultivating large quantities of AMF propagules becomes possible even in the soilless situation. But the deficiencies of this method comprise the need of building a specific aeroponic container, the requirement of huge amount of vaporized nutrient liquid with necessary work to watch and refresh them, and failure to avoid the problem of adulterated microbial and pathogen contaminants.

4. Transformed root organ cultivating method uses isolated plant roots genetically transformed by the Ri plasmid of *Agrobacterium rhizogenes* (Tepfer, 1984, *Cell*, Vol. 37, 959-967) to be able to grow rapidly and independently and be inoculated with pure AMF to become a symbiotic root system (Mugnier and Mosse, 1987, *Phytopathology*, Vol. 77, 1045-1050). It is an advanced cultivating method to acquire AMF propagules without adulterated microbial contaminants in specialized cultivating circumstances after pure AMF are inoculated into the transformed root organ.

Comparing to the potted, hydroponic and aeroponic cultivating methods are that all practice the solid type cultivating using the complete plant root system; the adoption of solidified medium is not integrally efficient enough and needs much more procedures in the regaining process by first liquefying the solidified medium and then filtering the liquefied solution to make final products. It costs high for mass production and cannot proceed on a large scale.

The most advanced method is Transformed root organ cultivation. This method uses isolated plant roots genetically transformed by the Ri plasmid of *Agrobacterium rhizogenes* (Tepfer, 1984, *Cell*, Vol. 37, 959-967) to be able to grow rapidly and independently and be inoculated with pure AMF to become a symbiotic root system (Mugnier and Mosse, 1987, *Phytopathology*, Vol. 77, 1045-1050). It is an advanced cultivating method to acquire AMF propagules without adulterated microbial contaminants in specialized cultivating circumstances after pure AMF are inoculated into the transformed root organ.

[0013] People are attempting to cultivate mycorrhiza through Transformed root organ cultivation method using various types of growth chamber as this is the most advanced method by which contamination free high quality can be maintained. The transformed root organ cultivating method can use liquid (solution) medium to cultivate the root system too. Three types of reactor/growth chambers have been described including the submerged style, rotating drum, and the airlift style as follows:

[0014] The submerged style indicates cultivating transformed root organs in shallow liquid medium by still placed cultivation (Nuutila et al., 1995, Plant Cell Rep., Vol. 14, 505-509). In practice, the depth of the liquid nutrient medium cannot be large in order to prevent the root organs from the asphyxiant counteraction that is not good for growth. Therefore the submerged style is unable to contribute to the incremental reproduction of AMF propagules on a mass-produced scale.

[0015] Rotating drum, also called the vibrating style, indicates cultivating the root organs in a predetermined quantity of the liquid medium and adding the amount of dissolved oxygen in the solution by spinning stir or vibration. The stress caused by spinning stir and vibration will inhibit the growth of root organs and decrease the incremental reproduction rate of AMF at the same time.

[0016] The airlift style indicates cultivating root organs in a container full of liquid nutrient medium and releasing bubbles continuously from the bottom of the container to facilitate breathing of root organs (Jolicoeur et al., 1999, Biotechnology and Bioengineering, Vol. 63, No. 2, 224-232). The method which obviously overcomes the asphyxiant problem met in the submerged style and is free from the excess mechanical
stress arising in the rotating drum style is a more feasible way in all methods adopting liquid medium. But sterile air should be injected persistently into the container, and the injection process usually costs high and allows for adulterated microbial contamination to occur. Meanwhile, both of the root organs and AMF therein are submerged continuously in the liquid medium during the process, and the liquid-full container is not a normal growth circumstance to the root organ and AMF though a large quantity of dissolved oxygen is obtainable in the container by injected air. Therefore the unit efficiency is lower than the submerged style.

[0017] Wang in US Patent No. 6759232 issued on July 6, 2004 described the method using one cultivation tank for mycorrhizal growth. The method has three steps including inoculation, providing the whole symbiotic root organs and AMF propagules with liquid medium for a temporary contact and in third step removing the liquid medium from symbiotic root organs and AM fungal propagules after cultivated by the liquid medium. However, in this method step 1 to step 3 needs to be repeated periodically to facilitate the mass production and sporulation of the arbuscular mycorrhizal fungi. Cultivation in large scale with such periodic repetition of above mentioned steps are difficult. Fortin et al in US Patent No. 5554530 issued on Sep 10, 1996 tried to grow endomycorrhizal spore cultivating in a two-compartment Petri dish and in small bioreactor. However, large scale cultivation was the problem and exchanges of gases are also limits the growth in solid media.

[0018] Weathers et al in US Patent No. 8114664 issued on Jan 15, 2009 described about flexible wall bioreactor for growth of hairy root. But in such flexible bioreactor contamination of bacteria and fungi were observed as growth cycle of in vitro hairy root culture was long.

[0019] Schmidt et al in US Patent application No. US2009/0023194A1 issued on Jan 22, 2009 described the advantages of use of ultrasonic mist generator for growth of biological material. The advantage of use of media in form of mist droplet consumes less nutrient medium than conventional cultivation methods. Furthermore,
contamination of the culture medium with bacteria is minimized by the exposure to medium vapor because they are damaged or even filled during the nebulization. [0020] Declerck et al in Patent application No. PCT/EP2009/050434 dated 15 Jan 2009 describes about a semi-hydroponics bioreactor to grow pre-mycorrhizal plant. In the proposed bioreactor the whole pre-mycorrhizal plant needs to transfer to plastic tubular hydroponics bioreactor. Aseptic transferred of whole plant into such reactor is a question and maintaining sterility for longer duration in such system is a problem. [0021] In conclusion, the conventional difficulty of keeping low cost compatible with high quality of final products is always a check point of cultivating AMF in vitro. Higher quality can be maintained by transformed root organ which is the main worldwide struggling development. But the generalized range is limited due to the high cost of this method. [0022] Therefore, how to overcome the problems met in the symbiotic mass cultivation of transformed root organs and AMF in order to lower cost, promote their production and maintain the steady quality of AMF species is in bad need and will become the main technological part to generalize this benefit microbial fertilizer. [0023] Different designs of bioreactors have been attempted for the commercial production of secondary metabolites from hair root culture with limited success but there is limitation of mass production of mycorrhizal fungi in pilot scale bioreactor. In the present invention a novel Mist-Bioreactor has been designed for successful growth of mycorrhizal associated hairy root culture.

OBJECTIVE OF THE INVENTION

[0024] The main objective of the invention is to provide a novel bioreactor to maximize aseptic mass production and up scaling of AM fungi by cultivating the fungal spores and propagules in a transformed hairy root of plant. The bioreactor includes a sterilizable vessel made up of stainless steel or glass, misting forming device and mist controlling system, with an option of a special type of inoculation assembly, basket to grow culture and culture holding mesh.
[0025] Another objective of the present invention is to provide novel and improved systems/apparatus for the growth and cultivation of AM Fungi affording efficient oxygen and nutrient distribution capacity throughout the reactor vessel, superior to conventional fermentors or bioreactors.

[0026] Another objective of the present invention is to provide improved oxygen and nutrient supply at low shear to prevent damage of shear-sensitive tissues of transformed hairy root to produce AM fungal propagules which would be contamination free, simple, inexpensive and effective using the novel pilot scale bioreactor.

[0027] These and other objectives, features and advantages of the invention will be seen from the following description and accompanying figures.

**SUMMARY OF THE INVENTION**

[0028] The present invention relates to a novel gas-phase (mist) bioreactor for in vitro aseptic mass production and up scaling of arbuscular mycorrhizal (AM) fungi by cultivating the fungal spores in a transformed hairy root of plant in a limited aseptic space and the bioprocess using the bioreactor comprising of: sterilizable vessel made of stainless steel or glass; mist forming device; mist controlling system, and with an option of a special type of inoculation assembly, basket to grow culture and culture holding mesh,

wherein liquid nutrient medium steadily floods in a form of mist inside the vessel and then drains out and recycles at a regular intervals.

[0029] One embodiment of the present invention is that gas-phase (mist) bioreactor is in-situ sterilizable stainless bioreactor having the capacity of the reactor as 25 litres and having all parts of the bioreactor made of SS316 which are in contact with the culture or media.

[0030] Another embodiment of the present invention is that the vessel made of glass is sterilized by using autoclave.
[0031] One embodiment of the present invention is that the capacity of glass bioreactor is 7 litres.

[0032] Further embodiment of the present invention includes that the in-situ sterilizable stainless bioreactor comprises of insulated vessel made of stainless steel SS316 with 240 Grit internal and 180 Grit the external surface finishing and has spiral jacket made of SS304, having the H/D ratio of the vessel 2:1 and the top plate of the vessel is made of SS316 and is bolted to the shell through Rothman Clamps and bottom dish is welded to the shell.

[0033] In another embodiment the H/D ratio of the glass vessel is 3:1.

[0034] Further embodiment of the present invention is that the working temperature range of the vessel is 110°-140°C and working pressure is 0.5-3.0 bar.

[0035] Another embodiment of the present invention is that the glass bioreactor may be operated in normal atmospheric pressure.

[0036] Another embodiment of the present invention is that the top plate of the stainless steel vessel consists of 10 ports.

[0037] One of the embodiments of the present invention is that the bottom part of the stainless steel vessel consists of drain valve and harvesting/sampling port and pH, DO, and temperature probes are attached to it. Further, pH probe is connected to pH transmitter and can be set through programmable logic controller (PLC); 25 mm DO probe is connected with DO Transmitter; CO₂ and ethylene gas analyser are connected to the exhaust line of the vessel.
[0038] Another embodiment of the present invention is that in the mist bioreactor system, one peristaltic pump is connected with media line to form mist inside the reactor and other extra pump is used for cleaning of vessel.

[0039] The other embodiment of the present invention includes that the mycorrhizal fungi may be grown in a basket or may be in a funnel shape single conical structure instrument.

[0040] Another embodiment of the present invention includes an inoculation assembly made up of stainless steel SS316, the assembly is closed cylindrical in shape with a flip disk is attached inside the assembly and the assembly can be fitted with the Triclover Clamp (TC) end of the top plate of bioreactor and can be sterilized separately after removing from the reactor to transfer culture into pre sterilized assembly.

[0041] The embodiment of the present invention includes the mist forming device, which consists of an atomizing spray nozzle or ultrasonic mister.

[0042] The preferred embodiment of the present invention includes that Atomizing spray nozzle is attached to side wall of the vessel. The nozzle may be attached to top plate of the reactor. The spray nozzle is consists of air and media line to generate mist inside the reactor vessel.

[0043] Another embodiment of the present invention consists of an Ultrasonic Mister, which may be used instead of atomizing nozzle. In the Stainless Steel Mist-Bioreactor the Mister device may be fitted with a special triclover clamp (TC) joint on the top plate of the bioreactor. The mister is capable of providing a volumetric throughput of 3-4 L min⁻¹ and of providing droplets having a diameter of approximately 15-20 μm. This droplet size may vary between 5 μm and 25 μm.
[0044] In another embodiment the air atomizing Misting device in Glass Mist-Bioreactor is fitted with a special arrangement inside the bottom of the bioreactor as per design described in the detailed description wherein air atomizing Misting system can be capable of providing a volumetric throughput of 0.1-2.0 ml min\(^{-1}\) and of providing droplets having a diameter of between 5 μm and 25 μm.

[0045] On embodiment of the present invention is that the said mister includes a pulsed output having a duty cycle and the mist is in form of droplets.

[0046] Another embodiment of the present invention includes that the total bioprocess is automatic operable type with process control by PLC, which has provision to set all the parameters in the desired process limit and also to display and record the data by Supervisory Control and Data Acquisition (SCADA).

[0047] One of the preferred embodiment of the present invention is that the said gas includes ambient air, ethylene, carbon dioxide or other gases.

[0048] The other embodiment of the present invention includes a bioprocess for aseptic mass production and up scaling of arbuscular mycorrhizal fungi (AMF) using the gas-phase (mist) bioreactor of the present invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

[0049] These and other features, aspects, and advantages of the present invention will become better understood with regard to the following description, appended claims, and accompanying drawings. The drawings include the following figures:

[0050] Figure 1. Drawing of the 25 Litre stainless Steel Nutrient Mist Bioreactor (Outer part).
[0051] Figure 2. Drawing of the 25 Liter stainless steel Nutrient Mist Bioreactor showing inoculation plates inside vessel.

[0052] Figure 3. Design of the top plate of 25 Liter stainless steel Nutrient Mist Bioreactor.

[0053] Figure 4. Design of lamp holder with connection detail attached with Vessel in stainless steel Nutrient Mist Bioreactor.

[0054] Figure 5 A and B. Design of culture growing plate inside stainless steel Nutrient Mist-Bioreactor. Figure 6. Design photograph of culture growing plate inside stainless steel Nutrient Mist-Bioreactor.

[0055] Figure 7. Inoculation assembly of stainless steel Nutrient Mist-Bioreactor (Outer design)

[0056] Figure 8. Inoculation assembly of stainless steel Nutrient Mist-Bioreactor (Internal design). Figure 9. Exploded view of Spray Nozzle system: 1-Retainer ring, 2- Air cap, 3-Fluid cap, 4-Gasket, 5-Spring, 6-Stainless steel ball, 7-Body, 8-Stainer gasket, 9-Stainer screen, 10-Stainer body.

[0057] Figure 10. Internal design detail of the Ultrasonic Mist forming assembly with fitment.

[0058] Figure 11. Bioprocess diagram of the operation of pilot scale stainless steel Nutrient Mist bioreactor for production of AM fungi

[0059] Figure 12. Growth of AM fungal hairy root in 25 Litre stainless steel Nutrient Mist-Bioreactor.

[0060] Figure 13 Glass Nutrient Mist Bioreactor with air atomizing system for Mycorrhizal fungal growth.

[0061] Figure 14. Air atomizing system for Glass Nutrient Mist Bioreactor (Exploded view).

DETAILED DESCRIPTION OF THE INVENTION
[0062] In the present invention a novel gas-phase (mist) bioreactor is described that reduces gas exchange limitations, does not oxygen limit the growth of arbuscular mycorrhizal (AM) fungal associated hairy root culture. The bioreactor may be in-situ sterilizable made of stainless steel material or made of glass, which is autoclavable. The bioreactor is capable of cultivating the AM fungal spores by using root organs of symbiotic host plant in a limited aseptic space using bioreactor where liquid nutrient medium steadily floods in a form of mist inside the vessel and then drains out and recycles at regular intervals. The description of the bioreactor is mentioned below capable for production of in vitro aseptic mass production of arbuscular mycorrhizal fungi (AMF).

[0063] The present invention relates to gas-phase (mist) bioreactor for in vitro aseptic mass production of arbuscular mycorrhizal (AM) fungal spores and roots in a transformed hairy root of plant in a limited aseptic space comprising of-

a) a sterilizable vessel made of stainless steel or glass;

b) mist forming device;

c) mist controlling system, and

d) with an option of a special type of inoculation assembly, basket to grow culture and culture holding mesh,

wherein liquid nutrient medium steadily floods in a form of mist inside the vessel and then drains out after condensation and recycles at a regular intervals.

**Description of the Gas-Phase (mist) Bioreactor**

[0064] **Vessel.** The bioreactor vessel is a sterilizable vessel made of stainless steel or glass.

[0065] The stainless steel vessel is in-situ sterilizable. The capacity of the reactor may be 25 Litre. The vessel is made of stainless steel SS316 with spiral jacket with insulation. The H/D ratio of the vessel is 2:1. Design of the said mist-bioreactor vessel is shown in Figure 1 & 2.
[0066] The other form of bioreactor vessel is made of glass. The capacity of the reactor may be 7 Litre with the H/D ratio of the vessel may be is 3:1. The vessel made of glass is autoclavable. Design of the said mist-bioreactor vessel is shown in Figure 13.

**Design parameters**

[0067] The working temperature of the stainless steel vessel is 110-140 Deg C and working pressure range is 0.5-3.0 bar.

**Material of Construction**

[0068] In Stainless Steel Mist-Bioreactor all parts in contact with the culture are made of SS 316. The jacket with spiral is made of SS 304. All the ‘O’ rings were made of ethylene propylene diene terpolymer (EPDM), silicone. The internal surface finishing of the vessel is 240 Grit and the external is 180 Grit.

**Description of the top plate**

[0069] The top plate of the stainless steel vessel is made of SS316 and is bolted to the shell through Rothman Clamps. The bottom dish is welded to the shell. The top plate of the Stainless Steel Mist Bioreactor consists of 10 ports as follows:

Two (2) numbers of inoculation ports extended inside the vessel with SS pipe upto the inoculation Teflon culture disk (Figure 3, P1, P2), 1 No of exhaust port (Figure 3, P3), 1 No of view glass (Figure 3, P4), 1 No sterile pressure gauge (Figure 3, P5), 1 no of safety valve (Figure 3, P6), 1 No for CIP cleaning (Figure 3, P7), 1 No for steam inlet (Figure 3, P8), 1 No of central port (Figure 3, P9) is to attach rotated shaft for culture holder, 1 additional port (Figure 3, P10) for other use. The top plate design drawing is mentioned in Figure 3. One Lamp Holder is attached with Top Plate (Figure 4). One illumination lamp is connected with this lamp holder to observe the growth inside bioreactor.
[0070] In the other form of bioreactor wherein the vessel is made of glass the top plate of the vessel is also made of stainless steel SS 316. The top plate of the vessel is bolted to the glass vessel with Clamps.

Bottom part
[0071] Bottom part of stainless steel vessel consists of drain valve (Figure 1, N11) and harvesting/sampling port (Figure 1, N12).

Probes attached to the vessel
[0072] pH, DO and temperature probes are attached to the bottom part of the vessel. pH probe is connected to pH transmitter (pH range 0-14) and can be set through PLC. 25 mm DO probe is connected with DO Transmitter (Range 0-100%). CO₂ and ethylene gas analyser are connected to the exhaust line of the vessel. pH, temperature and DO Probes are shown in the reactor design (Figure 1) as N8, N9, N10 respectively.

Peristaltic pumps
[0073] One peristaltic pump (3-30 rpm) is connected with media line to form mist inside the reactor and other extra pump (30-300 rpm) is used for cleaning of vessel.

Basket to grow culture
[0074] In the Stainless Steel Mist-Bioreactor, a two tier rack made of perforated Teflon sheet fitted with SS supporting ring (SS 316) is fixed into the 25 L reactor vessel and can be removed easily for cleaning. The central stem of the rack (basket) is made of SS rod (SS 316) connected from the centre of the top plate using mechanical seal. The rack can be rotated by manually rotating the central rod of the rack from outside of the vessel. Diameter of upper and lower racks is 160 mm and 190 mm respectively. The distance between two racks is 190 mm. The perforated Teflon plate for growth of culture was mentioned in Figure 5A showing culture Plate No. 1 and Figure 5B is Plate No. 2.
[0075] Mycorrhizal fungi may be grown in a funnel shape single conical structure instrument also. The funnel shape may be prepared using SS mesh (SS 316) to grow AM Fungi. The diameter of the cone may be 160 mm and length 100 mm. Such type of conical shape culture holder can create anchor to the mycorrhizal hairy root with the soft wall mesh. The design photograph of such culture basket is shown in **Figure 6**.

[0076] Inside the Glass Mist-Bioreactor a funnel shape single conical structure mesh is attached to grow mycorrhizal fungi. The funnel shape may be prepared using SS mesh. Such type of conical shape culture holder can create anchor to the mycorrhizal hairy root with the soft wall mesh (**Figure 13**).

**Inoculation assembly**

[0077] Inoculation assembly is made of stainless steel SS 316. Assembly is closed cylindrical in shape with a flip disk is attached inside the assembly. The assembly can be fitted with the TC end of the top plate of Bioreactor. Assembly can be sterilized separately after removing from the reactor to transfer culture into pre sterilized assembly. The design of the assembly is shown in **Figure 7**. The internal design of the inoculation assembly is shown in **Figure 8**.

[0078] The inoculation process in the Stainless Steel Mist-Bioreactor consists of two steps. Step-1 is inoculation to assembly and step-2 is attachment of assembly to main bioreactor for actual inoculation to vessel. The inoculation process is mentioned below.

[0079] AM fungal associated hairy root to be aseptically cut into small pieces inside laminar hood. Then culture is aseptically transferred to pre-sterilized inoculation assembly. Airflow is maintained at a range of 8-10 LPM into the vessel at the time of inoculation of culture to sterilized bioreactor. The inoculation assembly is attached aseptically to TC end of the reactor. Finally, the culture to be inoculated to bioreactor by flipping the assembly leaver.
Mist forming device

[0080] In the Stainless Steel Mist-Bioreactor atomizing spray nozzle is attached to side wall of the vessel. The nozzle may be attached to top plate of the reactor. The spray nozzle is made of brass and in combination of stainless steel SS 316. The spray nozzle is consists of air and media line to generate mist inside the reactor vessel. The attachment of atomizing spray nozzle is shown in Figure 1, N14, and Figure 9.

[0081] Ultrasonic Mister may be used instead of atomizing nozzle. The Mister device may be fitted with a special triclover clamp (TC) joint on the top plate of the bioreactor as per design (Figure 10). The assembly was designed with standard joint such a way that it can be fit with any other mist-bioreactor. The mister is capable of providing a volumetric throughput in a range of 3-4 L min⁻¹ and of providing droplets having a diameter of approximately 15-20 µm. This droplet size may vary between 5 µm and 25 µm. When independent air flow is required for growth such mister may be used. In such case mist formation is not dependent on air flow and in which smaller droplet size helps in better gas transfer for growth. The size of the droplets provided by mister should offer good aeration and control of the gas phase composition, reduces shear damage to the root bed and also reduce any chemical gradients within the bioreactor vessel during growth of the root bed on culture basket.

[0082] In case of Glass Mist-Bioreactor air atomizing misting device is fitted with a special arrangement inside the bottom of the bioreactor as per design (Figure 13). The assembly was designed with standard joint such a way that it can be fit with any other mist-bioreactor. The mister, which can be capable of providing a volumetric throughput of 0.10-0.2 ml min⁻¹ and of providing droplets having a diameter of approximately 5 µm. This droplet size may vary between 5 µm and 25 µm. The size of the droplets provided by mister along with air flow offer good aeration and control of the gas phase composition reduces shear damage to the root bed and also reduce any chemical
gradients within the bioreactor vessel during growth of the root bed on culture basket. The exploded view of the air atomizing Misting system is shown in Figure 14.

[0083] In one embodiment, the mister is fitted on top of the bioreactor and dimensioned such that the headspace distance between the mister and the root bed must be minimum distance of 15-20 cm to ensure uniform medium distribution.

The mister made from atomizing nozzle can be sterilized along with the bioreactor through in-situ sterilization. But once the ultrasonic mister is attached the steam sterilization is avoided. The mister is cold sterilized as follows. The mister is soaked overnight in 70% ethanol followed by pumping (25 mL min⁻¹) 500 mL of a solution of 10% bleach, then 100 mL sterile water, 500 mL of a solution of 70% ethanol, and finally a solution of 100 mL sterile water. An alternative sterilization method involves the following. Briefly, the mister head is washed with tap water to remove any large debris, dried at 60°C, wrapped in aluminum foil, and heated again at 60°C for 12-15 hours. Following this heating step, the mister head is heated again at 110-115°C for 2-3 hours. Before being inserted into the mist bioreactor, the misting head is cooled in a laminar flow hood. A third sterilization procedure may involve gas-phase sterilization using ethylene oxide.

[0084] The present invention also explains about a bioreactor along with culture mesh holder with mist forming device, which is designed as per mist deposition model in which root beds are treated as if they are fibrous filters. Mist cycle is optimized with duty cycle by regulating misting by switching On/Off in scaled up pilot bioreactor. However, it is known that providing for continuous misting is injurious to tissue growth. The duty cycle may be changed in case of use of different mister device.

[0085] In the mist deposition model, for a fixed droplet size Dₚ, the droplet capture efficiency (ηₜ) of the root bed is given by ηₜ=1−exp[(-4 Lₚ η_c) / (Dₚ(1−α))] where L is the length of the root bed, Dₚ is the diameter of the root, and η_c is the combined capture
efficiency due to impaction and interception (\(\eta_{\text{IMP-INT}}\)), and diffusion (\(\eta_{D}\)), and these efficiencies are all functions of \(D_p\).

[0086] The overall mass deposition efficiency (\(\eta_{OM}\)) of the root bed is the product of the root bed efficiency \(\eta_{B}(D_p)\) and the mass fraction \(m(D_p)\) of mist particles of diameter \(D_p\), summed over the aerosol size distribution data: \(\eta_{OM} = \sum \eta_{B}(D_p) \times m(D_p)\). The medium captured by the roots (\(V_{\text{dep}}, \text{mL d}^{-1}\)) is expressed as \(V_{\text{dep}} = 24 \omega Q \times \eta_{OM}\), where the factor 24 converts from hours to days, \(\omega\) is the duty cycle (min hr\(^{-1}\)) of the mist, and \(Q\) is the medium flow rate (mL min\(^{-1}\)) during the mist “on” cycle. \(V_{\text{dep}}\) is therefore a non-linear function of \(\omega\) (packing fraction of root bed).

[0087] The medium required to support the growth of the root bed (\(V_{\text{req}}, \text{mL d}^{-1}\)) depends upon the amount of biomass present, the growth rate \(\mu\) (d\(^{-1}\)), the apparent biomass yield of the growth-limiting nutrient \(Y_{X/S}\) (g DW biomass per g nutrient consumed) and the concentration of the limiting nutrient medium \(C_{S}\) (g L\(^{-1}\)). To maintain a desired growth rate \(\mu\), \(V_{\text{dep}}\) must be greater than or equal to \(V_{\text{req}}\).

Pipe Rack

Air connection:

[0088] Air inlet: Air inlet line is used for air feed into the system by means of spray through the mist generators. Air inlet line is connected with in-situ sterilizable absolute filter element (5” size, 0.2 \(\mu\)) with full draining stainless steel housing. Extra two supporting pre-sterilized filters (0.2 \(\mu\)) are also attached before in-situ sterilisable filter elements. Air compressor is connected with the airline of the bioreactor.

[0089] Airline of the bioreactor was constructed by serially connecting two numbers of pre-sterilized air filters (2 Nos, 0.2 micron, 1” and 5”, filters) before in-situ sterilizable absolute filter element (5” size, 0.2 \(\mu\)) with full draining stainless steel housing leading to vessel.
Air Exhaust:

[0090] Air exhaust line is also connected with in-situ sterilisable absolute filter element (5” size, 0.2 μ) with full draining stainless steel housing.

Exhaust Gas analyser:

[0091] CO2 and ethylene gas analyser are connected to the exhaust line of the vessel. These analysers are connected with isolation valves to avoid steam entry into the analysers.

Steam/Water connection:

[0092] Cooling water circulation line is connected to the bioreactor to control temperature through vessel jacket.

Control panel of the Mist-Bioreactor:

[0093] The control panel of the bioreactor consists of controllers, transmitters and other accessories.

PLC:

[0094] The total fermentation process is automatic operable type with process control by PLC, which has provision to set all the parameters in the desired process limit and also to display and record the data.

Supervisory Control And Data Acquisition (SCADA):

[0095] The system is designed to suit intellation based SCADA Software and interface system to control the fermenters as required by user. The software is operated through Windows computer. SCADA records all measured data and records developed Algorithm to feed nutrients as per the batch.
Algorithm for Mist control:

[0096] Algorithm is developed for controlled mist cycle during the batch. Program is also developed to regulate mist flow by controlling air and media into the feed. PLC is connected with laptop installed with SCADA software. All the data of the mist-bioreactor is recorded into the laptop through SCADA.

Utility systems:

[0097] Steam Boiler and air compressor are connected with mist-bioreactor system. Steam boiler may be a capacity of 10-15 KW and can produce steam 20-30 kg/hr with maximum pressure of around 5-10 bar. Oil free air compressor (1 HP) is connected with reactor through Polyurethane (PU) tubing. Controlled air pressure distribute further to instrumentation line and process line. Process line is connected to vessel through filter and spray nozzle whereas instrumentation line is used for controlling pneumatically operated valves controlled through PLC.

[0098] The Stainless Steel Mist-Bioreactor is connected and run as per self-explanatory drawing of the bioprocess mentioned in **Figure 11.** The media, form of mist is spread inside the reactor vessel. The deposited media from the reactor vessel is transferred to another reservoir for analysis and further re-circulated to main reservoir tank/bottle to reproduce mist in the mist-bioreactor.

[0099] The concept drawing of the Glass Mist-Bioreactor is shown in **Figure 13.** The bioreactor may be operated in normal atmospheric pressure.

[0100] To check the purity of the filtered process air going to the bioreactor vessel is tested further before entering into mist-nozzle by splitting the airline into two. One airline enters directly to vessel and other line leads to one media bottle containing sterile nutrient broth (NB) media with air purging facility.

[0101] Media line can be connected with bioreactor by connecting the bottle attached with pre-sterilized Media filters (0.2 u, 1” x2 Nos) with silicon tubing and needle assembly. The needle is pierced aseptically through septum of the Nozzle port to connect media line with Reactor.
Sugar containing modified minimal media can be prepared for AM fungal growth in bioreactor. Substantial growth is observed inside the bioreactor. Freshly growing white colour hairy root is observed. Hairy root tips came out from the mesh in branching pattern (Figure 12).

Advantages of the Invention

Gas-phase (mist) bioreactor for in vitro aseptic mass production of arbuscular mycorrhizal (AM) fungal spores and roots in a transformed hairy root of plant in a limited aseptic space is unique for mycorrhizal growth which includes the in-situ sterilizable Stainless steel vessel or autoclavable glass. The advantage of use of media in form of mist droplet consumes less nutrient medium than conventional cultivation methods. Furthermore, contamination of the culture medium with bacteria is minimized by the exposure to medium vapor because they are damaged or even filled during the nebulization. Mycorrhizal fungi can be grown in this mist-bioreactor with limited supply of media in four weeks’ time.

REFERENCES

Patent Citations:


Non-Patent Citations:
• Alok Adholeya, Pragati Tiwari and Reena Singh. (2005) Large-scale inoculum production of arbuscular mycorrhizal fungi on root organs and inoculation


WE CLAIM:

1. Gas-phase (mist) bioreactor for *in vitro* aseptic mass production of arbuscular mycorrhizal (AM) fungal spores, propagules and roots in a transformed hairy root of plant in a limited aseptic space comprising of-
   a) a sterizable vessel made of stainless steel or glass;
   b) mist forming device;
   c) mist controlling system, and
   d) with an option of a special type of inoculation assembly, basket to grow culture and culture holding mesh,

   wherein liquid nutrient medium steadily floods in a form of mist inside the vessel and then drains out after condensation and recycles at a regular intervals.

2. The bioreactor as claimed in claim 1, wherein the stainless steel vessel (as shown in Figures 1 and 2) is *in situ* sterilizable.

3. The bioreactor as claimed in claim 1, wherein the vessel made of glass is sterilized by using autoclave.

4. The bioreactor as claimed in claim 1, wherein the capacity of glass bioreactor is 7 litres.

5. The bioreactor as claimed in claim 1, wherein the capacity of stainless bioreactor is 25 litres.

6. The bioreactor as claimed in claim 5, wherein the vessel is made of stainless steel SS316 with spiral jacket with insulation.

7. The bioreactor as claimed in claim 2, wherein H/D ratio of the vessel is 2:1.

8. The bioreactor as claimed in claim 3, wherein H/D ratio of the vessel is 3:1.

9. The bioreactor as claimed in claims 5 and 6, wherein the top plate is made up of stainless steel SS316.

10. The bioreactor as claimed in claims 4 and 6, wherein the top plate of the vessel is bolted to the shell through Rothman Clamps and bottom dish is welded to the shell.

11. The bioreactor as claimed in claim 6, wherein all parts of the bioreactor in contact with the culture are made of SS316.
12. The bioreactor as claimed in claim 6, wherein the jacket with spiral is made of SS304.

13. The bioreactor as claimed in claim 6, wherein the internal surface finishing of the vessel is 240 Grit and the external is 180 Grit.

14. The bioreactor as claimed in claim 6, wherein working range of temperature of the vessel is 100-140 °C and working pressure is 0.5-3.0 bar.

15. The bioreactor as claimed in claims 6 and 7, wherein the top plate of the stainless steel mist-bioreactor consists of 10 ports (as shown in Figure 3) as follows:

   (i) Two (2) numbers of inoculation ports (P1, P2) extended inside the vessel from the top plate with SS pipe (SS 316) upto platform/mesh for the inoculation of culture,
   (ii) 1 No of exhaust port (P3),
   (iii) 1 No of view glass (P4),
   (iv) 1 No sterile pressure gauge (P5),
   (v) 1 no of safety valve (P6),
   (vi) 1 No for CIP cleaning (P7),
   (vii) 1 No for steam inlet (P8)
   (viii) 1 No of central port is to attach rotated shaft for culture holder (P9)
   (ix) 1 additional port for other use (P10).
   (x) One Lamp Holder is attached with Top Plate (Figure 4), wherein one illumination lamp is connected with this lamp holder to observe the growth inside bioreactor.

16. The bioreactor as claimed in claim 6, wherein the bottom part of the vessel consists of drain valve (Figure 1, N11) and harvesting/sampling port (Figure 1, N12).

17. The bioreactor as claimed in claim 6, wherein pH, temperature, and DO probes are attached to the bottom part of the vessel as shown in (Figure 1) as N8, N9, N10 respectively.

18. The bioreactor as claimed in claim 17, wherein pH probe is connected to pH transmitter (pH range 0-14) and can be set through PLC; 25 mm DO probe
is connected with DO Transmitter (Range 0-100%), CO₂ and ethylene gas analyser are connected to the exhaust line of the vessel.

19. The bioreactor as claimed in claims 1 and 6, wherein one peristaltic pump (3-30 rpm) is connected with media line to form mist inside the reactor and other extra pump (30-300 rpm) is used for cleaning of vessel.

20. The bioreactor as claimed in claim 6, wherein the basket to grow culture consists of –
   i) a two tier rack made of perforated Teflon sheet (mentioned in Figure 5A showing culture Plate No. 1 and Figure 5B is Plate No. 2) and wherein diameter of upper and lower racks is 160 mm and 190 mm respectively and the distance between two racks is 190 mm;
   ii) the said perforated Teflon sheet is fitted with SS (SS 316) supporting ring is fixed into the 25 L reactor vessel and can be removed easily for cleaning;
   iii) the central stem of the rack (basket) is made of SS rod (SS 316) connected from the centre of the top plate using mechanical seal wherein the rack can be rotated by manually rotating the central rod of the rack from outside of the vessel.

21. The bioreactor as claimed in claims 1 and 20, wherein mycorrhizal fungi may be grown in a funnel shape single conical structure instrument and wherein the funnel shape is prepared using SS mesh (SS 316) to grow AM Fungi.; the diameter of the cone is 160 mm and length 100 mm and such type of conical shape culture holder creates anchor to the mycorrhizal hairy root with the soft wall mesh (such culture basket is shown in Figure 6).

22. The bioreactor as claimed in claim 6, wherein the inoculation assembly is made up of stainless steel SS316, the assembly is closed cylindrical in shape with a flip disk is attached inside the assembly and the assembly can be fitted with the Triclover Clamp (TC) end of the top plate of bioreactor and can be sterilized separately after removing from the reactor to transfer culture into pre sterilized assembly (see Figures 7 and 8).
23. The bioreactor as claimed in claims 6 and 21, wherein the inoculation process in the bioreactor consists of two steps: Step-1 is inoculation to assembly and step-2 is attachment of assembly to main bioreactor for actual inoculation to vessel wherein the inoculation process is mentioned below: AM fungal associated hairy root aseptically cut into small pieces and culture is aseptically transferred to pre-sterilized inoculation assembly wherein airflow is maintained of 8-10 LPM into the vessel at the time of inoculation of culture to sterilized bioreactor.

24. The bioreactor as claimed in claim 4, wherein inside the glass reactor a funnel shape single conical structure SS mesh is attached to grow mycorrhizal fungi and the said conical shape culture holder creates anchor to the mycorrhizal hairy root with the soft wall mesh.

25. The bioreactor as claimed in claim 1, wherein the mist forming device consists of an atomizing spray nozzle or ultrasonic mister.

26. The bioreactor as claimed in claim 6, wherein the mister is fitted on top of the bioreactor and dimensioned such that the headspace distance between the mister and the root bed must be minimum distance of 15-20 cm to ensure uniform medium distribution.

27. The bioreactor as claimed in claims 6 and 25, wherein the atomizing spray nozzle is made of brass and in combination of stainless steel SS 316 and is attached to side wall of the stainless steel vessel or top plate of the reactor (Figure 1, N14, Figure 9), wherein the spray nozzle consists of air media line inside the reactor vessel.

28. The bioreactor as claimed in claims 6 and 25, wherein the Mister device fitted with a special triclover clamp (TC) joint on the top plate of the bioreactor (Figure 10) and is used when independent air flow is required for growth and is capable of providing a volumetric throughput around 3-4 L min⁻¹ and of providing droplets having a diameter varying between 5 μm and 25 μm.

29. The bioreactor as claimed in claims 4 and 25, air atomizing Misting device in glass bioreactor is fitted with a special arrangement inside the bottom of
the bioreactor as per design (Figure 13) and the air atomizing Misting system as shown in Figure 14 can be capable of providing a volumetric throughput around 0.1-0.2 ml min⁻¹ and of providing droplets having a diameter of between 5 μm and 25 μm.

30. The bioreactor as claimed in claims 4 and 29, wherein the bioreactor is operated in normal atmospheric pressure as per design mentioned in Figure 13.

31. The bioreactor as claimed in claims 1, 25, 28 and 29, the size of the droplets provided by mister offer good aeration and control of the gas phase composition, reduces shear damage to the root bed and also reduce any chemical gradients within the bioreactor vessel during growth of the root bed on culture basket.

32. The bioreactor as claimed in claim 1, wherein mist cycle is optimized with duty cycle by regulating misting by switching On/Off in scaled up pilot bioreactor and the duty cycle is changed in case of different mister device.

33. The bioreactor as claimed in claim 1, wherein the gas includes ambient air, ethylene, carbon dioxide or other gases.

34. The bioreactor as claimed in claim 6 wherein cooling water circulation line is connected to the bioreactor to control temperature through vessel jacket.

35. The bioreactor as claimed in claim 1, wherein the total bioprocess is automatic operable type with process control by PLC, which has provision to set all the parameters in the desired process limit and also to display and record the data by Supervisory Control and Data Acquisition (SCADA).

36. The bioreactor as claimed in claim 1, wherein the control panel of the bioreactor consists of controllers, transmitters and other accessories.

37. The bioreactor as claimed in claim 6, is connected and run as per the drawing of the bioprocess illustrated in Figure 11.

38. The bioreactor as claimed in claims 6 and 25, wherein the mister made from atomizing nozzle is sterilized along with the bioreactor through in-situ sterilization by steam.
39. The bioreactor as claimed in claims 1 and 25, wherein the ultrasonic mister when attached to the reactor is cold sterilized.

40. The bioreactor as claimed in claim 39, wherein in cold sterilization the mister is soaked overnight in 70% ethanol followed by pumping (25 mL min in 500 mL of a solution of 10% bleach, then 100 mL sterile water, 500 mL of a solution of 70% ethanol, and finally a solution of 100 mL sterile water.

41. The bioreactor as claimed in claims 1 and 25, wherein the mister head is washed with tap water to remove any debris, dried at 60°C, wrapped in aluminium foil, and heated again at 60°C for 12-15 hours and again mister head is heated at 110-115°C for 2-3 hours.

42. The bioreactor as claimed in claims 1 and 25, wherein the ultrasonic mister when attached to the reactor also includes gas-phase sterilization using ethylene oxide.

43. The bioprocess for aseptic mass production and up scaling of arbuscular mycorrhizal fungi (AMF) using the bioreactor as claimed in claim 1.
Figure 1
Figure 3
Culture holder (SS made) showing perforation

Culture holder (Teflon made) showing perforation

PERFORATED SS SHEET - 1

PERFORATED TEFLON SHEET - 1

Figure 5
Figure 7

- Steam inline
- Flip Plate
- Flip knob
- Inoculation assembly
- Steam outlet
- Attachment clamp to reactor vessel
Figure 8
INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2018/050266

A. CLASSIFICATION OF SUBJECT MATTER
C12M1/00 Version=2018.01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C12M1/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Total Patent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>whole document</td>
<td>6-43</td>
</tr>
<tr>
<td>Y</td>
<td>CN102783414A (DALIAN POLYTECHNIC UNIVERSITY) 21 November 2012 (21/11/2012) refer abstract</td>
<td>1-43</td>
</tr>
<tr>
<td>Y</td>
<td>IN283DEL2001A (COUNCIL OF SCIENTIFIC AND INDUSTRIAL RESEARCH) 08 January 2008 (08/01/2008) refer abstract, claim-1</td>
<td>1-43</td>
</tr>
<tr>
<td>Y</td>
<td>DE10063010C1 (ETAG KNOWLEDGE CENTER GMBH, DE) 25</td>
<td></td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. See patent family annex.

Date of the actual completion of the international search: 22-10-2018
Date of mailing of the international search report: 22-10-2018

Authorized officer: Akash Kumar
Telephone No. +91-1125300200

Form PCT/ISA/210 (second sheet) (January 2015)
<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>April 2002 (25/04/2002) refer claims 1-7</td>
<td>1-43</td>
</tr>
</tbody>
</table>