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Bacillus thuringiensis derived biopesticides from biowaste digestate at 290-L demonstration scale through solid-state fermentation

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ABSTRACT

This study faces the challenge of scaling up and validating the production of *Bacillus thuringiensis* (Bt) derived biopesticides at both pilot (100-L) and demonstrative scale (290-L), following a novel operation strategy for solid-state fermentation (SSF) based on two consecutive and differentiate aeration stages. Digestate and vegetable biowaste served as the carbon and nutrient source for Bt growth and sporulation. The operation strategy, which combines an anoxic and an aerobic phase, was successfully implemented at the pilot scale, achieving proper temperature control, which is crucial for SSF development at commercial scale. A total final concentration of spores was achieved on the order of 10^7 spores per gram of dry matter with a production ratio of 2.4 spores per inoculated Bt viable cell. Results at the demonstrative scale were hindered, likely due to the alteration of the anoxic environment during reactor sampling and the longer anoxic time. The fermented solids with biopesticidal properties, could be considered compost-like amendments exhibiting good maturity based on low respirometric indices and phytotoxicity. This study underscores the importance of addressing these challenges to enhance the sustainability of biowaste management practices and promote the transition towards a circular economy model.

1. Introduction

Municipal solid waste generation is one of the issues of concern related to population and economic growth (Fan et al., 2020). Biowaste is defined as "biodegradable garden and park waste, food, and kitchen waste from households, restaurants, caterers, and retail premises, and comparable waste from food processing plants" (Directive 2008/98/EC). Even though the biological treatment of source-selected urban biowaste is extensively applied in urban and peri-urban areas, as the Waste Framework Directive targets, actions should be done to drive the recovery of nutrients, organic matter, and potential value-added bioproducts from waste towards a circular economy model. In terms of waste management, actions like "turning waste into a resource" and "closing the loop" are highlighted actions for treating unavoidable biowaste and introducing it as raw materials for other processes, changing the perception of being a problem to being a resource (European Commission, 2019). In this sense, the European project DECISIVE ("A Decentralized

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Management Scheme for Innovative Valorization of Urban Waste") aimed to demonstrate the feasibility of decreasing the generation of urban biowaste and increasing the recycling and recovery of materials through decentralized management, valorizing biowaste in a short cycle, and moving into a zero-waste strategy.

At present, in some European regions, biowaste from urban and peri-urban areas is source-selected, collected, transported to a centralized biological treatment plant and treated using anaerobic digestion and/or composting processes (Abad et al., 2019). Two main products are obtained from anaerobic digestion systems: biogas, a biofuel gas rich in methane; and digestate, a carbon and nutrient-rich organic material classified as component material (CMC) for EU fertilizing products (Regulation (EU) 2019/1009). With the aim of enhancing and improving the system of biowaste management, treatment, and the further use of the products obtained, the DECISIVE project proposed a decentralized treatment based on micro-anaerobic digestion (mAD) coupled with a Stirling engine, and solid digestate valorization through Solid-State Fermentation (SSF) technology. In this sense, a wide range of products, such as heat, liquid fertilizer and solid fertilizer enriched with bioactive compounds were obtained.

The bioconversion of organic waste into value-added products offers numerous advantages (Dantroliya et al., 2022). SSF is a suitable technology to produce marketable bioproducts using a solid matrix including agricultural, urban, or agro-industrial organic waste as substrates (Schmidt et al., 2014). It is defined as the fermentation process that takes place in the absence or near absence of free water but with the appropriate moisture content to support microbiological growth. Its reaction media consists of a solid-gas-liquid system, as the fermentation substrate is solid, the gas is used as a continuous phase, and a thin layer of water holds the microorganism's growth (Thomas et al., 2013).

Previous studies have demonstrated the feasibility of valorizing the solid fraction of digestate using SSF to produce enzymes (Mejias et al., 2018; Santi et al., 2015), ameliorated fertilizer combined with fungal biomass (Alias et al., 2022), and biopesticides (Cerda et al., 2019; Mejias et al., 2020; Rodríguez et al., 2019). In these regards, promising results were reported using the solid fraction of digestate as a support matrix, carbon and nutrient source for *Bacillus thuringiensis* (Bt) growth and sporulation. Bt is a Gram-positive, sporulating, and facultative-aerobic bacterium that is widely used as a biopesticide. During its sporulation phase, it synthesizes a crystalline inclusion that contains δ-endotoxins or Cry proteins (Sanchis and Bourguet, 2009). Some studies have used SSF technology to produce Bt-derived biopesticides. In our previous work we established a novel two-stage aeration strategy for Bt based biopesticide production via SSF of biowaste digestate at lab scale (Mejias et al., 2020). Molina-Peñate et al., (2023) used two different digestates (from biowaste and sewage sludge) combined with the solid fraction after biowaste enzymatic hydrolysis with good results when working with 4.5 kg of organic matrix. Zhang et al. (2013) reported successful results when working with 4–8 kg of kitchen waste. However, the spore production decreased when the process was scaled up to 35 kg. This reduction in production is due to heat retention and temperature gradients occurring in solid matrices when increasing scale, which is the main challenge related to SSF scalability (Ashok et al., 2017; Rodríguez et al., 2021).An increase in temperature has direct consequences on the process, like water evaporation and issues in the maintenance of water activity, as well as problems related to the development of mesophilic microbial population. Scale-up studies for SSF are scarce in literature and most of the publications report studies using few grams of substrate. It is essential to address SSF scale-up in order to develop this technology sufficiently to help fill the gaps in the implementation of circular bioeconomy.

This study faces the SSF scale-up of a Bt-derived biopesticide production system from the solid fraction of biowaste digestate. We aim to integrate and validate at pilot (100-L) and demonstrative scale (290-L) the technological approach for digestate valorization producing Bt-derived biopesticides developed by Mejias et al. (2020) and patented procedure (Artola et al., 2021). The process was carried out by fermenting an organic mixture load of 25–70 kg per batch. Additionally, the quality of the fermented material was assessed in view of its potential use as soil amendment. In addition, attention was paid to the identification and quantification of microplastics in both the initial digestate and the fermented solids as this is a transversal arising issue in biowaste treatment.

2. Methodology

2.1. Strain and inoculum preparation

Bacillus thuringiensis var israelensis (CECT 5904) was obtained from Colección Española de Cultivos Tipo (CECT, Valencia, Spain). The strain was cryopreserved at −80ºC in cryovials which contain cryopearls impregned with Bt.

For pre-inoculum preparation, 100 mL of sterile Nutrient Broth Nº2 (Oxoid) was placed in a sterile 500 mL Erlenmeyer flask and inoculated with a cryopearl under sterile conditions. Once the pre-inoculum reached its exponential growth (18 hours), 1 mL aliquot was used to inoculate 380 mL of fresh media placed in a 1-L Erlenmeyer flask. A total of 14 1-L Erlenmeyer flasks were required for reaching 5-L Bt culture. After 20 hours, the inoculum was centrifuged (3500 rpm, 10 min, 25ºC) and the supernatant was discarded and placed in a sterile flask. The pellet obtained from 100 mL of culture was resuspended with 3 mL of supernatant. The resuspended pellet was recovered and diluted 1/10 with supernatant. The concentration of the processed inoculum was around 5×10^8 CFU mL^{-1}. No spores were detected at this point.

2.2. Substrate conditioning and inoculation

Digestate and fruit and vegetable biowaste (FVB) were provided by the solid waste treatment plant of Granollers (Barcelona, Spain). The selected biowaste used in this study came from a weekly local market. Before its use, FVB was shredded, and both materials (digestate and FVB) were hygienized separately (70ºC for 1 hour) as specified in the Commission Regulation (EU) No 142/2011. After the thermal pre-treatment, digestate and FVB presented the following characterization, respectively: total solids (%): 29 ± 2 and 11 ± 3; volatile solids (%, dry basis): 68 ± 1 and 91 ± 3; pH: 8.6 ± 0.3 and 4.6 ± 0.5; Dynamic Respirometric Index (DRI, g O_2 kg^{-1} dry

matter h^{-1}): 1.7 ± 0.5 and 4.6 ± 0.7.

2.3. Fermentation systems

Two bioreactors were used in this work and are shown in Fig. 1. The fermenters were cylindrical and made of stainless steel, equipped with a helicoidal mixer and a perforated plate to separate the solids from the air distribution chamber. At the bottom of the reactor, a perforated metal sheet supported the solids, forming an air distribution chamber. This design prevents air inlet obstructions and ensures even air distribution throughout the solid matrix. The air from the airflow meter was fed at the bottom, exited at the top, and was sent to an oxygen sensor. The 100- L reactor system (R100) has been described previously (Rodríguez et al., 2021) and was used in the GICOM lab facilities at the *Universitat Autònoma de Barcelona* (UAB). This reactor has a diameter/height ratio 0.575 and wall surface/volume 0.885 m^2/m^3.

The demonstrative reactor of the DECISIVE H2020 project (R290) was designed by UAB and *Aeris Tecnologias Ambientales* (Cerdanyola del Vallès, Barcelona, Spain). The reactor was installed in the waste treatment plant of the *Consorci per a la Gestió dels Residus del Vallès Oriental* (Granollers, Barcelona, Spain). It consisted of a 290-L vessel (diameter/height ratio 0.678 and wall surface/volume 0.664 m^2/m^3), equipped with a dual airflow supply (60–600 NL h^{-1} rotameter, PT-11/PVC Tecfluid; and 300–3100 NL h^{-1} volumetric airflow meter, Tecfluid M21), oxygen sensor (O2-A2, Alphasense, UK), fix temperature prove (Pyro-Alloy TR-NNKp-250), and helicoidal stirrer with a 1 kW engine and a frequency inverter. The scheme of the reactor can be found in Mejias (2020). Even though the reactor was equipped with a helicoid stirrer, mixing could not be applied successfully as a strategy for bed-temperature homogenization. Previous experiences reported the solid mixture was not homogenized ideally due to the sludge-like characteristics of the substrate, such as plasticity.

Regarding the aeration system, an inlet airflow connection was installed at the bottom of the reactor. Inlet air diffused between the pores of a metallic net (2 mm pore size) that sustained the soil and passed through the solid-packed bed. The outlet gas exiting from the top of the reactor was channeled through a water trap and ended up in an oxygen sensor box for online oxygen concentration monitoring. The temperature was monitored at different points of the packed bed using iButton Devices (Thermochron, UK). Specific oxygen uptake rate (sOUR) was calculated according to the following Eq (1) (Almeira et al., 2015):

$$sOUR = F \cdot \left(0.209 - y_{O2}\right) \cdot \frac{P \cdot 32 \cdot 60 \cdot 1000a}{R \cdot T \cdot DW \cdot 1000b}$$

Where sOUR is the specific oxygen uptake rate (g O_2 kg^{-1} DM h^{-1}); F, the airflow rate into the reactor (mL min^{-1}); y_{O2}, oxygen molar fraction in the exhaust air (mol O_2 mol^{-1}); P, the pressure of the system assumed constant at 101325 (Pa); 32, oxygen molecular weight (g O_2 mol^{-1} O_2); 60, conversion from minutes to hours; 1000a, conversion from mL to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, the temperature at which F is measured (K); DW, dry weight of solids in the reactor (g); 1000b, conversion from g to kg.

Fig. 1. Images of the bioreactors. A) R100 (in front) and R290 (back) at GICOM's facilities in UAB; B) R290 in Granollers (Barcelona, Spain) biowaste treatment plant during the execution of the experiments reported herein.

2.4. Operational strategy

The operational guidelines were followed from Mejias et al. (2020). More specifically, the mixture of digestate, FVB, and wood chips acting as a bulking agent was set at 47:28:25 % (wet weight basis). Since substrates were not sterilized, the environmental conditions to boost Bt competitiveness among other autochthonous microorganisms were considered a key factor. Hence, the operational strategy developed in the abovementioned study was applied to both operating scales (100-L and 290-L) in order to validate it at pilot and demonstrative scale. The operational strategy aimed to promote *Bt* growth and sporulation by using two aeration stages during the fermentation of digestate and FVB. The first stage, a micro-aerated phase, involved limiting the airflow to maintain oxygen levels below 5 %, which reduced heat accumulation in the solid matrix and created a competitive environment favoring *Bt* growth over other microbial communities. After 24–30 hours, a second fully aerated phase was introduced, with oxygen levels above 17 %, to encourage *Bt* sporulation and the production of biopesticide crystals. Fermentation durations ranged from three to five days, depending on the experiment.

2.5. Sampling and analytical methods

For the R100, samples were taken at the end of the fermentation process and for the R290, samples from three positions (bottom, middle, and top of the packed bed reactor) were taken daily throughout batch fermentation via sampling ports. Dry matter (DM), organic matter (OM), and pH were analyzed following the standard procedures (US Composting Council, 2001). The determination of viable cells and spores was performed as described by Ballardo et al. (2016). Briefly, a solid-liquid extraction (1:10, w/v; 180 rpm for 20 min) was done using a Ringer solution. Serial dilutions were prepared and plated onto Petri dishes with Nutrient agar medium (Oxoid). For spore determination, a thermal shock was needed to lysate viable cells. Thus, an aliquot from the liquid extraction was placed at 80ºC for 10 min and then placed into ice. The sample was then serially diluted and plated. Petri dishes were incubated at 30ºC for 20 hours. Results were expressed as CFU or spores per g^{-1} DM. The sporulation ratio (%) indicated the relation between the spore and total CFU concentration. The production ratio was calculated dividing the final spore count by the initial viable cells count.

The phytotoxicity of the fermented product was evaluated following the guidelines of (Komilis and Tziouvaras, 2009). An aqueous extract from the solid was obtained by adding 20 mL of water per 10 g of sample (v/w, on a wet weight basis). The extract was centrifuged and filtered through a 0.45 μm filter. The liquid part was then placed in several Petri dishes with filter paper discs together with 10 cucumber or radish seeds at room temperature for 7 days. A control was performed with distilled water. The germination index was calculated considering the relative seed germination and the relative root growth.

Dynamic Respiration Index (DRI) and total oxygen consumption in 4 days (AT_4) where analyzed according to Ponsá et al. (2010) as well stablished stability parameters for compost and compost-like amendments.

2.6. Microplastic identification and quantification

Microplastics (MPs) identification and quantification from the solid organic samples were performed in collaboration with the Institute for Energy and Environmental Technology (IUTA) of Duisburg (Germany). MPs were identified and quantified using thermal extraction desorption gas chromatography-mass spectrometry (TED-GC-MS). Analytical conditions were taken from (Eisentraut et al., 2018). Before TED-GC-MS, a density separation was required for sample conditioning and MP concentration. A saturated NaCl solution was prepared (1.2 kg NaCl/800 mL ultrapure water) and mixed with a weighed dried solid sample. The sample was vigorously mixed for 10 min with a stirring rod, followed by a sedimentation process of 8 hours. The supernatant was then skimmed off with a 50 μm sieve, dried overnight at 60ºC, and finally homogenized using a mortar and pestle.

In the TED-GC-MS analysis, samples are heated in a thermogravimetric analyzer (TGA) unit (TGA 2 with autosampler, Mettler Toledo, Germany) in a nitrogen atmosphere. 70 μl alumina crucibles were used to hold samples during the pyrolysis, following the set parameters: 2 minutes isothermal start at 25 °C, N_2 flow 20 mL min^{-1}. Then 25 – 600ºC at 10ºC min-1, N_2 flow 50 mL min^{-1}. Terminating with a 3 min isotherm at 600ºC, N2 flow of 50 mL min^{-1}. The nitrogen gas is used to purge the decomposition products out of the TGA unit and to transfer them into a 3.5-inch thermal coupling tube (Gerstel GmbH & Co KG, Germany), which is coupled to the decomposition product gas flow only in selected temperature ranges. After the adsorber is coupled with the decomposition products, it is transported via the autosampler arm (MPS, Gerstel GmbH & Co KG, Germany) to the thermal desorption unit (TDU) (Gerstel GmbH & Co KG, Germany). Here, the decomposition products are thermally desorbed and mobilized, cryo-focused on a cooled injection system (CIS4, Gerstel GmbH & Co KG, Germany), separated through a gas chromatograph (GC7890, Agilent, USA) with a (HP 5 ms Ultra Inert 30 m x 250 μm x 0.25 μm, Hewlett Packard, USA) column, and detected in the mass spectrometer (MSD 5973 N, Agilent, USA).

The adsorber is first heated up in the TDU from 50 – 300ºC at a rate of 40 °C min^{-1} which terminates with a 300ºC isotherm for 5 minutes, using splitless mode and a helium atmosphere. This carries the decomposition products from the solid phase into the cooling injection system to be cryo-focused from an initial temperature of −120ºC to 270ºC at a rate of 12°C sec^{-1}. The decomposition products then enter the GC column, where they are separated in the GC oven with a temperature range of 40 – 300ºC at a rate of 5ºC min-1, which then ends with an isothermic step at 300ºC for 4 minutes with a helium purge flow of 3 mL min^{-1}. The separated products can then enter the MS detector, which is set to the following (source 230ºC, quad 150ºC, energy 70 eV, low mass 30, high mass 450).

3. Results and discussion

3.1. Process and operation strategy scale up to 100- L

The operation strategy consisted in two different consecutive aeration stages to provide a first microaerobic phase followed by a fully aerobic environment after 24 hours (Mejias et al., 2020). This operation strategy was successfully applied at System R100 on a fermentation load of 25 kg. Fermentation was performed in duplicate (SSF1 and SSF2), and spore concentrations were only analyzed once at the end of the process, this is, the fermentation reactor was not opened throughout the fermentation duration (72 hours). Fig. 2 shows temperature, oxygen, and airflow profiles for SSF1 and SSF2. Micro aeration was applied for the first 24 hours (0.08 L min^{-1} kg^{-1}) leading to low oxygen contents below 5 % at the end of this period and slow temperature rise. After increasing airflow (0.2 L min^{-1} kg^{-1}), packed-bed temperature decreased. Maximal temperature difference between different material layers in the bioreactor was 8ºC for replicate SSF1. After 72 h, total spore count was on the order of 10^6 spores per gram of DM and a ratio of 0.13 spores to initial CFU was obtained (Table 1), being these values below previous results at lab scale (Mejias et al., 2020).

When performing the process duplicate SSF2 (Fig. 2), the temperature increase was faster, likely due to the higher biodegradability of the digestate batch. For this reason, aeration was stopped, promoting low, near-to-zero oxygen levels within the bioreactor. At process time 24 h, aeration was re-started, leading to a temperature rise and oxygen levels over 15 % in the out gases. Maximum temperature range observed within the reactor was 7ºC. This more restrictive oxygen conditions led to a higher spore to initial CFU ratio of 2.4 (Table 1) and total spore count on the order of 10^7 spores g^{-1} DM, thus confirming the effectiveness of the proposed strategy. Although these results are positive, production is still lower than previous results at lab scale using constant temperature, due to temperature dynamics affecting overall performance. Implementing strategies to manage temperature gradients would facilitate the scale-up process.

Results show how the operation strategy proposed by Mejias et al. (2020) was successfully scaled up to 100- L bioreactor and promoted the Bt spore production reaching a spore to initial CFU ratio value higher than 1.

3.2. Scale-up to a 290-L demonstration scale and effect on packed-bed temperature profiles

The airflow rate upscaling criterion was set by maintaining the same specific airflow rates as those used in the 100-L pre-demonstration scale reactor (SSF1): 0.08 L min^{-1} kg^{-1} that translated in a total airflow of 5.4 L min^{-1}. However, this scale-up

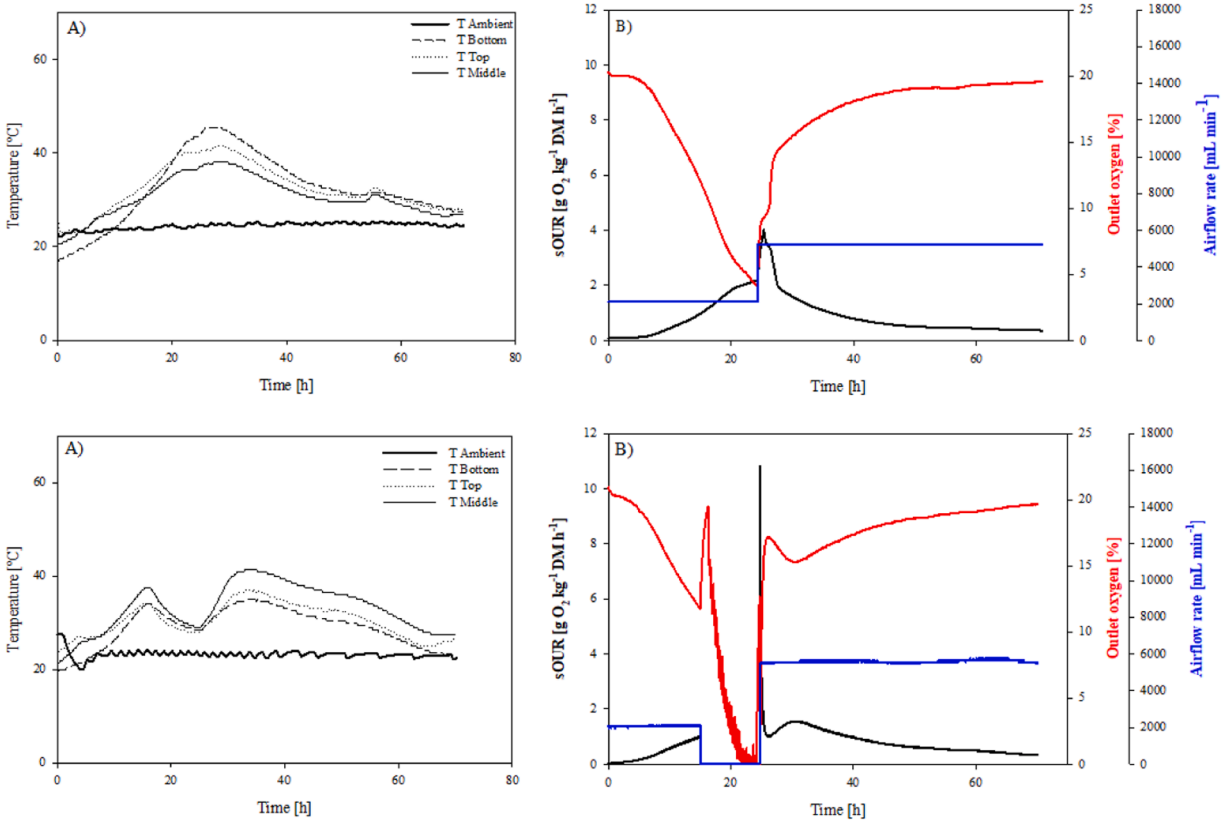

Fig. 2. Temperature (A) and respiration (B) profiles for replicates in R100 SSF1 (microaeration, up) and SSF2 (aeration interrupted, down).

Table 1
Production yields of biopesticide at different reactor scales tested.

	SSF 1	SSF 2	SSF 3	SSF 4	SSF 4 replicate
Reactor volume [L]	100	100	290	290	1.6
Aeration condition 1st stage	Micro-aeration	Micro + non-aeration	Non-aeration	Micro + non-aeration	Micro + non-aeration
Initial CFU [CFU g^{-1} DM]	1.7×10^7	7.6×10^6	2.1×10^7	2.7×10^7	3×10^7
Final CFU [CFU g^{-1} DM]	2.7×10^6	2.6×10^7	n.a.	n.a.	6.6×10^6
Final spore [spore g^{-1} DM]	2.2×10^6	1.8×10^7	9.7×10^5	1.3×10^6	4.7×10^6
Sporulation ratio (%)	82	68	n.a.	n.a.	71
Spores to initial CFU ratio	0.13	2.4	0.05	0.05	0.16

n.a., not analyzed

criterion was not adequate since limiting oxygen conditions were not fully achieved in the initial phase (minimum O_2 values of 10.4 %), which impacted overall process performance, particularly affecting temperature profile evolution. As shown in Fig. 3A, temperature sensors at different axial points in the packed bed recorded significant temperature gradients during fermentation, leading to thermophilic temperatures (above 45°C) at 5 of the 15 monitored points. A maximum temperature gradient of 39.5°C was observed at 37 hours. As expected, lower temperatures were recorded at the bottom of the packed bed due to convection, while biogenic heat accumulated in the medium and upper parts of the bed. This led to a non-homogeneous heat distribution, which could affect Bt growth and the production of the targeted product. The lack of Bt growth was most likely due to the thermophilic temperatures and the failure to achieve anoxic conditions in the vessel.

As the diameter of a bioreactor increases, the effectiveness of cooling through its walls diminishes (Perez et al., 2019). For instance, the external surface-to-volume ratio of the R290 bioreactor is only 75 % of that of the R100 bioreactor, resulting in significantly reduced heat dissipation capacity. This explains the broader temperature range and higher temperatures observed in the R290. To compensate for this reduction in surface-to-volume ratio, higher aeration rates or alternative cooling mechanisms are necessary. For example, Pitol et al. (2016) adjusted air temperature to effectively manage temperature and optimize enzyme activity in a 200 L SSF system. Similarly, Biz et al. (2016) used a humidification column, while Nagel et al. (2001) combined continuous reactor mixing, wall cooling, and evaporative cooling. Consequently, additional energy will be required to maintain the temperature within the desired range, whether through enhanced aeration or by implementing one or more of these cooling strategies. This additional energy requirement and associated costs should be considered when evaluating the scale-up strategy. Alternatively, working with a limited oxygen supply can keep metabolic activity at low levels, thus reducing the heat release. This two-stage aeration strategy could potentially offer energy savings compared to the cooling methods mentioned above.

3.3. Airflow rate strategy validation at a demonstrative scale

Two experiments were carried out testing two modifications on the aeration regime applied (namely SSF3 and SSF4), focusing on mitigating the temperature rise over Bt vegetative growth cycle (first 24 hours) through micro- or non-aeration periods (Table 1). SSF 3 consisted in not providing air during the first stage (24 hours) for reaching both crucial operational conditions, which were anoxic conditions and avoiding temperature increase. SSF3 experiment performed as expected in terms of oxygen profile, reaching a minimum oxygen concentration of 7.1 % at 25 hours. Temperature within the reactor during the anoxic phase was maintained between 20 and 30ºC (Fig. 3B), which resulted in an encouraging achievement since the batch was performed in hot season when higher air temperatures can lead to a higher packed bed temperature' increase. The increase in temperature occurred during the aerated phase. The temperature ranged from 27 to 55ºC, with a maximum temperature gradient within the reactor of 22.5ºC at 40 hours of the process. Even though mesophilic temperatures were maintained during the Bt growth phase, the viable cell and spore count did not achieve the expected values (Fig. 4). The viable cell concentration slightly increased in the anoxic phase (from 2.1 to 2.7 $\times 10^7$ CFU g^{-1} DM, as an average value). However, the abundance of other microorganisms in the aerated phase samples prevented Bt quantification. Concerning the spore concentration, maximum values of 2.9 and 2.4 $\times$ 10^5 spores g^{-1} DM were monitored at the top part of the packed bed at 48 and 72 hours. An important spore production gradient was observed across the three sampled points. This low final spore concentration affected the final spore yield, achieving an average yield of 0.05 produced spores per inoculated CFU.

A second batch, SSF4, was performed with the aim to promote oxygen consumption and extend the anoxic phase, expecting higher Bt growth values and higher probabilities of increasing the spore yield. In this sense, the specific airflow rate introduced was reduced to 70 % (0.03 mL min^{-1} g^{-1}) and applied over the first 5 hours to activate the biological activity of the substrate. This modification helped to shorten the lag phase preceding the degradation of organic matter. The air supply was cut afterward, and the anoxic phase was extended up to 44 hours after the process started. Once the aeration was turned on, an oxygen value of 2.5 % was monitored, indicating the success of the implemented action. It should be highlighted that the amount of inoculum required for this demonstrative scale (290- L) was around 5 L, therefore, future work should explore the standardization and reproducibility of inoculum preparation process in a liquid bioreactor. Alternatively, sequential batch strategies to avoid the need of large volumes of inoculum could be explored (Sala et al., 2021).

In terms of temperature evolution, the extension of the non-aerated stage also caused a delay in the temperature increase, which occurred when a total aerobic environment was achieved (Fig. 3C) similar to SSF3. The temperature was kept between 20 and 30ºC during the first 44 hours of the process, which should be a suitable temperature condition for Bt growth. Maximum punctual

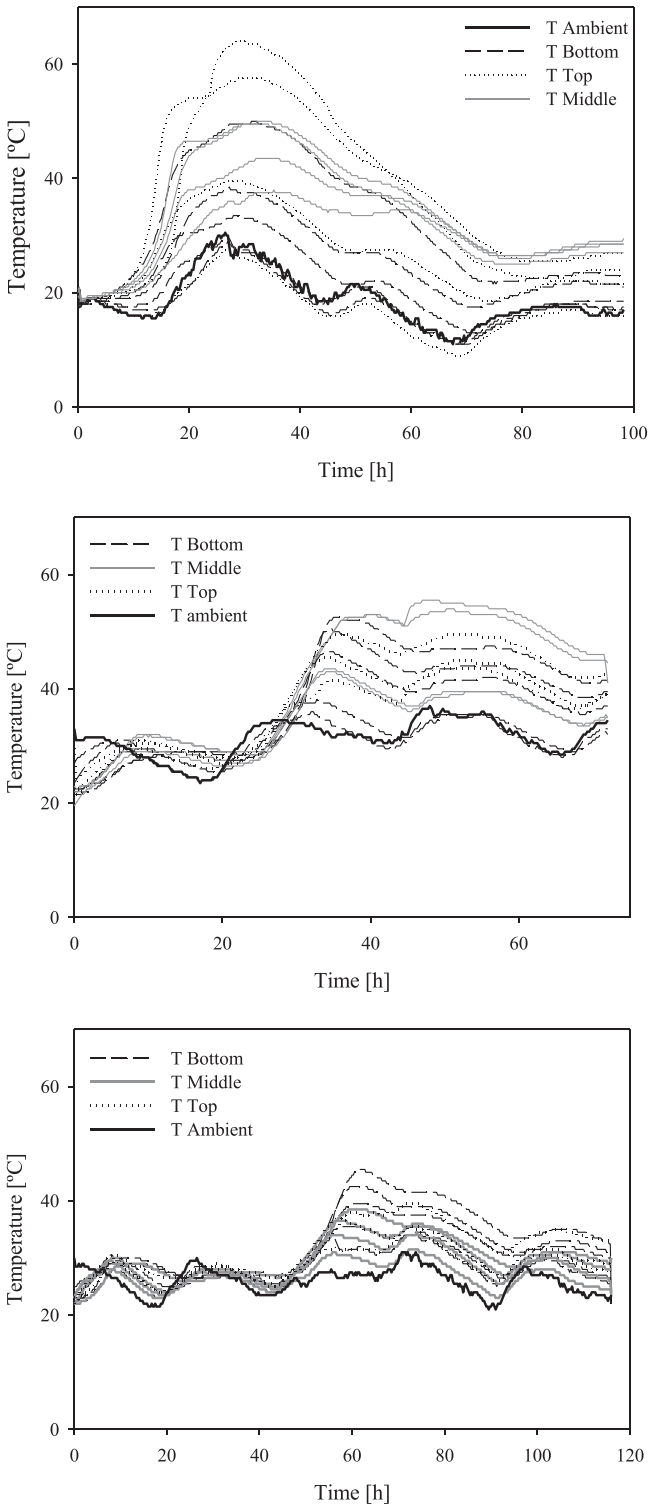

Fig. 3. Packed bed temperature profiles at demo scale in R290-L: A) constant aeration; B) and C) SSF 3 and SSF 4, replicates first 24 hours of the process without air supply.

temperatures of 46°C were monitored at the bottom part of the packed bed once the second stage started. Even though ambient temperatures ranged from 20 to 30°C, no thermophilic conditions were achieved over the second stage. This could be a side-effect of higher organic matter degradation on the anoxic phase, leading to less available biodegradable carbon in the second stage, and

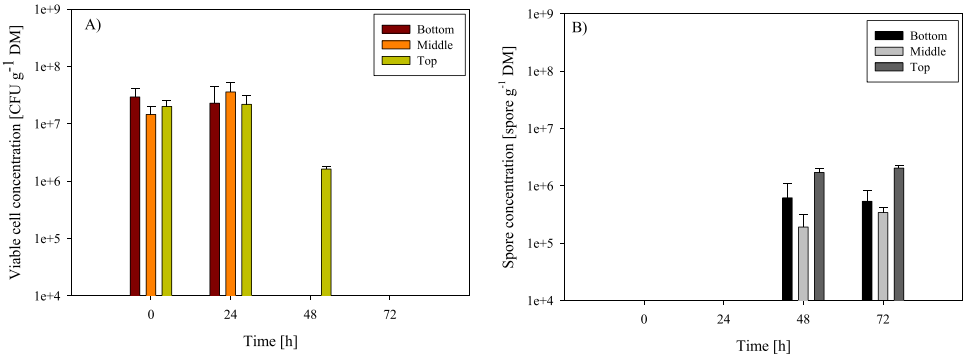

Fig. 4. CFU and spore production of SSF 3. Bt Viable cell concentration (A) and Bt Spore concentration (B).

consequently, not reaching thermophilic temperatures. Thus, better reproduction of controlled-like conditions in terms of temperature was achieved in SSF4.

The oxygen and temperature conditions achieved in SSF4 promoted the Bt growth, reaching values close to the previously reported results at laboratory scale (6.9, 8.6, and 2.4-fold increase at 22 hours on the bottom, middle, and top parts, respectively) (Fig. 5A). However, this value was reduced at 28 hours (from 1.2 $\times 10^8$ to 5.2 $\times 10^7$ CFU g^{-1} DM) and significantly reduced at 43 hours (5.9 and 2.2 $\times 10^6$ CFU g^{-1} DM). Besides, it was not possible to identify Bt in the subsequent sampling because of the presence of other microorganisms. One hypothesis for this reduction in CFU concentration was the alteration of oxygen conditions on the reactor headspace when sampling. Even though Bt is an anaerobe facultative bacterium, implying a surviving capacity in anaerobe conditions for a certain time, previous results demonstrated that in the studied working conditions (digestate and biowaste not sterilized but hygienized) the anoxic environment for boosting Bt growth is the fundamental advantage versus other autochthonous microorganisms. The alteration of these conditions when opening the bioreactor for sampling could have negative effects on the targeted bacteria growth. In addition, opening the reactor in the complex environment of a real waste management facility poses an extra challenge with stronger contamination issues.

The loss of Bt vegetative cells before the sporulation phase caused the reduction of the spore potential production (Fig. 5B). Moreover, the sporulation was delayed since spores were first monitored at 70 hours of sampling. This phenomenon was in accordance with Karim et al. (1973), who observed a slowdown of spores' formation rate when working at a dissolved oxygen setpoint of 5 %. Final spore production yield was 0.05 produced spores per initial CFU at 116 hours.

Limited bibliography reports the production of Bt-derived biopesticides at a representative industrial scale by SSF. In Ballardo et al. (2020) similar spore concentration was reported, working with home composters of 400-L. A solid Bt-enriched inoculum was used to inoculate a mixture of fruit and vegetable leftovers and pruning waste. The spore concentration progressively decreased, from 5.9 $\times$ 10^7 to 7.8 $\times$ 10^5 spores g^{-1} DM at 40 days. The reduction of the final spore concentration was also observed by Zhang et al. (2013). A loss of four orders of magnitude (from 10^{10} to 10^6 spores g^{-1} DM) was monitored when increasing the fermentation media from 4 to 35 kg. The main attributed reason was the metabolic heat and CO_2 retention on the packed bed when increasing the medium mass, inhibiting bacterial growth.

In addition to 290-L reactor, a control fermentation was carried out at 1.6-L laboratory reactors, reproducing also the operation performed in SSF4 (Table 1). The same trend was observed, detecting an increase of one order of magnitude of viable cell concentration (from 3x10^7 to 4 $\times 10^8$ CFU g^{-1} DM) at 22 hours, but this value decreased until values between 6 $\times$ 10^6 and 1 $\times$ 10^7 CFU g^{-1} DM. Interestingly, this decrease was not reported in our previous work (Mejias et al., 2020) when the reactors where only sampled once the fermentation stopped. A sporulation ratio of 71 % was obtained, with a concentration of 4.7 $\times$ 10^6 spores g^{-1} DM. These results were slightly higher compared with the pilot plant performance. Nonetheless, the spore yield was the lowest obtained at the 1.6-L scale (0.16

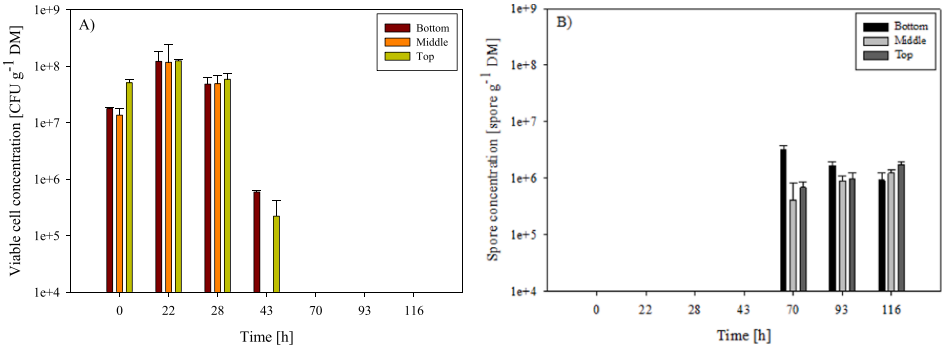

Fig. 5. CFU and spore production of SSF 4. Bt Viable cell concentration (A) and Bt Spore concentration (B).

produced spores per inoculated CFU) compared with the 7.3 yield previously reported (Mejias et al., 2020). Thus, the extension of the non-aerated stage, the entrance of the atmospheric air when sampling, or a combination of those two factors could alter process performance and subsequent spore production.

3.4. Quality of the fermented material

Finally, the quality of the fermented material was evaluated as a preliminary step toward assessing its potential use as a soil amendment. The stability and maturity of the fermented material were analyzed using respirometric indices (DRI and AT_4) and germination indices. The germination test was used to analyze the phytotoxicity of the solid. DRI and AT_4 values of 0.4 ± 0.1 g O_2 kg^{-1} DM h^{-1} and 30 ± 7 g O_2 kg^{-1} DM were obtained, respectively, representative of a very stable material (Barrena et al., 2011). Furthermore, spore concentration was maintained constant along the respirometry test ($1.4 \times 10^6 \pm 3.6 \times 10^5$ spore g^{-1} DM). Regarding the germination test, germination indices of 177 % and 170 % for cucumber and radish seeds were obtained, respectively. Germination indices above 100 % indicate a beneficial effect on seed growth. On the contrary, values lower than 100 % indicate phytotoxicity, affecting seed germination and root growth (Komilis and Tziouvaras, 2009). Therefore, fermented material could be considered mature material.

Eight samples from different points of the biowaste treatment plant and the SSF process, including fresh and processed materials, were analyzed to identify and quantify MPs. Table 2 shows the polymer mass results. Polyethylene, polystyrene, polyethyleneterephthalate (PET), and polypropylene were identified.

Polyethylene was the most abundant polymer in all samples. The principal hypothesis of polyethylene input in these samples is the presence of plastic bags in the centralized biowaste collection scheme. Those plastic bags are opened and ripped mechanically at the treatment plant, and not all plastic fragments are recovered during the mechanical pre-treatment or post-treatment for compost. However, the biowaste samples analyzed were taken from a biowaste pile without plastic bags, and with no visual presence of plastics. This suggests that this MPs contamination could occur in previous stages of food production, handling, marketing, and disposal. He et al. (2018) studied the occurrence of MPs in farmlands. The majority of MPs found were polypropylene and polyethylene, indicating that this contamination may happen from plastic mulching in farmland, or when using sludge as fertilizer. This practice is commonly employed in agriculture. However, MPs degradation in soils is very slow, showing a 0.1–0.4 % of weight loss of PE after 800 days, and a 0.4 % of weight loss of PP after one year of soil incubation (He et al., 2018).

It is important to remark that, even though a representative amount of dry organic substrate was taken, only a few mg of the extracted and concentrated MPs were analyzed through TED-MS-GC, potentially losing part of this representative characteristic. The ratio polymer fraction/sample was also calculated, as a comparable value. Digestate was the material with a higher polymer fraction (6.16 %). This value was significantly reduced after the hygienization process (0.5 %). The reduction could be associated with some thermal degradation of polymers during hygienization step, or due to a lack of homogeneity of the samples.

Moreover, MPs concentrated on the anaerobic digestion reactor, and as consequence, in the solid fraction of digestate since the polymer fraction increased from 1.28 % in the pulper tank to 6.16 % in the fresh digestate. Nevertheless, this anaerobic digestor is fed with different types of biowastes collected (with different ratios of impurities), not only with fruit and vegetable biowaste. This explains why the MPs concentration of the pulper tank was higher than the biowaste used in this study.

Regarding the SSF process, the active microbial population of the process did not significantly promote MPs degradation, since initial and final SSF samples showed very similar polymer fractions (from 1.55 to 1.45). Lastly, comparing the final fermented material and the compost produced in the centralized biowaste treatment plant, the compost presented a lower polymer fraction. Nonetheless, compost is refined after the composting process to recover the bulking agent material, and some polymers could be also removed from this organic matrix. Hence, this polymer fraction should be considered when using the final fermented material or compost as fertilizer in fields for environmental health, affecting soil organisms because of their ingestion or accumulation (Sajjad et al., 2022; Urra et al., 2019). These results highlight the importance of ubiquitous contamination by MPs, collection systems and plastic fragmentation in waste treatment facilities, all together compromising the quality of bioproducts and potential valorisation of biowaste.

4. Conclusions

A solid-state fermentation strategy combining anoxic and aerated phases was proved efficient at a representative scale (100- L)

Table 2
TED-GC-MS analysis results for microplastics quantification.

Polymer	FVB	D	H-FVB	H-D	PT	MbSSF	MaSSF	C
Dry sample amount (g)	27	80	23	90	30	45	125	150
Polyethylene (mg)	8	3428.3	50.9	392	372.1	497.3	1588.5	1072.7
Polystyrene (mg)	3.3	559.2	11.6	8.7	8.3	198.8	226.4	38.2
PET (mg)	0.7	943.1	1.1	17.5	4.9	n.a.	n.a.	22.9
Polypropylene (mg)	0.7	n.a.	2.3	30	n.a.	n.a.	n.a.	20.5
Polymer fraction/sample (%)	0.05	6.16	0.29	0.5	1.28	1.55	1.45	0.77

FVB=Fruit and vegetable biowaste; D=Digestate; H-FVB= Hygienized FVB; H-D= Hygienized digestate; PT=Pulper Tank; MbSSF=Mixture before SSF; MaSSF=Mixture after SSF; C=Compost. n.a. stands for not analyzed.

leading to 2.4 Bt spores produced per CFU inoculated. The importance of applying the strategy in a systematic way and avoiding the perturbation of process conditions when sampling was highlighted at a demonstrative scale (290- L). Final product presented high stability and low phytotoxicity and could be suitable to be applied as organic amendment with biopesticide properties. Ubiquitous microplastics contamination can compromise biowaste valorization. Addressing microplastic contamination at multiple stages (collection, treatment, and bioproduct refinement) is crucial to ensure environmental sustainability and security when applying those products on soils and crops. This study provides technical evidence about the technical feasibility of implementing waste valorization systems to produce high-value products, thus contributing to the implementation of circular bioeconomy strategies.

CRediT authorship contribution statement

Laura Mejias: Writing – original draft, Visualization, Investigation, Conceptualization. **Teresa Gea:** Writing – review & editing, Supervision, Conceptualization. **Esther Molina-Peñate:** Validation, Supervision. **Raquel Barrena:** Writing – review & editing, Supervision, Conceptualization. **Daniel Ruiz:** Validation, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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