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Sevastianos Roussos**

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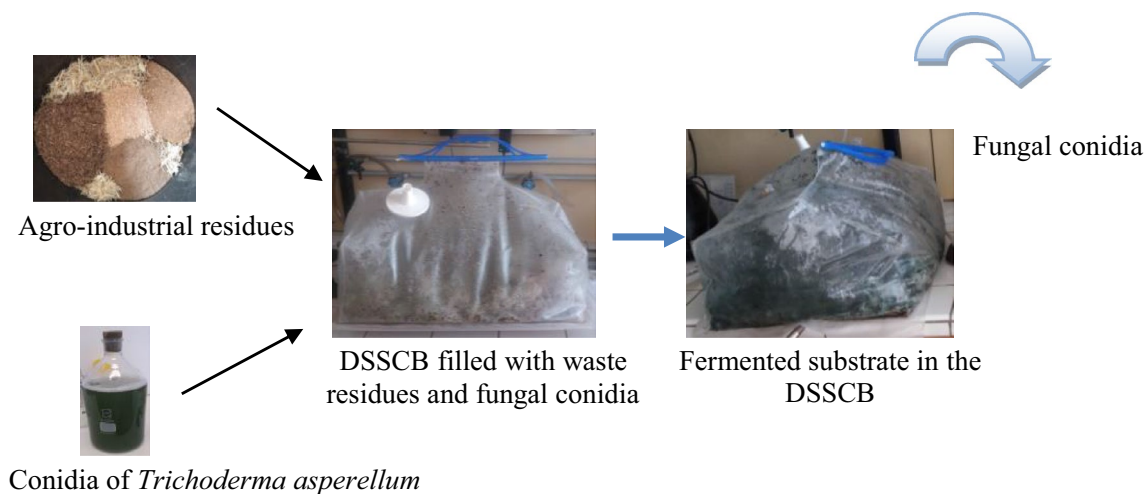
Ynoussa Maïga^{1,2} · Quentin Carboué¹ · Rayhane Hamrouni^{1,3} · Marie-Stéphane Tranier¹ · Yassine Ben Menadi^{1,4} · Sevastianos Roussos¹

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Abstract

A new prototype of a disposable solid-state culture bioreactor (DSSCB) belonging to the packed-bed category is developed for the production of fungal conidia. The bioreactor has the advantages of being autoclavable, lightweight and disposable. With a working volume of 39.52 L, it can be used for large-scale production of fungal conidia. The DSSCB was successfully tested under forced aeration for the production of conidia (2.1×10^{10} /g dry substrate in 140 h) from *Trichoderma asperellum*, a biocontrol fungus. A mixture of sugarcane bagasse, wheat bran, olive pomace, potato flakes and vine shoots was used as substrate. The DSSCB allowed an overall production of 1.26×10^{14} conidia. Based on its configuration, the fermented substrate can be easily transported and used in either solid or liquid form, for biocontrol application. This is a significant advance in the field of biomass production in solid-state culture as problems associated with the decontamination of the bioreactor prior to its reuse can be avoided. The study demonstrated that the DSSCB is suitable for the production of conidia using *T. asperellum*.

Graphic Abstract



Keywords Conidia · Solid-state culture · *Trichoderma asperellum* · Water stress

✉ Ynoussa Maïga
ynoussa.maiga@gmail.com

Extended author information available on the last page of the article

Statement of Novelty

Many bioreactors have been developed for the production of bio-active products; however, they are limited by their small size or by difficulties linked to their operation and maintenance. This study reports the development of a disposable solid-state culture bioreactor (DSSCB) with a working volume of 39.52 L, allowing the easy transport of fermented products which can be used in either solid or liquid form, for application in remote farmlands. The DSSCB successfully and massively produced conidia of *Trichoderma asperellum*, an important biocontrol agent. This is the first report of conidia production in solid-state culture using a disposable bioreactor working at this scale (18 kg of wet medium).

Introduction

Solid-state culture (SSC) is generally defined as the culture of microorganisms on a solid medium in the absence (or near absence) of free water, but with enough moisture to support the growth and metabolism of the microorganisms [1, 2]. When it uses agro-industrial residues as substrates for the production of bio-active products of commercial interest, this process can be classified as a waste management technology [3]. This technology is receiving renewed attention because of the growing need for new metabolites in various industries, such as agriculture, health, food and cosmetics [4]. One of the driving forces in the area of agriculture is that, at the global level, there is an increased desire to reduce the use of chemical pesticides. For example in France, the Ecophyto II + plan aims to reduce the use of chemical pesticides in agriculture by 50% by 2025, while offering new alternatives to farmers [5]. As another example, in West Africa, a study conducted in Burkina Faso on pollution by pesticides showed that 30% of the wells in agricultural zones contain water unsuitable for consumption and 36% of the vegetables grown have pesticide levels above sanitary standards [6].

Filamentous fungi are good candidates for biocontrol since they can protect crops against phytopathogens and reduce the use of chemical pesticides. *Trichoderma* strains are very popular, mainly because of their ability to act as highly efficient biofertilizers and biopesticides. They are grown using SSC, for which their metabolism is well adapted. Several studies have investigated the influence of environmental factors on bioprocesses involving SSC [7, 8]. Many of the fungal biocontrol agents are applied

in the field in the form of conidia (asexual spores of the fungi) because of their small size and their ability to be massively produced in a dormant stage [9]. One essential variable for the conidiogenesis is the aeration rate and in SSC systems, a possible way to induce and extend conidiogenesis, leading to high sporulation indexes, is through the application of a water stress [10]. Applied at the moment of maximum biomass production, water stress results from stopping progressively the wet air influx—needed for mycelial development—and to replace it by dry air with an increased aeration rate. As a consequence, the medium is progressively dried and this detrimental change will be perceived by the fungus as a nutritional stress (indeed, fungal nutrition is carried out through absorbtrophy, which requires the presence of an aqueous film surrounding the fungal cells to occur). As a physiological response, the fungus thus stops its vegetative growth and initiates its asexual reproduction, forming conidia [7]. Besides, the evaporation of excess water releases free spaces in the medium, increasing the overall sporulation index. Water stress thus works as an inducer of conidiogenesis, and when carried out progressively, the drying of the medium can extend conidia production for several days, as long as the minimal fungal metabolic requirements are fulfilled [2]. Not all SSC processes involving the growth of a fungus lead to increased conidia production when operated under water stress; however, the conidia obtained in this way exhibit an increased viability for a longer period as low hydration is necessary for the conidia to stay dormant. This process also prevents potential undesirable microorganisms from contaminating the medium [11].

So far, several SSC bioreactors have been designed for the production of fungal metabolites and biomass [10–14]. However, the use of traditional SSC bioreactors is complicated due to the need to sterilize the bioreactor between successive cultures, the fact that it consists of several parts that must be assembled and the high cost due to the necessity to use stainless steel. Therefore, there is a need for easy-to-use, efficient, and less cumbersome reactors. Accordingly, the present study aims to present a new design of a large disposable SSC bioreactor (DSSCB) belonging to the packed-bed category, which can efficiently process 6 kg of substrate (on a dry weight basis), which is ergonomic and space-saving due to its small size and weight (641 g). The particular metabolic activity that was chosen for this demonstration is the production of conidia from the biocontrol agent *Trichoderma asperellum*, cultivated on a mixture of agro-industrial byproducts undergoing water stress at the end of the vegetative growth.

Materials and Methods

Configuration of the Disposable Solid-State Culture Bioreactor (DSSCB)

The critical restrictions behind the present innovation were:

- the bioreactor must be efficient to produce biological control agents like conidia at a scale superior to 5 kg dry substrate (DS),
- the bioreactor must be inexpensive and ergonomic (easy-to-use to carry out SSC in a laboratory with basic equipment and easy to handle to safely bring fermented material in remote agricultural areas),
- the bioreactor should allow the safe storage and conservation of fermented material (no downstream transfers of material should be needed before the use of the material at the application location).

Due to these constraints, the DSSCB is specifically designed to produce biomass and metabolites of biological origin. It is compact and made of a flexible plastic (polyethylene terephthalate) that can be easily sterilized (when it is necessary to operate in asepsis). The bioreactor is a bag with two main compartments. Its characteristics are summarized in Fig. 1, where the numbers indicate the different components. The first compartment is the base (10 in Fig. 1), which serves as an aeration system (and which can be used to apply water stress by blowing dry air through the bed) and a support for the bioreactor. The second compartment is the fermentation chamber (20 in Fig. 1), which has a working volume of 39.52 L ($L \times W \times H$: $0.52 \times 0.40 \times 0.19$ m). These two compartments (the base and the fermentation chamber) are made of the same flexible plastic. To allow aeration during the culture, the base has an inlet orifice (12 in Fig. 1) that can be connected to a source of gas, like dry or wet air. This inlet is connected to the central aeration channels (11 in Fig. 1). The distribution of the air to the fermentation chamber occurs through three cylindrical aeration channels in the base; these channels are parallel to one another and are perforated on the top, where they connect with the fermentation chamber (110 in Fig. 1). These aeration channels also communicate with each other through orifices (111 in Fig. 1). This configuration allows uniform distribution of air to the fermentation chamber.

The two compartments are separated by a micro-perforated grid (30 in Fig. 1) that is fixed to the base, being located on top of the aeration channels, between the aeration holes and the fermentation chamber. It has several functions: it supports the solid substrate and improves aeration of the fermentation chamber by preventing solid particles from clogging the aeration holes. It is also used

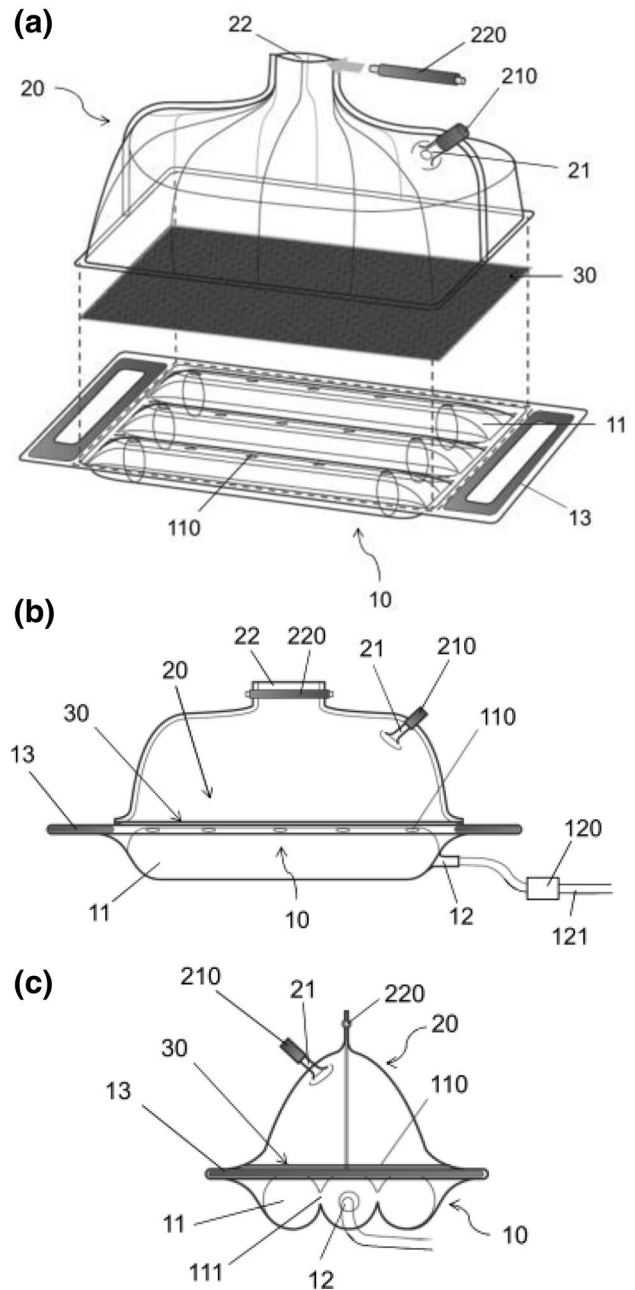


Fig. 1 Schematic representation of the bioreactor **a** Exploded perspective view. **b** Front view. **c** Side view. 0: basal portion; 11: aeration channel; 12: inlet orifice; 13: handles; 20: fermentation chamber; 21: outlet orifice; 22: opening; 30: micro-perforated grid; 110: aeration hole; 111: communication orifice; 120: air filter; 121: supply duct; 210: air filter; 220: removable fastening device

as a filter, especially when one wants to recover compounds contained in the fermenting material by leaching them with a liquid. This liquid passes through the aeration holes, from the fermentation chamber into the base. It is made of semi-rigid polyethylene terephthalate and thereby reinforces the base. Its meshes are of the order of 200 μm .

The base has handles (13 in Fig. 1) to facilitate the manipulation of the bioreactor. The fermentation chamber has an opening at the top (22 in Fig. 1) that is used for introducing and removing solid material. This opening is equipped with a removable fastening device (220 in Fig. 1). The bioreactor is also equipped with removable air filters (120, 210 in Fig. 1) located at the inlet hole of the base and at the outlet orifice (21 in Fig. 1) of the fermentation chamber. In addition, the recirculation of liquid from the base to the fermentation chamber is made possible by connecting the inlet orifice of the base with the outlet orifice of the fermentation chamber. Finally, the bioreactor cost is 30 €.

Operation and Recovery of Fermented Substrates from the DSSCB

Operation Process

The operation of the DSSCB consists of the following steps:

- Solid, dry, or previously moistened substrate (impregnated with a culture medium suitable for the growth of the microorganism) is placed in the fermentation chamber (20) on the micro-perforated grid (30).
- The loaded bioreactor is then sterilized, for example, by autoclaving or ionization. This step can be omitted if the process does not require sterile conditions.
- To start the culture, a liquid suspension of the microorganism is introduced via the opening to inoculate the medium. To ensure uniform distribution of this inoculum, the whole is mixed (i) using a sterile forceps introduced through the upper opening and (ii) by carrying out rotational movements after closing the bioreactor. It is also possible to mix the substrate and the liquid suspension in a sterile container before filling the DSSCB.
- During the culture phase, the solid medium is aerated through the ventilation system of the base, for example, by injecting air (dry or wet), via the inlet orifice (12) of the base, using the filter to maintain asepsis of the system, if necessary. The humidity of the inlet air can be controlled, enabling the application of a specific water stress to favor the fermentation process.
- Once the time set for fermentation has elapsed, the fermented substrate is dried by aerating with dry air—then it can be directly used or stored for some time in the bioreactor before use. It can also be transported within the bioreactor, if appropriate.

Recovery of Fermented Products of Interest

If the product of interest is the fermented solid substrate, it can be removed directly from the fermentation chamber

through its large opening. It is also possible to recover the useful product in liquid form using the following steps:

- a liquid is introduced into the fermentation chamber through its large opening;
- the liquid is mixed with the fermented product inside the fermentation chamber;
- the enriched liquid of microorganisms and metabolites flows to the base;
- the enriched liquid is recovered through the hole of the base.

Implementing a Fermentation with the DSSCB for the Production of Conidia

Fungal Strain

Trichoderma asperellum G3 from the collection of “Biotechnologie Environnementale et Chimie-metrique, IRD/IMBE (Marseille, France)” was used. The apical growth of the strain on potato dextrose agar (PDA) varied from 16 to 18 mm/day and its sporulation index on sugarcane bagasse supplemented with nutrient medium was 1.12×10^8 spores/g of dry substrate [16]. The conidia were stored in a 5 mL glass bottle at 4 °C on PDA.

Implementing the SSC

In order to determine the suitability of the DSSCB for the production of conidia, SSCs were conducted at laboratory scale in Erlenmeyer flasks (5 g DS) and at pilot-scale using the DSSCB (6 kg DS and a bed height of 15 cm). A mixture of agro-industrial waste was used: grapevine shoots (33.33%), sugarcane bagasse (10%), wheat bran (33.33%), olive pomace (13.33%) and potato flakes (10%). The solid substrate was moistened at 50% (wet basis) with distilled water and sterilized at 121 °C for 2 h. The fungal conidia were produced using Erlenmeyer flasks with PDA. Then, the suspension of conidia was prepared using distilled water and mixed into the sterilized solid substrate to give an inoculation density of 2×10^7 conidia/g DS and a moisture content of 66% (wet basis). These culture conditions are adapted from the ones used by Hamrouni et al. [17] for the production of secondary metabolites, conidia, and enzymes using the same strain. The inoculated medium was subsequently transferred into the bioreactors before being incubated for one week at 25 °C. A zero time sample was collected just before incubation and stored at – 20 °C for analysis. SSCs were carried out in flasks without forced aeration while the DSSCB fermentation was carried out under forced aeration conditions using air at the rate of 10 to 45 L/min (1 atm and 25 °C). From the beginning of the process until 23 h, water-saturated air (made possible by passing the air through a humidifier) was used at the rate of 10 L/min to initiate the germination of the

conidia. After 23 h, the humidifier was disconnected and dry air was used to aerate the bioreactor. From 23 to 47 h, the aeration rate was gradually increased to 45 L/min to apply a progressive water stress. Then, after 50 h, the aeration rate was progressively reduced to 25 L/min. During the process, the mass of the fermenting substrate inside the DSSCB was monitored using an electronic platform balance (KERN & SONS GmbH, Germany) and the temperature was measured using a meat thermometer (OWIM GmbH, Germany) in the core of the bed at 7.5 cm depth. Samples were collected from the entire flask and the fermentation medium of the DSSCB through core sampling on all of the bioreactor bed section at five points (the center corresponding to a first sample and the four angles subsequently mixed together, corresponding to a second sample). The sampling was performed over time to evaluate moisture content, pH, and content of conidia. Laboratory scale experiments were carried out in duplicate.

Microscopic Observations of the Fermentation Medium in the DSSCB

Samples were dyed with cotton blue (callose-specific dye) and observed under a microscope in order to follow the development of the fungus (germination of conidia, growth of the mycelium and conidiogenesis) and to confirm that the substrate was not contaminated by any other microorganism. A conidium is considered germinated when the germinated tube is equal to or greater than the diameter of the conidium.

pH and Moisture Content Assessment

One gram of fermented substrate was added to 25 mL of distilled water and triturated using an Ultra Turrax homogenizer (IKA, China). The pH of the suspension was then determined using a pH meter (HANNA, Italy).

Moisture contents (on a wet basis) were determined after drying for 24 h at 105 °C.

Conidia Content

The conidia content was evaluated by mixing 1 g of sample and 50 mL of distilled water containing Tween 80 (0.01%). Then, 1 mL was diluted appropriately and the conidia counted using a Mallassez cell.

Results and Discussion

Evolution of *Trichoderma* Grown on a Mixture of Agro-Industrial Waste in the DSSCB

The conidia germinated after 20 h and began to elongate to form the mycelium. After 23 h, all of the conidia had

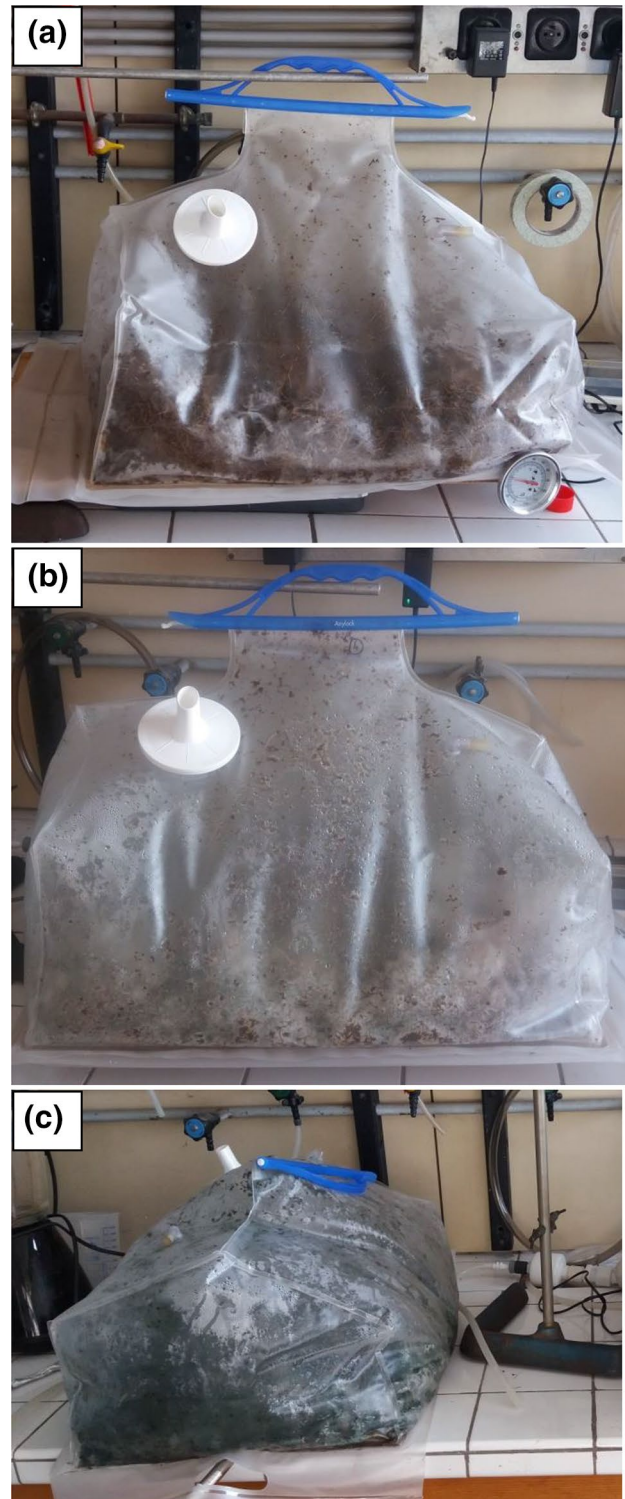


Fig. 2 Macroscopic view of the DSSCB showing the changes in color and the aspect of the fermenting substrate (a–c)

germinated, marking the end of the germination phase. Conidiogenesis began after 48 h. This evolution was marked by notable macroscopic changes of the fermenting substrate,

including the gradual appearance of a white mycelium and then green conidia (Fig. 2a–c).

Evolution of Physical Parameters

At the laboratory scale, the variation of the physical parameters was negligible and no significant change was observed for the pH, temperature and moisture content (data not shown). However, at the pilot scale, the evolution of the core temperature, pH, moisture content, and overall bed weight changed during the SSC (Fig. 3). The process started at 25 °C with a low aeration rate of 10 L/min during 23 h to initiate the germination of conidia and vegetative growth. This vegetative growth generated an increase of temperature of which the peak reached 48 °C after 44 h of process. Afterward, the temperature decreased as the

aeration rate increased to 45 L/min after 47 h, suggesting a possible efficient heat removal through water evaporation as the water stress is implemented. This decreased temperature could also be the consequence of a reduced metabolic activity following the temperature increase. This hypothesis could be confirmed with a monitoring of the metabolic activity through, for example, oxygen uptake rate measurement. The temperature notably reached a minimum value of 22 °C after 68 h, before increasing again until 31 °C (140 h) as the aeration rate is progressively reduced to 25 L/min (starting from 50 h). The moisture content and overall bed weight decreased, particularly after 50 h of process passing from 66 to 34% and 18 to 9.7 kg—respectively—at the end of the process. Regarding the pH, there was only a slight increase from 6.4 to 7.0.

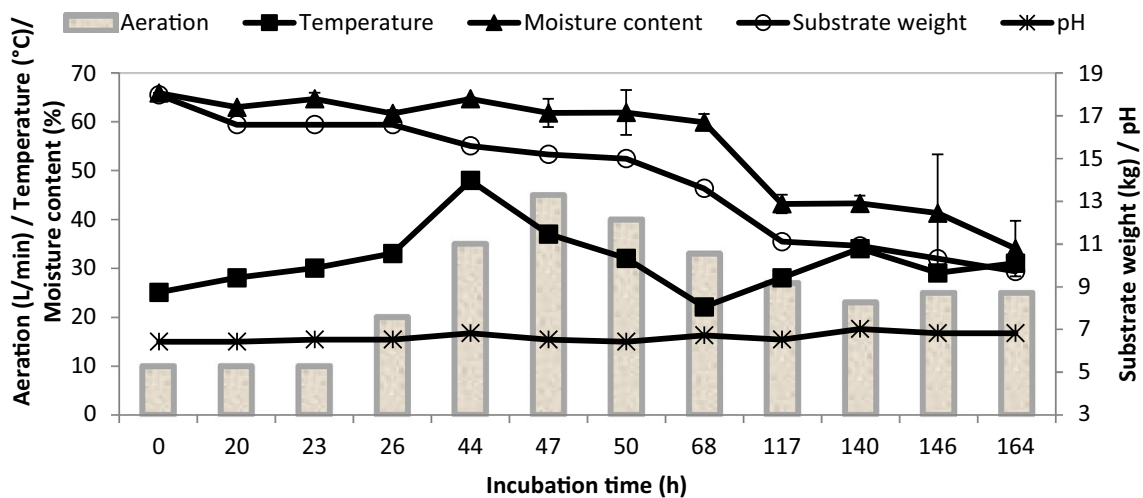
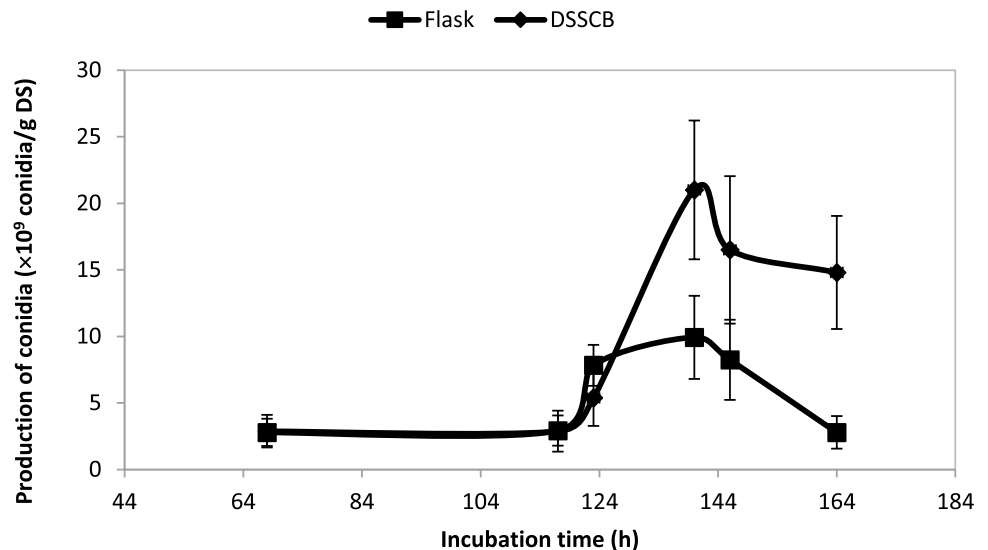


Fig. 3 Evolution of physical parameters in the DSSCB

Fig. 4 Profiles of conidia produced by *Trichoderma asperellum* grown on a mixture of agro-industrial waste in flask and in the DSSCB



Production of Conidia

At both scales, the conidia production followed a similar tendency as the conidia indexes remained constant until 117 h before increasing (Fig. 4).

The conidia production started at the same moment in the DSSCB as well as the flasks and the maximum contents were obtained after 140 h of fermentation. The highest conidia content was obtained using the DSSCB ($2.10 \times 10^{10} \pm 5.21 \times 10^9$ versus $9.92 \times 10^9 \pm 3.12 \times 10^8$ conidia/g DS for the flasks). The forced aeration and the water stress characterized by the reduction of the moisture content could have enhanced the production of conidia in the DSSCB. This difference in conidia contents is consistent with reported results that demonstrated a positive effect of aeration on *T. asperellum* conidia production [18]. It is thus most likely that the positive effect of aeration on conidiogenesis overcame the detrimental effect of the temperature rise at the larger scale.

Using the same strain of *T. asperellum*, Hamrouni et al. [19] obtained conidia production of $8.55 \times 10^9 \pm 4.00 \times 10^6$ conidia/g DS, in a packed-bed bag bioreactor containing 9 kg of moist medium. In comparison, our production yield is almost 2.5 times higher. However, it is worth mentioning that the conditions they used were optimized for both 6-pentyl-a-pyrone and conidia production, that their medium contained *Jatropha* cake and olive oil in addition to the mixture we used, and that they observed a moisture content decrease of only 10% in their bioreactor. Considering the total initial quantity of dry medium processed in the DSSCB, the overall conidia/bioreactor value is 1.26×10^{14} . A single bioreactor would allow to treat almost 10^6 plants (with a treatment application of 10^8 conidia per plant [20]).

Comparison of the DSSCB with Existing Bioreactors

One of the disadvantages of SSC is related to the difficulty in removing the metabolic heat generated during the culture, which is directly linked to the growth rate of the microorganism. This situation can lead to high temperatures that are detrimental to the process organism, particularly at higher scales of production. The various types of bioreactor designs have different efficiencies with respect to heat removal; Mitchell et al. [21] distinguish four classes of batch-operated bioreactors, depending on whether they have forced aeration or not and are agitated or not. Typically, in a tray bioreactor—static and without forced aeration—the bed height is a critical factor to avoid the formation of detrimental temperature gradients [22]. Using such a bioreactor, Jou and Lo showed that the height of the bed should not exceed 1 cm to get an optimal growth rate for a *Trichoderma* sp. strain [23]. Such conformation needs large production surfaces which are not suited to our restrictions.

The DSSCB falls in the static packed-bed bioreactor category, which is characterized by the presence of forced aeration that aids in the replenishment of oxygen and mitigates accumulation of heat and carbon dioxide. It usually consists of an enclosed cylindrical glass or metal tube or drum in which the substrate is inoculated. These bioreactors are reusable and require the transfer of the fermented material at the end of the process, a step during which the operator may be in contact with the biological material or that may contaminate the surrounding lab. In addition, each reuse necessitates a preliminary meticulous cleaning of the bioreactors. Moreover, pilot-scale SSCs often require separate sterilization of the solid medium and the bioreactor before the inoculated medium is aseptically introduced in the sterile bioreactor [24]. The DSSCB bypasses these time-consuming steps as the medium can be autoclaved directly inside the device. Besides, it protects the user from exposure, as the bioreactor does not have to be emptied and can be stored directly (after the fermented material has been properly dried in situ by aerating the bed with dry air).

The temperature rise in packed-bed bioreactors is unavoidable for an aerobic process carried out on more than 5 kg DS. Various strategies of heat removal exist including the use of complex cooling devices—such as cooling plates or agitation systems involving mechanical blades or rotating drum-type apparatus. These cooling devices were not considered in the case of the DSSCB. Indeed, they would not be disposable and would therefore add duration to the already time-consuming pre- and post-fermentation steps (more elements to clean). For this reason, heat dissipation in the DSSCB mainly relies on the forced air aeration. Therefore, microorganisms able to tolerate a rise of temperature (when this occurs) without negatively impacting the final yields would be preferable for this bioreactor.

Several studies have reported the production of conidia using bag-type bioreactors. For example, De la Cruz-Quiroz et al. [25] obtained a maximum sporulation index of 1.2×10^9 conidia/g DS using *T. asperellum* grown on corn cob in a bioreactor consisting of a polyethylene bag, operated without forced aeration. Although this index is high, it is lower than the one we obtained (2.1×10^{10} conidia/g DS) probably because of the influence of aeration. In addition, the scales are different as they carried out the SSC on only 15 g of solid medium [25] compared to 6 kg DS used in our study. The results obtained with the DSSCB are therefore encouraging.

Limitations and Perspectives of the DSSCB

The configuration of the DSSCB seems suitable for the production of conidia by *T. asperellum* grown under water stress as we obtained a good overall conidia index. However, this high yield is accompanied by an important temperature rise

that may be detrimental to the production of some compounds. Indeed, the synthesis of some secondary metabolites is very sensitive to temperature variations [26]. Following this idea, producing high quantities of secondary metabolites through aerobic processes with the DSSCB could generate lasting temperature gradients that may inactivate the biomass. This temperature increase, due to the fungal metabolism, is also the consequence of the compaction of the substrate that causes air channeling across the bed and subsequent heat and mass heterogeneities [15]. A way to partially reduce the compaction effect is by introducing a solid material acting as an inert support in the medium, providing desirable physical properties like porosity to the bioreactor bed [27]. However, the most efficient way to prevent the appearance of mass and energy gradients is through the application of agitation specifically adapted to the fungus in such a way as not to disrupt its structures. Hence, one of the major limits of this bioreactor is the absence of a proper system of agitation, although the bioreactor is light enough (18 kg) to allow the use of a simple intermittent agitation system (with a swinging shelf or even performed manually). In any case, additional experiments are necessary to evaluate the performance of the bioreactor for the production of various compounds including enzymes, conidia, and secondary metabolites, using different microorganisms.

Conclusion

The DSSCB is a new bioreactor design that allows the fermentation of up to 18 kg of solid substrate (wet weight basis) under forced air conditions for the production of conidia ($2.10 \times 10^{10} \pm 5.21 \times 10^9$) of *T. asperellum*, a fungal strain used in biocontrol. This result is higher than the one obtained using flasks at the laboratory scale ($9.92 \times 10^9 \pm 3.12 \times 10^8$ conidia/g DS). Taking into account the quantity of solid substrate used for the test, up to 1.26×10^{14} conidia are estimated to have been generated. Based on the configuration of the bioreactor, these conidia can be carried easily and used in either a solid or a liquid formulation for biocontrol in crops. This is a significant advance in the field of metabolite production in solid-state culture as problems associated with the sterilization of the bioreactor prior to its reuse are avoided. To validate the developed bioreactor, it is necessary to test its suitability with other strains and for the production of secondary metabolites.

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Authors Contribution QC and MST carried out the initial work on the DSSCB. YM and RH carried out the experiments. YM led the writing

of the manuscript with support from all authors. BMY participated in the initial work on the DSSCB. SR initiated and supervised this research

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Data Availability The data and bioreactor are available.

Compliance with Ethical Standards

Conflict of interest The authors declare that there are no conflicts of interest.

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Affiliations

Ynoussa Maïga^{1,2}  · Quentin Carboué¹ · Rayhane Hamrouni^{1,3} · Marie-Stéphane Tranier¹ · Yassine Ben Menadi^{1,4} · Sevastianos Roussos¹

¹ Aix Marseille Univ, Avignon Université, CNRS, IRD, IMBE, Marseille, France

² Université Joseph KI-ZERBO, Laboratoire de Microbiologie et de Biotechnologie Microbienne, 03 BP 7021 Ouagadougou, Burkina Faso

³ Univ. Manouba, ISBST, BVBGR-LR11ES31, Biotechpole Sidi Thabet, 2020 Ariana, Tunisia

⁴ BioIntrants Technologie, Zone Industrielle Bouteffeha, Beja, Tunisia