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Abstract

Background/Aim: A new prototype of Solid-State Fermentation Bioreactor, namely "Zymotis-2 ", was developed to produce fungal spores.

Main Methods and Major Results: A fermentation process for fungal spores, and hydrolase enzymes (endo and exoglucanases, amylases) production by *Trichoderma asperellum* DWG3, *Aspergillus niger* G131 and *Beauveria bassiana* was scaled-up from flasks and glass Raimbault column packed with 20 g of solid substrates (dry weight) to 5 kg of solid substrate by using the new Zymotis-2 bioreactor. Fungi strains growth using a mix of vine shoots, wheat bran, and olive pomace was tested under similar experimental conditions in Zymotis-2 bioreactor, column bioreactor and flasks in a parallel fermentation system. Overall, significant spore production on Zymotis-2 bioreactor was obtained, achieving $22.01 \pm 0.01 \times 10^9$ spores/g DM $16.30 \pm 0.07 \times 10^9$ spores/g DM, and $3.30 \pm 0.07 \times 10^9$ spores/g DM for *B. bassiana*, *T. asperellum* DWG3, and *A. niger* G131, respectively. Forced aeration increased the endoglucanases, exoglucanases and amylases activities for *T. asperellum* DWG3 but *B. bassiana* and *A. niger* G131 were affected negatively by the aerated process, showing the lowest enzyme activities.

Conclusions and Implications: In conclusion, a high yield of spores was obtained at 137 h of cultivation time, confirming the validity of the new Zymotis-2 bioreactor to produce virulent spores at low cost by *T. asperellum*, *B. bassiana* and *A. niger* G131.

KEYWORDS
Aspergillus niger, *Beauveria bassiana*, biopesticides, enzymes, hydric stress, secondary metabolites, *Trichoderma asperellum*

1 | INTRODUCTION

Solid state fermentation (SSF), is a biotechnological process involving the use of solid material under controlled conditions in the absence of free running water.[1] It is a very ancient process primarily used to meet human needs. Typical examples of SSF are fermentation of rice by *Aspergillus oryzae* to initiate the koji process and *Penicillium roquefortii* for cheese production. Nowadays, SSF is widely used in the entire fermentation industry, particularly for the production of enzymes (cellulases, lipases, chitinases), secondary metabolites (mycotoxins, polyketides like 6-PP and peptaibols etc.), spores (inoculum and biopesticides) and biomass.[2] Such technology is an alternative offering high spore

Abbreviations: SSF, solid state fermentation; IMBE, institute mediterranean of biodiversity and marine ecology and continental; PDA, potato dextrose agar; DM, dry matter; DNS, dinitrosalicylic acid; PCA, principal component analysis; F, flasks; R, Raimbault columns; U, Zymotis-2 bioreactor

production for different species of filamentous fungi, including species of *Trichoderma*, *Beauveria*, *Aspergillus*, *Metarhizium*, and so on, with a minimal cost of separation. The selection of SSF as a process of cultivation is not only driven by qualitative and quantitative consideration, but it is also advantageous in terms of economical and ethical concerns related to environmental protection. Thus, SSF technology has an economical interest according to agricultural byproducts, as culture medium in SSF is cheaper than using synthetic substrates.[3–5] In fact, SSF allows the use of different agro-industrial byproducts rich in cellulose which are usually disposed without any pre-treatment, such as plant bagasse, husk and fruit seeds, representing a potential source of sugars and energy for fungal development.[6] In laboratory SSF process, most bioreactors designs used include Erlenmeyer, flasks, Raimbault columns and polyethylene bags. However, information available on bioreactors design and adequate conditions for fungal cultivation is scarce.[7]

Another crucial step is extrapolation of large-scale. The most important environmental parameters related to bioreactors operation are temperature and moisture content,[5,8] which are both tightly associated to the metabolic activity of the microorganisms. To overcome such limitations, several configurations of SSF bioreactors have been proposed. Arora et al.[9] classified the existing bioreactors in four categories: (i) tray bioreactor, (ii) packed-bed bioreactor, (iii) air pressure pulsation bioreactor, and (iv) intermittent or continuously mixed bioreactor. Problems related to non-tolerance of agitation, heat and CO_2 produced during fermentation by microorganisms may inhibit cell growth and affect the production of metabolites.[1]

In 1993, an advanced version of packed-bed bioreactor designated as 'Zymotis-1' was proposed by Roussos et al.[10] to overcome issues of heat rise during SSF process. It consisted of adjacent sections containing the fermenting media separated by cooling plates in which cooling water was circulated to control the heat generation during the process. It was shown that the temperature control during the active growth phase was not efficient and that increase in aeration rate had to be adopted.[10] Therefore, removal of heat and CO_2 from the fermented substrate by the forced aeration could be a possible alternative to increase the growth of fungi during scale-up.

In this vein, a new prototype bioreactor designated as Zymotis-2 for a disposable SSF Bioreactor was developed for the production of fungal metabolites. The bioreactor has the advantages of being autoclavable, lightweight and disposable. With a useful volume of 0.04 m^3, and weight of 641 g, it can be used for large scale production of fungal metabolites that can be carried easily for biocontrol applications in remote areas.

Hence, the aim of the present study was to evaluate a novel large-scale solid-state fermenter facilitating scale up studies at 16.5 kg substrate (fresh material). A process for spore production and cellulolytic enzymes (endoglucanases, exoglucanases and amylases) by *T. asperellum* DWG3, *A. niger* G131 and *B. bassiana* using a mix of vine shoots, olive pomace and wheat bran as substrates in Zymotis-2, Raimbault column bioreactors and flasks under identical conditions in parallel fermentation is described. Aspects related to the effect of forced aeration and related spore are also discussed.

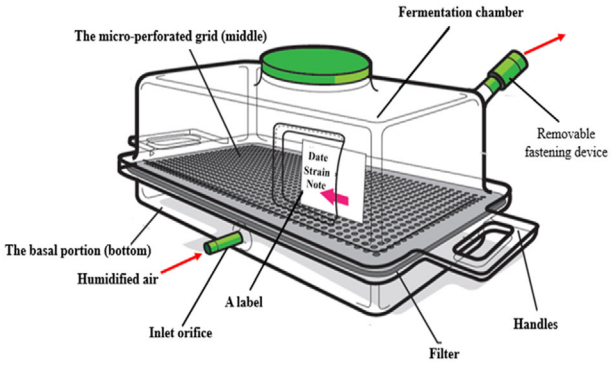

FIGURE 1 Schematic representation of Zymotis-2 bioreactor

2 | MATERIALS AND METHODS

2.1 | Microorganisms and culture conditions

Fungal strains of *B. bassiana*, *A. niger* G131, and *T. asperellum* DWG3 were provided by the Institute Mediterranean of Biodiversity and Marine Ecology and Continental (IMBE), Aix-Marseille University, France. Fungal strains were inoculated and grown on potato dextrose agar (PDA), and incubated at 30°C for 5 days at the same conditions. For the stock culture and preservation of the strain, the slants were preserved at 4°C in a 5 mL bottle on PDA.

2.2 | SSF scale up

- Representation of Zymotis-2 bioreactor: The design of the bioreactor has been patented due to a range of innovative features, reported in a previous work by Maiga et al.[11] Zymotis-2 is specifically designed to produce biomass and metabolites of biological origin (antioxidants, antibiotics, antifungals, etc). The bioreactor looks like a bag (flexible plastic material) with two main compartments (Figure 1). The first compartment is composed by the basal portion that serves as support for the bioreactor and an aeration system (application of water stress for example). This compartment has an inlet orifice that can be connected to a source of air/gas, preferabley dry or wet air, to promote the growth of microorganisms. The second compartment is a fermentation chamber (52 × 40 × 19 cm), intended to receive the solid substrate moistened with a culture medium suitable for the growth of the strain. A micro-perforated grid is fixed to the basal portion which is used as support for the solid substrate and as a filter for recovering fluids contained in the fermentation chamber in the basal portion, restraining the solid substrate.
- SSF: Flasks (250 mL), Raimbault columns (diameter of 2.5 cm and height of 20 cm) and Zymotis-2 bioreactor (20 × 40 × 60 cm) were tested. The substrate was integrated by a mix of vine shoots (50%), wheat bran (30%), and olive pomace (20%) on dry weight basis as support. Mixed substrates were sterilized at 121°C for 30 min. After cooling, the substrate was inoculated with a spore suspension of

TABLE 1 Aeration conditions of the SSF on Zymotis-2

Time incubation [hours]	RVU	Aeration [L min^{-1}]
0–24	50	5–16
24–48	150	20–30
72–120	326	80
120–144	326	80

each strain at a concentration of 2×10^7 spores g^{-1} dry matter (DM). These steps were performed in order to achieve an initial moisture content of 66% (fresh weight) to initiate the fermentation process. Cultures were carried out at 25°C in Raimbault glass columns and in 250 mL flasks packed with 40 g of solid (DM). 16.5 kg of inoculated substrate was placed in a Zymotis-2 and incubated at 25–30°C for 6 days. Fermentations were controlled with the application of forced humid air during all processes.

- Forced air conditions: The aeration was generated by an air compressor and humidified by passing the air through distilled water. A flowmeter was used to adjust the initial aeration rate at 20 mL min^{-1} for columns. Flasks were not hermetically closed to allow oxygen flow by diffusion. For Zymotis-2 aeration conditions are showed on Table 1.
- RVU: The air injected through the system was measured as Renewal of the Useful Volume (RVU). An RVU is the rate of air passing through the Useful Volume (VU) of substrate per hour.
- Aeration: Is the amount of air passing through the substrate. It was generated by an air compressor and humidified by passing the air through distilled water.
- Sampling, treatment, and analytical determinations: To monitor the fermentation process, samples (5 g) were collected at various intervals from each bioreactor. Three grams were used to determine pH and moisture content and for morphological and microscopic observation on fungal growth, while the remaining 2 g were placed in Falcon tubes and frozen at -20°C for further analyses.
- Moisture determination: For dry weight determination, 1 g of fresh fermented material was introduced into an oven at 105°C for 24 h and then re-weighted to estimate the percentage of lost water by the following equation:

$$Moisture\,(\%) = \frac{(W - D)}{W} \times 100$$

W: Substrate fresh weight and D: dry weight
All results are expressed as g of DM.

2.3 | Spore counting

- Spore counting: One gram of each sample from the fermented substrate (fresh weight) was added to 100 mL of diluted Tween 80 (0.01%) in an Erlenmeyer flask and the mixture was stirred for 15 min for homogenization. After appropriate dilution, the spores were counted using a Mallassez cell. The results are expressed as the spores per gram of dry matter (spore/g DM). Each counting was performed in triplicate. The results are expressed as % spore = mean SD.

2.4 | Reducing sugar assessment

Extraction of reducing sugar and enzyme solution: One gram of fermented material (fresh weight) was collected and mixed with 20 mL of distilled water. The suspension was homogenized by ultrasounds (Ultra-turax) for 1 min. Then, the mixture was centrifuged (5000 × g, 3 min at 4°C) and 1 mL of the clear supernatant was used for the assessment of reducing sugar, and activity of amylase, endoglucanases, and exoglucanases.

Reducing sugar level was spectrophotometrically determined by the dinitrosalicylic acid (DNS) method described by Miller,[12] using glucose as a standard.

Samples (1.0 mL) and DNS reagent (3.0 mL) were introduced in a test tube, vortexed and placed in a bath at 100°C for 5 min. Reaction was stopped by placing the tube in an ice frozen bath. Calibration curve was based on 0 to 1 g L^{-1} of glucose and the absorbance was measured at 575 nm.

2.5 | Amylase activities

The amylase activity was performed according to the method described by Singh et al.[7] Amylase activity was estimated by analyzing reducing sugar released during hydrolysis of 1% (w/v) starch in 0.1 M phosphate buffer (pH 7) by enzyme solution incubated at 50°C for 10 min.

One unit of amylase activity was defined as the amount of enzyme required for the liberation of 1 μmol of reducing sugar as glucose per minute under standard assay conditions.

2.6 | Endoglucanase and exoglucanase activities

Endoglucanase activity was determined, according to Nava-Cruz et al.,[13] by analyses of reducing sugar released during hydrolysis of 1% sodium carboxymethyl cellulose in 50 mM citrate-phosphate buffer (pH 4.8) by enzyme solution incubated at 50°C for 30 min.

Exoglucanase activity was performed by using filter paper Whatman (1 cm × 5 cm) in sodium citrate buffer (50 mM, pH 4.8) at 50°C for 60 min, according with Mandels et al.[14]

Enzymes determinations mentioned above were stopped on a bath with ice for 5 min.

An enzyme activity was defined as the amount of enzyme that catalyzes the release of 1 μmol of glucose per minute under the assay conditions. The activity of the enzyme is expressed as units per gram dry matter (U g^{-1} DM).

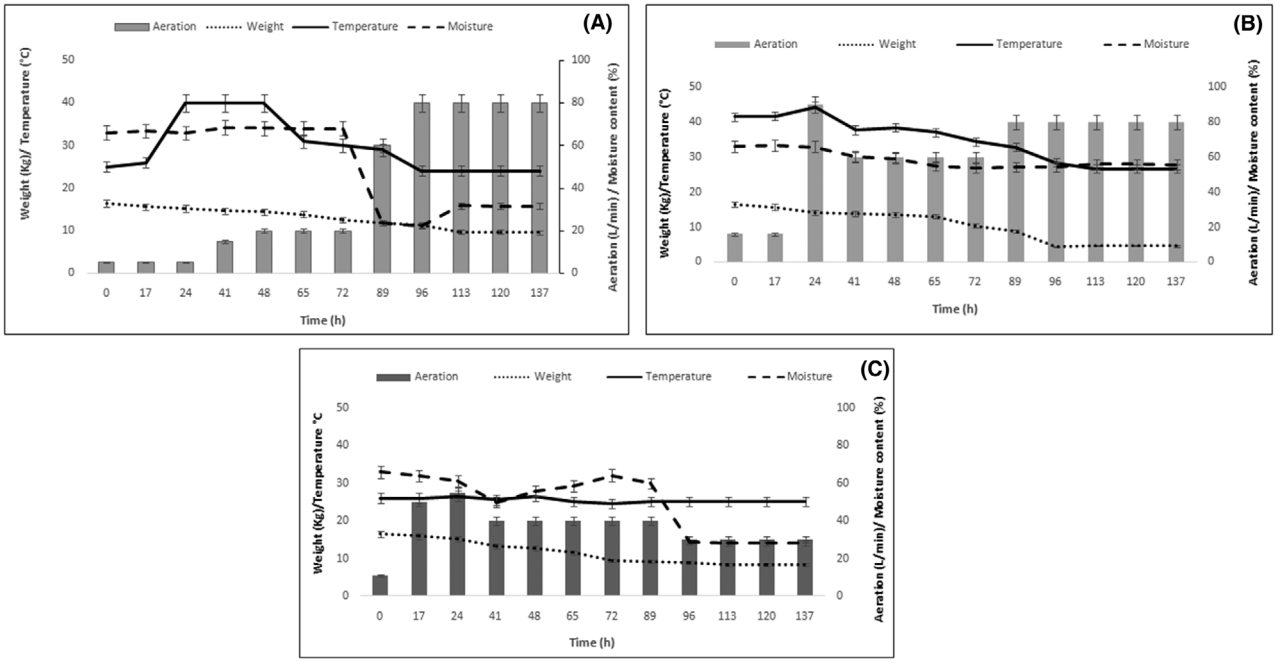

FIGURE 2 Evolution of fermentation parameters in Zymotis-2 bioreactor; (A) *T. asperellum* DWG3, (B) *B. bassiana*, (C) *A. niger* G131

2.7 | Experimental design and statistical analysis

- The experimental design was integrated by the evaluation of three fungi strains (*T. asperellum* DWG3, *B. bassiana* and *A. niger* G131 under three different culture conditions (flasks 250 mL, Raimbault columns and Zymotis-2). All treatments were carried out in triplicate. The experiments were designed and analyzed statistically by ANOVA. Specifically, three-way ANOVA was applied for physicochemical parameters and microbiological analysis, Duncan's multiple range test was used to determine significant differences among results (coefficients, ANOVA tables and significance ($p < 0.05$) were computed using Statistical v. 10.0).
- Principal component analysis (PCA) is a tool currently used in chemometrics and were described in a previous study.[15] Software Chemometrics analysis was performed using Unscrambler X V.10.3 (CAMO/Software, Oslo, Norway).

3 | RESULTS

3.1 | Evolution of fermentation parameters on Zymotis-2

The evolution of the aeration, along with other parameters on Zymotis-2 are presented in Figure 2. For *T. asperellum* DWG3, the aeration was set at 5 L min^{-1} to initiate the conidia germination (Figure 2A). From 24 to 48 h of fermentation, it was gradually increased to 20 L min^{-1} to facilitate the elongation phase. After 41 h, the aeration was progressively induced to 80 L min^{-1}. The initial weight of the substrate used for the fermentation was 16.5 kg and it was progressively reduced to a final value of 9.62 kg at the end of the process with 80 L min^{-1} of aeration. The initial temperature was 26°C and then it increased up to 40°C at 24–48 h. The temperature remained constant at about 30°C from 65 to 89 h of incubation and then decreased to 24°C at the end of the process (137 h). The water content varied from 66% at the beginning of the process to 31.62% at the end of culture.

Regarding parameters variation for *B. bassiana*, the weight of the substrate was 16.5 Kg of substrate at the beginning of the fermentation on Zymotis-2 bioreactor (Figure 2B). During the experiment, it decreased to reach a value of 14.26 Kg, remained constant from 41 to 65h and then decreased to a final value of 4.74 Kg. In terms of moisture evolution during the fermentation process in Zymotis-2 bioreactor, a slight variation of the initial value (from 66%–55.80%) was noticed. Aeration was gradually increased from 16 L/min at the begin of the experiment to 80 L min^{-1} at the end of the fermentation.

For *A. niger* G131, the aeration was set at 11 L min^{-1}, from 17 to 24 h of fermentation and it was gradually increased to 55 L. After 41 h, the aeration was progressively reduced to 30 L min^{-1} (Figure 2C). The temperature of the substrate was about 26°C at the beginning of the fermentation, while during the experiment, it increased slightly to reach a maximum value of 48°C, then decreased to 24°C and increased to a final value of 25°C.

The initial weight of the substrate used for the fermentation was 16.5 kg, and it was progressively reduced to a final value of 8.26 kg at the end of the process (Figure 2C).

The water content varied from 66% at the beginning of the process to a lower value of 28% after 137 h of fermentation.

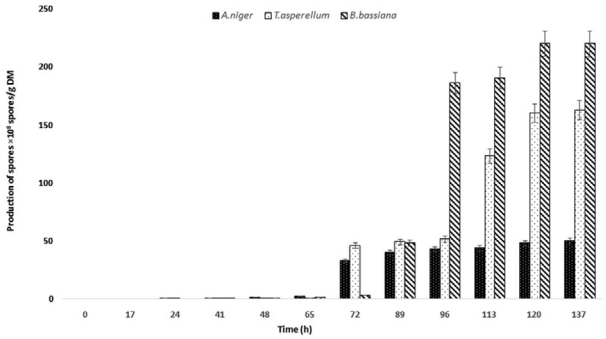

FIGURE 3 Kinetics of spore production under SSF on Zymotis-2

3.2 | Production of fungal conidia on Zymotis-2

Figure 3 summarizes the kinetics of spore production by the three strains tested in SSF using Zymotis-2 bioreactor. The results obtained showed an important concern associated with the high spore production by *B. bassiana*, reaching over $220.01 \pm 0.01 \times 10^8$ spores per g DM at 120 h of culture (Figure 3). Although a long phase without spore formation was observed, the production of spores initiated from the 89 th h of cultivation and then increased to reach the maximum concentration. At the end of the fermentation (137 h), a slight stationary phase was noted, while the spore concentration remained at the same levels.

A spore formation for up to 65 h was observed in *T. asperellum* DWG3, but sporulation was documented only after the first 72 h of incubation. However, the mycelium was noticed after 41 h culture. The spore production increased exponentially from 89 to 96 h of culture (Figure 3), while the highest quantity of conidia was observed at 120 h ($163.00 \pm 0.07 \times 10^8$ spores per g DM) and remained constant until the end of the experiment.

Spores production by *A. niger* G131 started at 72 h of culture ($33.0 \pm 0.07 \times 10^8$ spores per g DM) and it remained constant until 96 h. Then, a steep rise was exhibited to reach the maximum value of ($137.20 \pm 0.07 \times 10^8$ spores per g DM) at the end of fermentation.

3.3 | Determination of the enzyme activities on zymotis-2

For all strains tested, production of enzymes started after 17 h of incubation (Figure 4A, 4B, 4C). For *T. asperellum* DWG3, the highest endoglucanases activity was observed at 120 h of incubation on Zymotis-2 (20.64 ± 0.01 U g^{-1} DM) (Figure 6A).

A slight increase of exoglucanses activity was noticed and the greatest value was noticed at 65h (10.75 ± 0.14U g^{-1} DM at 65 h).

Amylases activity of 4.59 ± 0.01 U g^{-1} DM at 24 h was observed by *T. asperellum* DWG3, the conditions applied on Zymotis-2 bioreactor resulted in the optimum amylases activity, leading to 27.23 ± 0.01 U g^{-1} DM at 137 h.

In comparison with *T. asperellum* DWG3, *B. bassiana* resulted in no significant ($p > 0.05$) production of endoglucanases and exoglucanases.

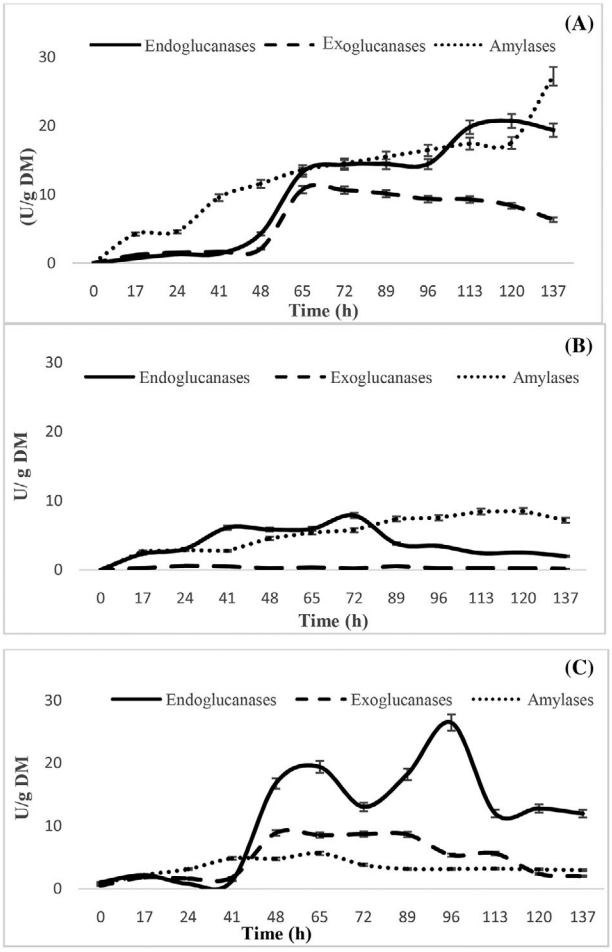

FIGURE 4 Profiles of endoglucanase, exoglucanases and amylases activities produced by *T. asperellum* DWG3 (A), *B. bassiana*, (B) and *A. niger* G131(C) grown on Zymotis-2 bioreactor

The highest endoglucanases activity value (7.89 ± 0.05 U g^{-1} DM) was documented at 72 h of incubation. The production of exoglucanases initiated after 17 h of incubation. *B. bassiana* resulted in an exoglucanases activity of 0.61 ± 0.07 U g^{-1} DM at 24 h. Then, the exoglucanases production decreased to 0.18 ± 0.29 U g^{-1} DM at the end of the experiment (Figure 4B).

A gradual increase in the amylases activity of *B. bassiana* was observed, reaching up to at 8.46 ± 0.01 U g^{-1} DM after 113 h, but a slight decrease down to 7.19 ± 0.07 U g^{-1} DM was noted at the end of incubation period.

On other hand, significant ($p < 0.05$) differences on endoglucanases activity were observed by *A. niger* G131, as a maximum endoglucanases activity (26.49 ± 0.01 U g^{-1} DM) at 96 h of culture was observed (Figure 4C).

The highest exoglucanases activity was obtained at 48 h with a value of 8.91 ± 0.03 U g^{-1} DM.

The maximum value of amylases activity was recorded (5.67 ± 0.07 U g^{-1} DM) after 65 h of cultivation. Then, the enzyme production was slightly decreased and remained stable until the end of the experiment.

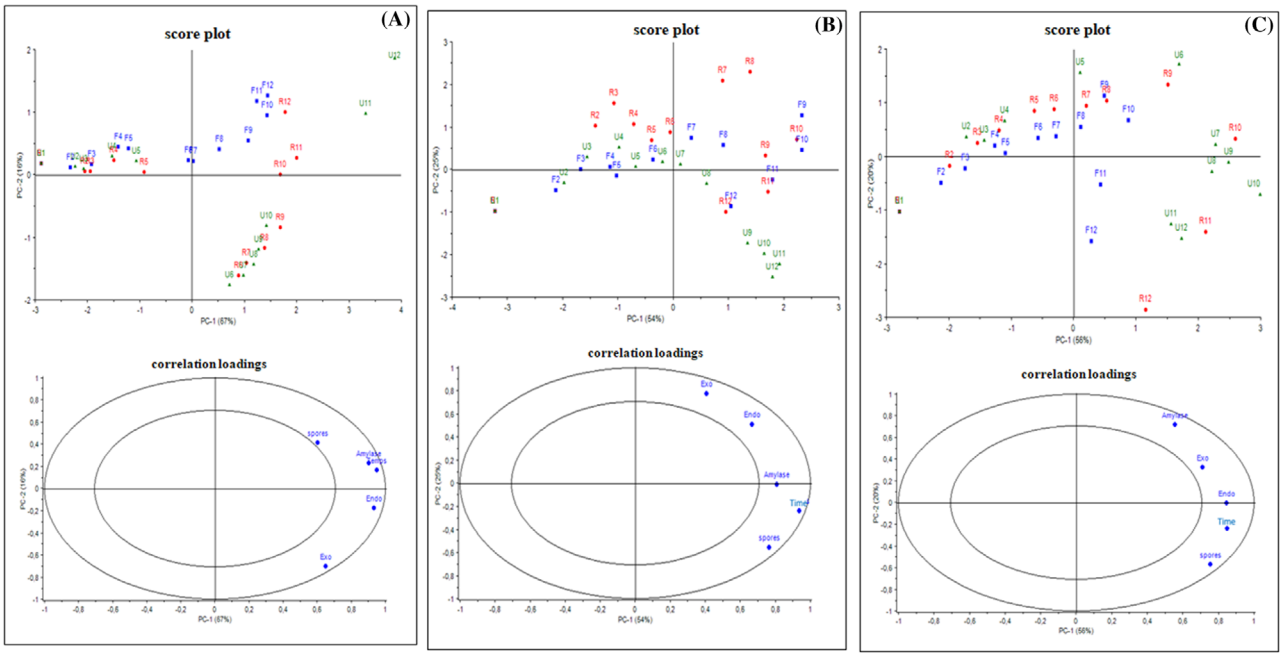

FIGURE 5 PCA on the enzymes and spore production related to the bioreactors variations by *T. asperellum* DWG3 (A), *B. bassiana* (B) and *A. niger* G131 (C)

3.4 | Correlation between strains spores and enzymes production using Zymotis-2, Raimbault columns and flasks by chemometric analyses

3.4.1 | *T. asperellum* DWG3

As a complement of the observation of the kinetic production, another possibility to analyze the data is to use a PCA approach to observe the spores and enzymes production behavior under different experimental conditions (kinetics of production related to the bioreactors used) and compare them. Figures 5A show the score plot and the correlation loadings (PC1 = 67% and PC2 = 16% of explained variance) obtained with three enzymes (endo and exoglucanases and amylases) and spores' production. For short times (0 to 48 h of fermentation corresponding to: F1, R1, U1, F2, R2, U2, F3, R3, U3, F4, R4, U4, F5, R5, U5), enzymes production in the three bioreactors is grouped. Another group corresponds to the Raimbault columns and the Zymotis- 2 bioreactor with greater production of exoglucanases between 65–96 h (the points associated 6 and 9). At the timepoints 11 and 12 related to 113 and 120 h, the Zymotis-2 bioreactor is characterized by greater production for spores, amylases and endoglucanases. At these timepoints, the Raimbault columns and the flasks showed the same behavior.

3.4.2 | *B. bassiana*

The cluster of samples in (PC1, PC2) score plot suggests a temporal evolution on PC1 axis with an explained variance of 54% (Figure 5B). This PC1 axis gathers in its negative part the first 65 h of fermentation (time points 1 to 5).

PCA applied to all compounds (enzyme and spores) produced (PC1 = 54% and PC2 = 25% of explained variance) led to a good separation based on the bioreactors tested.

Flasks and Raimbault columns bioreactors were correctly separated. However, for Zymotis-2, the data corresponding to the kinetics are clustered along positive part of the PC1, indicating that an increase of aeration due to Zymotis-2 application is characteristic of a higher metabolic system and thus compounds production.

The associated correlation loading (Figure 5B) showed that spores are produced in higher quantities in Zymotis-2 for a culture duration ranging between 89 and 137 h corresponding to the time points 8 and 12.

3.4.3 | *A. niger* G131

For short times of fermentation (F1, R1, U1, F2, R2, U2, F3, R3, U3, F4, R4, U4, F5, R5, U5) enzymes and spore production for the three bioreactors are grouped. The interpretation of the correlation loadings allows highlighting the correlations existing between the responses variables. Two groups appear: For time points 5 and 8, the Zymotis-2 bioreactor is characterized by greater production of enzymes (amylases exoglucanases and endoglucanases). At these timepoints, the same behavior in Raimbault columns and in flasks was observed (Figure 5C). Then, there is a grouping for the Raimbault columns and the Zymotis-2.

4 | DISCUSSION

4.1 | Impact of physicochemical parameters on fungal growth on Zymotis-2

Information on bioreactors design and conditions on biomass production by fungus, as well as on the particularities of a certain process on the onset of the fermentation development is scarce. Indeed, the generation of metabolic heat which is excessively increased during the fermentation process is one of the disadvantages of SSF. In this study, a novel SSF configuration was proposed to control such thermal effects. Our process involved application of forced aeration to achieve production of a large amount of viable microbial biomass and/or metabolites, including fungal spores. In addition, it was possible to apply a water stress to the solid support during the spore production phase that stimulated their production, maintained the spore production phase for several days and limited the contamination of the culture medium by other microorganisms. Thus, Zymotis-2 was successfully tested under forced air conditions for the production of spores and fungal cellulosic enzymes by three candidate strains: *T. asperellum* DWG3, *B. bassiana* and *A. niger* G131.

In the present culture system, aeration was the fundamental parameter to induce fungi metabolic activity and sporulation. A low aeration rate of 50 L h^{-1} was settled at the beginning of the fermentation process to initiate the germination of spore. Once germination was completed, aeration rate was increased to 20 and then to 80 L h^{-1} to promote fungal growth and enhance microbial metabolism resulting in the release of heat. The application of forced aeration had the objective of providing the air necessary for fungal growth and the elimination of the carbon dioxide generated during the process. Aeration with dry air is intended to achieve water stress not only to promote conidiogenesis, but also to have a dry fermented product at the end of the process that is easy to handle and use in several applications. The effect of forced aeration on antifungal metabolites, enzymes and spore's production from *T. asperellum* strains was evaluated in a previous work[16] and the results suggested that forced aeration on SSF systems leads to production of high amounts of spores, lytic enzymes and secondary metabolites like 6-pentyl-alpha-pyrone.

Moisture was another important parameter that was studied during Zymotis-2 process, since it indicates the ability of the material to absorb water. Several studies have described the effect of the initial substrate moisture content on the fungal sporulation, enzymes activity and secondary metabolites production with value-added products by different fungal strains cultivated under SSF. Most researchers suggested that a moisture content lower than 55% and higher than 80% are not favorable for fungal growth on SSF.[17] Moreover, an excessively humid environment could be a factor for creating the culture conditions for unwanted microorganisms, increasing the risk of contamination.[10]

In accordance to our findings, a moisture range 40%–60% was evaluated by De la Cruz et al.[18] in order to induce sporulation of *Trichoderma asperellum* using orange peel residues as a substrate. The authors mentioned that (1) no fungal growth was observed at moisture level lower than 40%, probably due to the lack of nutrient diffusion, (2) 70% moisture resulted in stacking of the solid material without fungal growth, and (3) 69.0% was the optimum level of moisture for the development of *T. asperellum*.

The optimum moisture values for SSF depend on the physical structure of the solid substrate (for example, the surface area and the porosity of such support of culture) and on the intrinsic characteristics of the microorganism used.

4.2 | Comparison of fungal spore production and cellulosic enzymes with previous bioreactors

Zymotis-2 bioreactor with forced aeration had a positive effect on spore production, reaching up to $220.01 \pm 0.01 \times 10^8$ *B. bassiana* spores per g DM. Comparing the results obtained in Raimbault columns and flasks, the differences in the profile may be attributed to a desiccation process, possibly owe to stress by the culture conditions, causing, thus, an increase in the spores concentration. Therefore, the forced aeration conditions may have enhanced the production of spores by fungi in Zymotis-2 bioreactor, facilitating the fermentation of large substrate quantity.

Noticeably, the production of spore to be used in biological control or food processing by SSF is a topic currently under investigation, evaluating several approaches, including fungal strains, mixture substrates, bioreactor designs. In this vein, Hamrouni et al.[2] reported 85.48×10^8 spore per g DM of *T. asperellum* TF1 using a mixture of agroindustrial wastes in plastic single used bioreactor. A polyethylene bioreactor was evaluated by De la Cruz-Quiroz et al.[18] to enhance spore production from *T. asperellum* and corn cob as substrate, obtaining 1.4×10^9 spores per g DM. The same substrate was also evaluated by Kancelista et al.[19] on SSF, reporting a yield of 3.13×10^9 spores per g DM. Likewise, a production of 1.8×10^8 spore per g was also reported during cultivation of *Clonostachysrosea* on the same bioreactor described by De la Cruz-Quiroz et al. [18]. On other hand, Singh et al. [20] investigated spore production of *Trichoderma harzianum* under SSF conditions using a bag bioreactor and tea leaves as substrate. A 4.4×10^6 spores per g DM yield was obtained. Similarly, Roussos et al.[10] recorded 3.61×10^{10} and 3.25×10^{11} spores per g DM by *T. harzianum* using sugarcane bagasse as support/substrate in SSF on Raimbault columns and Static bioreactor 'Zymotis', respectively.

According to Hamrouni et al.,[21] forced aeration has a high influence on the stimulation of fungal sporulation of *Trichoderma* strains. They tested a mixture of waste substrates and three *T. asperellum* strains and evaluated an aeration rate of 60 mL min^{-1} g DM^{-1} on Raimbault columns, resulting in 2.23×10^9 spores per g DM at 120 h of culture by *T. asperellum* TF1.

Spore production on Raimbault columns with forced aeration from six *Trichoderma strains* was also evaluated by De la Cruz-Quiroz et al.[22] and a mean production of 1×10^9 spores per g DM was observed, indicating that the forced aeration had no positive effect on the spore production for any of the 6 *Trichoderma* tested.

Jin and Custis[23] reported 1.2×10^{11} spores per g DM of *T. harzianum* using rice grain as a substrate and plastic bags bioreactor. The authors presented good results under the experimental conditions and their values were relatively similar to our present work.

Spore production by *B. bassiana* under SSF using Zymotis-2 was also validated by index sporulation. The highest quantity of spores was noted when using flasks followed closely culture on Raimbault columns. Such a behavior suggests that the production of spores from the genus *Beauveria* is not related with the air application, probably due to a minimal oxygen demand and thus sporulation may not be oxygen-limited. This outcome has a direct impact on the technical feasibility for the spore production by *B. bassiana*, as under these culture conditions it is possible to use flasks, bag bioreactor or Zymotis-2 bioreactor. These results confirm those previously obtained by Dalla-Santa et al.,[24] who tested fungal sporulation by *B. bassiana* on SSF using a mixture of agroindustrial wastes (60% potatoes wastes and 40% sugarcane bagasse) in Erlenmeyer flasks and column-type bioreactor (with forced aeration). They suggested that the forced aeration had no effect on stimulation of *B. bassiana* sporulation (maximum levels were 1.07×10^{10} spores per g DM after 10 days of incubation in Erlenmeyer flasks).

Additionally, Xie et al.[25] reported a maximum concentration of 2.70×10^{9} spore per g obtained by *B. bassiana* cultivated on rice as carbon source. They concluded that sporulation of *B. bassiana* was affected by the metabolic heat and heat decreased spore production.

Pham et al.,[26] studied spore production from *B. bassiana* under SSF using polished white rice and polyethylene bag bioreactor. They reported a maximum spore production of 1.07×10^{10} spores per g DM after 10 days of incubation.

The results of the studies on comparative production of endoglucanases, exoglucanases and amylases enzymes by *T. asperellum* DWG3, *B. bassiana*, and *A. niger* G131 in parallel fermentations under identical conditions suggested that application of aeration acted beneficially on the production of enzymes by *T. asperellum* DWG3. Enzyme biosynthesis was monitored during *T. asperellum* growth on Zymotis-2 bioreactor, followed by the culture of the fungi on Raimbault column and flasks.

However, production of endoglucanases and exoglucanases by *B. bassiana* was negatively affected by Zymotis-2 bioreactor and Raimbault columns compared to the enzymatic activity determined on flasks. In contrast, air application represented an increment on amylases activity.

Several reports have been published on cellulolytic enzymes production from different *Trichoderma* sp. De la Cruz Quiroz et al.[22] reported a maximum exoglucanases activity (3.14 U g^{-1} at 24 h) by *T. asperellum* T2-31 after 24 h of incubation using SSF on a mixture of sugarcane bagasse, wheat bran, chitin, potato flour, and olive oil. In the present study, a maximum value of 10.86 U g^{-1} DM endoglucanases activity by *T. asperellum* after 65 h of SSF on Zymotis-2 bioreactor was reported. Likewise, Roussos et al.[10] have reported endoglucanases activity of up to 128.03 and 71.85 IU g^{-1} DS when *Trichoderma harzianum* was grown on a mixture of sugarcane bagasse, wheat bran and mineral medium using 'Zymotis-1' bioreactor and Raimbault column respectively. The difference in the enzymatic activity can be explained by the nature of the strain and the composition of the solid medium.

In this context, a screening study was performed by De la Cruz-Quiroz et al.[18] to identify influential factors on endoglucanases produced by a strain of *T. asperellum* using corn cob as substrate and a polyethylene bioreactor. The factors tested were: pH (6.0), inoculum (1×10^{7} spores per g), temperature (24°C), moisture (66%), inducer (1%) and time (5 days). They mentioned that moisture, pH and inoculum were the factors that mainly determined endoglucanases production. Following the same strategy, Hamrouni et al.[21] investigated optimal formation of carboxymethyl cellulase by growing 3 *T. asperellum* strains on different culture medium at the same physicochemical conditions and concluded that enzymatic activity depends on the composition of the culture medium and the nature of the strain.

There is a lack of information regarding *B. bassiana* and hydrolase enzymes production on SSF. Petlamul et al.[27] revealed that *B. bassiana* had the ability to release cellulase enzyme for cellulose degradation and evaluated cellulase enzymes activity when *B. bassiana* was grown on different substrates in the presence of glucose on the media.

Previously, Dhillon et al.[28] achieved endocellulase activities up to 30.06 and 58.16 IU g^{-1} ds with *Aspergillus niger* (120 h) and *T. ressei* (96 h), respectively, using rice straw as solid substrate. The mixture of the strains resulted in an increased cellulase activity of 74.00 IU g^{-1} ds.

Likewise, previous studies have indicated production of cellulosic enzymes by *A. niger* strains. Lakshmi and Narasimha[29] reported 5.55 and 64.00 U mL^{-1} h^{-1} of carboxymethyl cellulase activity after cultivation of *Aspergillus* strains in Czapek-Dox broth medium amended with 1% cellulose. A production of 43.32 U mL^{-1} $h^{-1}$was also recorded in *Penicillium* culture in the same medium.

4.3 | Advantages of Zymotis-2 bioreactor

Zymotis-2 bioreactor can be used for several applications, including the production of biopesticides for agriculture, biorefineries like cellulosic ethanol production from lignocellulosic biomass, food additives, digestive enzymes (amylases, cellulases, phytases, etc.) for animal feed, detergents, and so on. The spore production by SSF using Zymotis-2 bioreactor is a relatively easy process, it requires personnel with no experience, and therefore technology transfer to farmers is feasible.

Additionally, Zymotis-2 bioreactor is considered advantageous regarding the storage of the fermented products. Indeed, at the end of culture, the dried fermented product can be stored inside the fermentation chamber and the whole mixture can be transported wherever is needed. It is also possible to introduce liquids into the fermentation chamber, such as water, buffers or solvents and mix with the fermented substrate. The enriched liquid with the microorganism and metabolites can be collected into the basal portion.

Finally, Zymotis-2 bioreactor has the ability to increase the storage and viability time of the biomass, by allowing an aseptically gas exchange between the microbial strains and the external environment.

5 | CONCLUSION

In conclusion, a significant increase on spore production by *T. asperellum*, *A. niger*, and *B. bassiana* strains on Zymotis-2 bioreactor at a mixed substrate, consisting of vine shoot, wheat bran and olive pomace loads, was reported. The results suggested an operational stability of Zymotis-2 bioreactor for the SSF to produce spores in a 137 h of process. Moreover, the forced air supply into SSF allowed control of dry velocity and contributed to spore production with a high sporulation index and yield. The culture conditions tested confirmed the validity of the new Zymotis-2 bioreactor, since spores production of BCA's microorganisms is essential to be used as biopesticides. Application of SSF, agro-industrial residues and Zymotis-2 bioreactor is expected to facilitate development of novel formulations to be used in field crops, decreasing process costs. Finally, although there is available data on spores fungi topics in the literature, continuous investigation on enhancement of bioactive metabolites production by filamentous fungi, using Zymotis-2 bioreactor through evaluation of different kinds of agro-industrial wastes, product formulation and viability of spores is always an intriguing issue.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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DATA AVAILABILITY STATEMENT

The data are available by request from the corresponding author.

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