Universitat Autònoma de Barcelona

A STEP TOWARDS BIOWASTE DIGESTATE VALORIZATION: PROCESS DEVELOPMENT FOR Bt-DERIVED BIOPESTICIDES PRODUCTION THROUGH SSF AND PERFORMACE AT DEMONSTRATION SCALE

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Teresa Gea, profesora titular, i Raquel Barrena, investigadora postdoctoral, del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

Certifiquen:

Que la biotecnòloga i màster en enginyeria biològica i ambiental Laura Mejias Torrent ha realitzat sota la nostra direcció el treball amb títol "A step towards biowaste digestate valorization: process development for Bt-derived biopesticides through SSF and performace at demonstration scale" que es presenta en aquesta memòria i que constitueix la seva tesi per optar al Grau de Doctor en Ciència i Tecnologia Ambiental per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, es presenta a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada tesi, signat el present certificat.

Dra Teresa Gea

Dra Raquel Barrena

Bellaterra, octubre 2020

A la meva família.

Agraïments

I bé... ja ha arribat el dia. Tanco els ulls i sembla que fos ahir quan vaig entrar als laboratoris del DEQBA per primera vegada. L'aventura que havia de ser d'uns mesos (una noia que està acabant la carrera i vol fer pràctiques extracurriculars) ha acabat amb tota una experiència de més de quatre anys.

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OVERVIEW

In the framework of the European project DECISIVE (grant agreement No 689229) the present thesis is focused on the valorization of digestate from the decentralized management of the organic fraction of the municipal solid waste through the solid-state fermentation (SSF) technology. Previous work focused on the proof of concept of the idea highlighted the viability of producing *Bacillus thuringiensis*-derived biopesticides using digestate as a principal substrate.

From these results, a first assessment at a laboratory scale (0.5-L) using the design of experiments' methodology was performed for determining the more relevant parameters in the fermentation. Temperature and the use of biowaste as co-substrate were identified as key parameters for the process. This effect was studied at 1.6-L, confirming the need of adding co-substrate for increasing the production yields. At that point, the relevance of oxygen levels in the firsts hours of fermentation was identified and highlighted. As a result, an aeration strategy was developed with the aim of maximizing the spore production.

This strategy was validated at a prototype reactor (22-L) using two different strains: Bt var. *kurstaki* and Bt var. *israelensis*. Promising results were observed when the process was performed on batch mode. However, the final production was significantly reduced when working on fed-batch or sequential batch mode.

Lastly, the developed operation strategy was implemented at the 290-L pilot reactor, trying to achieve an adequate environment for boosting Bt growth and sporulation. The quality of the fermented material was assessed in terms of spore concentration, solid maturity, and microplastics identification and quantification.

This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No 689229.

RESUMEN

Dentro del marco del proyecto europeo DECISIVE (grant agreement No 689229) esta tesis se centra en la valorización del digestato obtenido de la gestión descentralizada de la fracción orgánica de los residuos municipales (FORM) mediante la tecnología de fermentación en estado sólido (FES). Trabajos anteriores enfocados a la prueba de concepto destacaron la viabilidad de producir biopesticidas derivados de *Bacillus thuringiensis* (Bt) utilizando digestato como principal sustrato.

A partir de estos resultados, se realizaron una serie de diseños de experimentos a escala laboratorio (0.5-L) con el objetivo de determinar los parámetros con más relevancia en la fermentación. La temperatura y la adición de FORM como co-sustrato se identificarion como parámetros clave del proceso. Su efecto se estudió a escala superior (1.6-L), confirmando la necesidad de añadir co-sustrato para mejorar los rendimientos de producción. En este punto, también se identificó y destacó la importancia de los niveles de oxígeno durante las primeras horas de proceso. Como resultado, se desarrolló una estrategia de aeración para maximizar la producción de esporas.

Esta estrategia se validó en un reactor prototipo (22-L), utilizando dos cepas diferentes: Bt var. *kurstaki* y Bt var. *israelensis*. Los resultados fueron muy prometedores cuando el proceso operaba en bach, pero la producción final disminuía significativamente cuando se operaba en fed-batch o batch secuencial.

Finalmente, esta estrategia de producción se implementó en el reactor piloto de 290-L, intentado lograr en ambiente favorable para incrementar el crecimiento y esporulación de Bt. Se estudió la calidad del sólido fermentado en referencia a la concentración de esporas, madurez del sólido, e identificación y cuantificación de microplásticos.

Este proyecto ha recibido fondos del programa de investigación e innovación Horizon 2020 de la Unión Europea según el Grant Agreement No 689229.

RESUM

Dins el marc del projecte europeu DECISIVE (grant agreement No 689229) aquesta tesi es centra en la valorització del digestat obtingut de la gestió descentralitzada de la fracció orgànica dels residus municipals (FORM) mitjançant la tecnologia de fermentació en estat sòlid (FES). Treballs anteriors enfocats a la prova de concepte van destacar la viabilitat de produir biopesticides derivats de *Bacillus thuringiensis* (Bt) utilitzant digestat com a principal substrat.

Partint d'aquests resultats, es van realitzar una sèrie de dissenys d'experiments a escala laboratori (0.5-L) per determinar els paràmetres amb més rellevància en la fermentació. La temperatura i l'addició de FORM com a co-substrat es van identificar com a paràmetres clau del procés. Aquest efecte es va estudiar a una escala superior (1.6-L), confirmant la necessitat d'afegir co-substrat per tal de millorar els rendiments de producció. En aquest punt, també es va identificar i destacar la importància dels nivells d'oxigen durant les primeres hores de procés. Com a resultant, es va desenvolupar una estratègia d'aeració per tal de maximitzar la producció d'espores.

Aquesta estratègia va ser validada a un reactor prototip (22-L), utilitzant dos soques diferents: Bt var. *kurstaki* i Bt var. *israelensis*. Els resultats van ser molt prometedors quan el procediment es realitzava en batch, però la producció final es veia reduïda significativament quan es treballava en fed-batch o batchs seqüencials.

Finalment, aquesta estratègia de producció es va implementar al reactor pilot de 290-L, intentant aconseguir l'ambient favorable per incrementar el creixement i esporulació de Bt. La qualitat del material fermentat va ser analitzada en termes de concentració d'espores, maduresa del sòlid, i identificació i quantificació de microplàstics.

Aquest projecte ha rebut finançament del programa de recerca i innovació Horizon 2020 de la Unió Europea segons el Grant Agreement No 689229.

CHAPTER 1: INTRODUCTION

1.1. The issue of biowaste and the circular economy concept

Municipal solid waste generation is one of the issues of concern related to population and economic growth (Van Fan et al., 2020). For many years, biowaste was disposed of in landfills, mostly under non-controlled conditions. It is the most inexpensive but also the worst environmental option, with major environmental issues in terms of greenhouse gas (GHG) emissions and air, ground, and water pollution (Demirbas, 2011; Van Fan et al., 2020). In 1999, the Landfill Directive 1999/31/EC was published, which set up a strategy to reduce its final disposal to landfills. Actions such as recycling, composting, biogas production, or materials/energy recovery were proposed in this document. The main part of the urban organic waste concerns biowaste, which 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. It does not include forestry or agricultural residues, manure, sewage sludge, or other biodegradable waste such as natural textiles, paper or processed wood" (Directive 2008/98/EC). Nowadays, the biological treatment of source selected urban biowaste is one of the most implemented technologies in urban and peri-urban areas, as targets the Waste Framework Directive (European Commission, 2010).

Around 88 million tonnes of food waste are generated every year in Europe, including not just inedible parts, but also edible parts. Households were classified as the major contributors on this food waste in 2012, estimating a food waste generation of 92 kg per person per year (Fusions, 2016). Limited natural sources are also wasted for its production. In this sense, the European Commission launched the EU Platform on Food Losses and Food Waste to achieve the Sustainable Development Goal (SDG) 12.3 of food loss and waste reduction targets without compromising food safety, feed safety and/or animal health (European Commission, 2019). Since the resources of the planet earth are

limited, the exponential growth of the economy following a linear model is unsustainable (Lieder and Rashid, 2016). Along these lines, a transformation of the economy is needed.

The transition from a traditional linear economy (take-make-dispose) to a circular economy, where the value of materials and resources are maintained as long as possible, is a necessary step towards a more sustainable, zero-waste economy. Geissdoerfer et al. (2017) defined the concept of Circular Economy as "a regenerative system in which resource input and waste, emission, and energy leakage are minimised by slowing, closing, and narrowing material and energy loops. This can be achieved through long-lasting design, maintenance, repair, reuse, re-manufacturing, refurbishing, and recycling". In December 2015, action plans were released to implement the circular economy concept in product design, production processes, consumption habits, and waste management (European Commission, 2015). From then to the present time, 54 actions have been completed or are being implemented (European Commission, 2019b). In terms of waste management, actions like "turning waste into resources" and "closing the loop" are being highlighted. The main idea of these actions is to reduce waste generation, and for those wastes that cannot be avoided, to treat and introduce them as raw materials for other processes, changing the perception of being a problem to being a resource.

In this sense, the European project DECISIVE ("A Decentralized management Scheme for Innovative Valorization of urban biowaste") proposes to develop and demonstrate a decentralized biowaste management model, engaging citizens to be part of this model, to promote circular economy behavior, and to close the loop of the organic waste recycling.

1.2. DECISIVE project

The European project DECISIVE started in September 2016 with 13 partners involved (Table 1).

Table 1: DECISIVE partners consortium.

Partner organization name	Country	Status
Irstea – Research unit GERE	France	Research Institute
Universitat Autònoma de Barcelona (UAB) – Composting research group	Spain	University
Aarhus University – ENVS (AU)	Denmark	University
Hamburg University of Technology (TUHH) – AWW-BIEM Groups	Germany	University
Fundació ENT	Spain	Non-Profit organization
Innovative Technological Systems S.r.l. (ITS)	Italy	SME
Aeris Tecnologias Ambientales S.L.	Spain	SME
ACR+	Belgium	Non-Profit organization
Agència de Residus de Catalunya (ARC)	Spain	Public authority
PSUtec SPRL	Belgium	SME
Suez-Environment (SE)	France	Large Enterprise
GEOMAR Helmholtz Center for Ocean Research	Germany	Research Institute
reFARNERS	France	SME

The objective of this project is to demonstrate the feasibility of decreasing the generation of urban biowaste and increasing the recycling and recovery of materials through a decentralized management, valorizing biowaste in a short cycle, and moving into a "zero-waste" strategy.

At present, in the urban and peri-urban areas, biowaste is collected and transported to a biological treatment plant and treated using anaerobic digestion and/or composting processes (Abad et al., 2019). Anaerobic digestion consists of an anaerobic fermentation, where the easily biodegradable organic matter is converted into biogas through three different steps (hydrolysis, acidogenesis, and methanogenesis) and under the action of different microorganisms (Breeze, 2018). The target product from this process is biogas, however, another by-product is also obtained, digestate. Digestate use on land as a fertilizer was not supported by the legislation of the EU based on the end-of-waste criteria (Directive 2008/98/EC). This means that digestate was considered a waste, and had to be managed according to the specific legislation of the sector (Di Maria and Sisani, 2019). Nevertheless, the Regulation 2019/1009 aims to promote the use of recycled or organic materials for fertilizing purposes, in which the use of digestate as fertilizer is under study.

Composting can be defined as "the biological decomposition and stabilization of organic substrates, under conditions that allow the development of thermophilic temperatures as a result of biologically produced heat, to produce a final product that is stable, free of pathogens and plant seeds, and can be beneficially applied to land" (Haug, 1993).

In this centralized scheme, the collection and transportation of biowaste to the waste treatment facility is expensive, time-consuming, and energy-intensive (Cofie et al., 2014). Besides, this model has been criticized for its inadequate resource recycling rates due to poor source-separation, inefficient plant operation, or the risk of recycling harmful

substances (Särkilahti et al., 2017). Along these lines, some studies compared the biowaste treatment through decentralized management instead of centralized management. Benefits of the decentralized management are related to decentralized electricity generation and heat conversion, closing the loop in areas such as islands or remote villages, and the enhancement of citizen participation, increasing awareness on waste generation and improving source separation (Panaretou et al., 2019; Breitenmoser et al., 2019).

Therefore, DECISIVE project aims to study the implementation of this circular concept. The entire system should be considered as a biorefinery unit, valorizing all waste streams generated in the whole process (Figure 1). This project is divided into two steps: a definition of decentralized management concept, and consolidation of decision support tools platform. Furthermore, it includes the definition, construction, and development of two demonstration sites. As regards the technological part of the demonstration sites, two innovative solutions are proposed for biowaste treatment and valorization: eco-designed micro-anaerobic digestion (mAD), and solid-state fermentation (SSF).

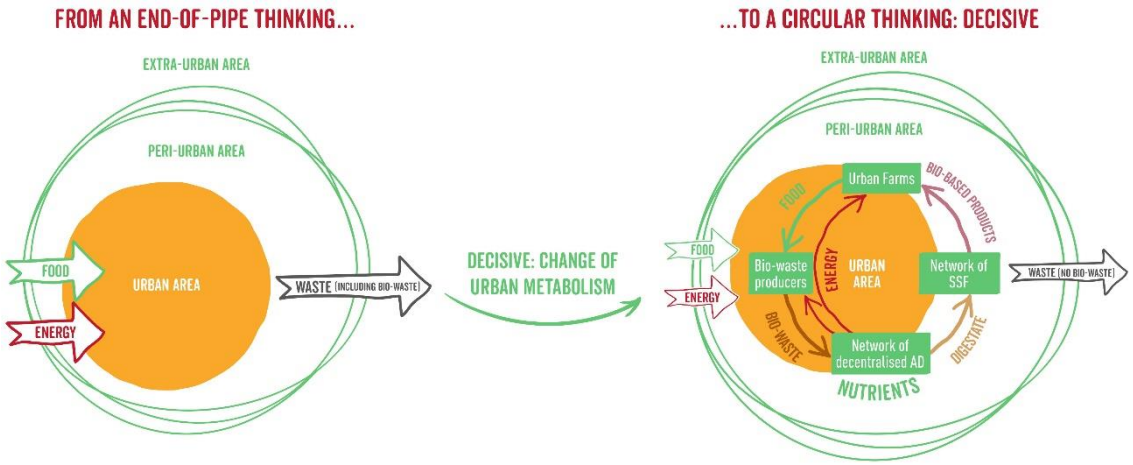

Figure 1: Closing the loop in biowaste management scheme of DECISIVE project.

The anaerobic digestion (AD) is not a new technology, as it has been used for several years on an industrial scale, treating biowaste, sludges, agro-industrial wastes, manures, and other biodegradable waste (Lora Grando et al., 2017; Abad et al., 2019; Tabatabaei et al., 2020). However, micro-AD is mainly used in developing countries. In Europe, AD is generally restricted to larger scale plants (Walker et al., 2017). On the other hand, SSF is a prominent alternative for processing and transforming several residues into value-added products of different characteristics. However, it is not fully implemented on industrial scales (Farinas, 2015; Cerda et al., 2019). In this scheme, SSF units are proposed for digestate valorization.

1.3. Solid-state fermentation

SSF is a promising technology to produce marketable bioproducts using a solid matrix. It has been defined as the fermentation process that takes place in the absence, or near absence, of free water but needing the appropriate moisture content to support microbiological growth (Thomas et al., 2003). 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 the thin layer of water holds the microorganism growth (Cheng, 2013).

The interest in this technology has increased during the last decade, as urban, agricultural, and industrial organic wastes can be used as substrate for the fermentation process, thus reducing the bioproducts production cost, and favoring SSF to be economically viable (Schmidt et al., 2014). At the same time, waste is seen as a raw material for this technology, contributing to the circular economy thinking.

A wide range of bioproducts can be produced through SSF from a range of substrates using different bioreactor configurations. Arora et al. (2018) classified SSF reactors into four types: tray bioreactor, packed bed bioreactor, intermittent or

continuously mixed SSF bioreactor, and air pressure pulsation bioreactor. The selection of the most appropriate configuration mainly depends on the substrate, the microorganism, and the final product.

Tray bioreactor: metallic, plastic or wood trays have been used for many years for fermented food products such as tempeh, miso, or soy sauce (Mitchell, 2006) (Figure 2). The scalability of this configuration relies on increasing the area and/or increasing the number of trays, implying a considerable space requirement. In these systems, mixing can be done manually.

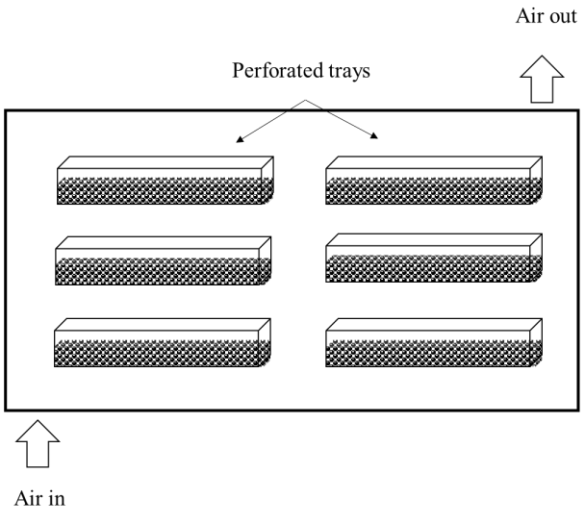

Figure 2: Tray bioreactor.

Méndez-González et al. (2020), produced a biological control agent derived from *Metarhizium* genus using trays, studying the effect of the airflow rate. Conidia production was closely related to the airflow rate regime when working in trays, as a decrease from $1.19x10^9$ to $0.43x10^9$ conidia g^{-1} dry matter was obtained when the airflow rate was increased from 0.1 to 1 L Kg^{-1} min^{-1}, but also presenting with the highest viability. In a different study, successful pectinase production was achieved in tray bioreactors using orange pomace and sugarcane bagasse (Mahmoodi et al., 2019).

Packed bed bioreactors: they are normally built as a cylindrical plastic, steel, or metal tube/drum, where all substrates are packed inside (Figure 3). Although forced aeration is supplied to improve heat and oxygen transfer, it can also lead to a pressure drop, air channeling, and bed compaction.

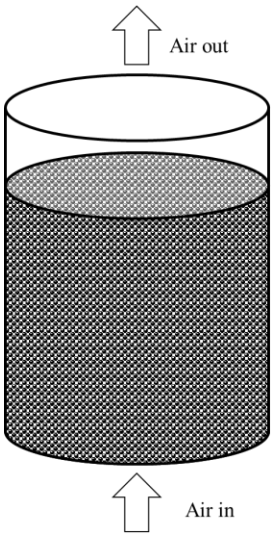

Figure 3: packed-bed bioreactor.

Packed-bed bioreactors are the most modelled configuration to obtain kinetic parameters, and heat and mass transfer responses. In Pessoa et al. (2019), a computational fluid dynamics (CFD) simulation was processed to describe temperature profiles at various heights within the packed bed in three different scenarios. Ranjbar and Hejazi (2019) used a 2-dimension model to study the effect of temperature and moisture content on kinetic parameters of *Pseudomonas aeruginosa*, using a 3-L packed bed bioreactor.

Intermittent or continuously mixed bioreactors: these types of reactors comprise a moderate agitation and forced aeration system in order to maximize convective transfer, as the transfer area of solids is maximized (Figure 4). However, these configurations are not suitable if the target microorganisms are damaged due to shear stress. For example, it would not be suitable for fungi growth, as mycelia would be squashed (Mitchell, 2006).

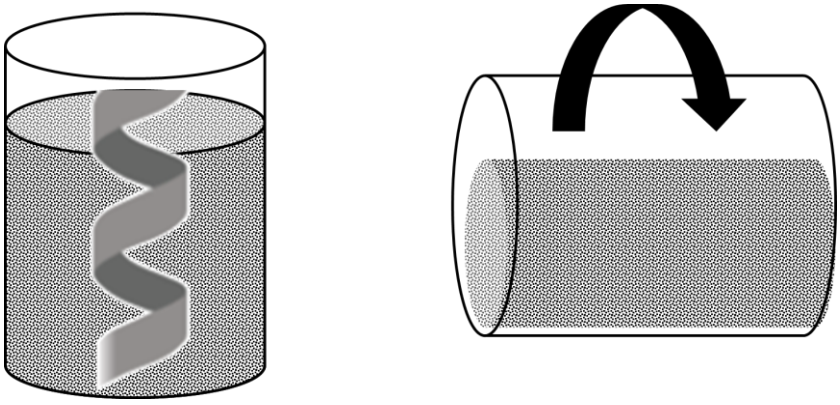

Figure 4: mixed SSF bioreactors.

In da Paula Eduardo et al. (2007), a 40-L horizontal rotating drum bioreactor was used to produce protein by *Monascus ruber* grown on rice. An increase of the specific airflow rate did not achieve any benefit to the protein production, while on changing the agitation pattern from 10 turns per day to 1 turn every 2 hours, the production was doubled. Similar trends have been observed by other authors. Finkler et al. (2017) used an intermittent agitated packed bed reactor to produce pectinases, being loaded with a total of 30 kg of substrate. The best agitation regime was set with 3 mixings per day, observing more uniformity on pectinase production through the packed bed.

Air pressure pulsation bioreactors: their main characteristic is the use of periodic pulsation of air pressure, which can be combined with a forced aeration regime to promote microbial activity (Figure 5). This configuration is also known as gas double-dynamic solid-state fermentation (GDD-SSF). Air pressure pulsation is used to increase oxygen concentration in the bed, while the decompression phase induces the removal of carbon dioxide (Arora et al., 2018). Solid mixing is also achieved by air circulation. Drawbacks of this configuration are related to the water losses from the solid substrate due to the high

frequency of pressure pulses, affecting the water activity and, in consequence, the microbial activity (Chen, 2013).

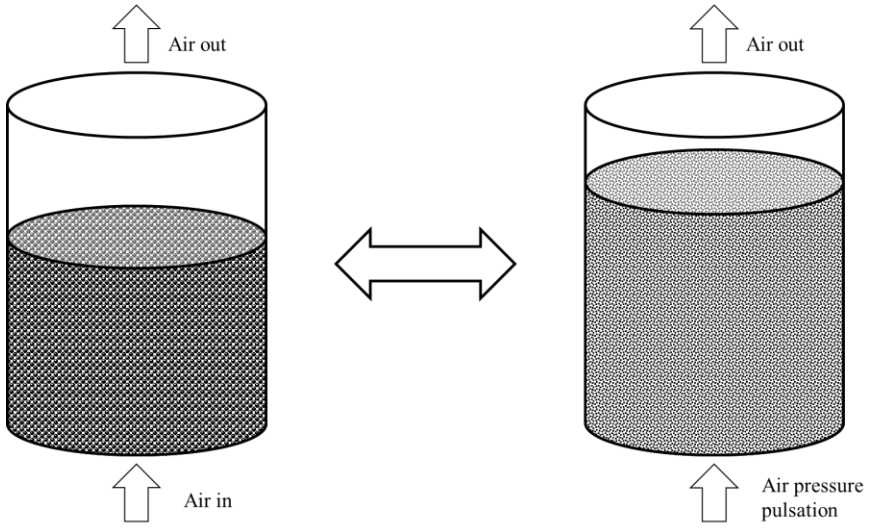

Figure 5: Air pressure pulsation bioreactor.

GDD-SSF has shown to be an alternative reactor for industrial-level processes to avoid the shear forces caused by agitation systems of other configurations (He and Chen, 2012). Although successful enzymatic productivities were achieved with this system, its main disadvantage was the low substrate loading (15 kg of the dried substrate in an 800-L reactor).

1.4. Scale-up challenges

The principal phenomena occurring within the substrate bed are the following:

- Metabolic heat production related to microbial growth.
- Conduction as a response to temperature gradients. Energy goes from warmer regions to colder regions. Depending on the process and the bioreactor, this conduction may exist in one, two, or three dimensions.
- Diffusion of O_2, CO_2, and water vapor that diffuse within the inter-particle spaces in response to concentration gradients.

- Convective heat transfer when the bed is forcefully aerated. Energy is transferred from the solid phase to air, increasing the temperature of outlet air. This convective phenomenon can lead to the formation of temperature gradients along the substrate bed, with the top of the bioreactor being the warmest region.
- Evaporation of water from the solid phase into the air phase. It depends on the saturation of the air and the increase in air temperature. The degree of evaporation depends on the water saturation of the air. However, even if inlet air is saturated, air is getting warmer along the substrate bed. Hence, the water-holding capacity of the air increases and evaporation occurs. Evaporation involves the progressing drying and obtaining of a moisture gradient along the bed height.

Although the basis of submerged fermentation (SmF) is well established and developed, it cannot be applied to process scale-up for SSF (Singhania et al., 2009). SSF scale-up adversities are mainly related to intense heat generation and the difficulty to remove the waste metabolic heat from the solid matrix (Ashok et al., 2017; Soccol et al., 2017). Heat removal of SmF systems is easier than in SSF because thermal properties of the continuous aqueous phase (thermal conductivity and heat capacity of liquid water) are superior to those moist solid beds with inter-particle air. Furthermore, applying mixing as a strategy to remove heat from the packed bed is not always possible (Mitchell, 2006). Thus, the best way for cooling the substrate is from the outer surface (Ashok et al. 2017). An increase in temperature has direct consequences on the process, like water evaporation and problems in the maintenance of water activity, as well as problems related to mesophilic microbial population development. For that reason, scale-up key parameters have to be related to heat removal and maintenance of water activity, indispensable for

the correct survival and function of microorganisms (Soccol et al., 2017). In practice, water activity is very complex to determine, and moisture content of the solid is measured.

Some references have studied how operating parameters, such as the height of substrate bed, inlet airflow temperature, or superficial velocity influence metabolic heat removal, to maintain the proper conditions for microorganism growth (Mitchell et al., 1999; Pitol et al., 2015; Finkler et al., 2017).

Mitchell et al. (1999) studied the effect of bed height, superficial velocity, and inlet air temperature using a dynamic mathematical model of heat transfer in a packed-bed bioreactor, developed by Sangsurasak and Mitchell (1995). It was observed that the maximum temperature achieved in a 1-m high packed-bed was negatively proportional to the superficial flow velocity applied to the system, varying the specific growth rate. A positive correlation was also observed between critical bioreactor height and superficial velocity. Critical height can be defined as the height where critical temperature is achieved (temperature above optimum temperature which negatively affects the process). A higher critical height was obtained when a lower specific growth rate was analyzed, being related to lower metabolic heat production. The height of the substrate bed is limited by the critical temperature set, being a key design factor for scale-up. Furthermore, inlet air temperature can significantly affect the critical height, the most appropriate strategy being to set the inlet air temperature a few degrees below the optimum temperature. Pitol et al. (2015) studied the scale-up of the production of pectinases in a packed-bed bioreactor from 12 g to 30 kg of dry substrate. Temperatures above 47ºC were reached within the solid bed, resulting in a decrease in pectinase activity. Pitol et al. (2015) overcame this heat removal problem by improving the ratio of wheat bran/sugarcane bagasse, ensuring a good porosity, and changing inlet air temperature from 32ºC to 24ºC when a metabolic heat production occurred. The best results were achieved by applying

these conditions, and setting the bed height to 40 cm, with 1840 U kg^{-1} h^{-1} being obtained at 10 hours. In Finkler et al. (2017), agitation was proposed to remove heat from the substrate bed. Starting from the same substrate mixture as Pitol et al. (2015) and maintaining the inlet air temperature at 30ºC, fermentations with intermittent agitation showed a smaller axial temperature gradient than those fermentations without agitation.

Not less important, problems related to biomass separation and product recovery should also be considered (Singhania et al., 2009). Biomass estimation is essential for the kinetics studies, which enhance the understanding of dynamics for microbial growth and product formation, increasing the productivity and product yield (Jugwanth et al., 2020). However, its separation and quantification when working in SSF systems is not simple, and involves the need of using indirect methods for biomass estimation (Steudler and Bley, 2015). Moreover, SSF can be considered as a low-cost technology, as solid organics waste and natural supports can be used as substrates. However, the product recovery is complex, and it involves an extra cost of the process. Therefore, an evaluation is needed to study the economic viability of the process (Singhania et al., 2009).

1.5. Previous work in selecting the target bioproduct for digestate valorization within the DECISIVE project

To select the most appropriate bioproducts to be produced for digestate valorization, a lab-scale screening study was performed using 0.5-L packed-bed bioreactors. Potential bioproducts were chosen based on the research group knowledge, with hydrolytic enzymes, biosurfactants, and biopesticides being selected (Abraham et al., 2014; Jiménez-Peñalver et al., 2016; Ballardo et al., 2017; Cerda et al., 2017).

Digestate characterization presented with a significant amount of lignocellulose, being a potential source for cellulase and xylanase production. Mejias et al. (2018) studied

the production of cellulases and xylanases by exploring three different inoculation strategies: (i) performing the SSF with the autochthonous microbiota, (ii) applying a bio-augmentation strategy with *Trichoderma reesei*, and (iii) using a sequential batch operation to select a specialized inoculum. Nonetheless, low enzymatic activity, compared with the literature, was obtained in the three different systems. Proteolytic activity was also detected in the sequential batch operation mode. Its action against the target enzymes was one of the hypotheses of the low cellulase and xylanase recovery. The other hypothesis contemplated was that digestate pH (between 8 and 9), was not adequate for cellulase production.

Cerda et al. (2019b) attempted the production of biosurfactant and biopesticides. *Starmerella bombicola* was used as inoculum for sophorolipids production. The initial pH was too alkaline for *S. bombicola* growth, which requires a starting pH of around 6. Maximum sophorolipids production was obtained when the digestate was supplemented with oleic acid, achieving a production of 0.02 g sophorolipids g^{-1} DM. However, this production was too low compared with the 0.18 g SL g^{-1} DM obtained by Jiménez-Peñalver et al. (2016). Hence, the production of hydrolytic enzymes and biosurfactants were discarded within the DECISIVE project.

Lastly, the feasibility of producing *Bacillus thuringiensis*-derived biopesticides was studied using digestate as a sole source of organic matter and nutrients. *Bacillus thuringiensis* var *kurstaki* was used as inoculum. First trials were focused on lab-scale fermentations using sterile and hygienized digestate. In both cases, the bacillus was able to survive and sporulate, reaching a maximum spore concentration at 72 hours. Consequently, biopesticides were selected as a target product for the DECISIVE project, and the following work was related to understand, optimize, and scale-up the process.

1.6. Biopesticides

Over the years, chemical pesticides have made a great contribution in the fight against numerous pests including fungi, weeds, and insects. However, their indiscriminate use has left consequences such as insecticide resistance, and human health and environmental problems. Consequently, eco-friendly alternatives are needed to produce sustainable agricultural products (Mnif and Ghribi, 2015). Biopesticides can be classified into three groups: biochemical pesticides, microbial pesticides, and semiochemicals (Chandler et al., 2011; Gao et al., 2020).

Biochemical pesticides are natural substances, such as secondary metabolites, or enzymes, that have pesticide properties. They have low mammalian toxicity and rapid degradation. Semiochemicals refer to those chemical signals produced by one organism, like pheromones, that change the behavior of organisms of the same or different species. Lastly, microbial pesticides include bacteria, fungi, oomycetes, viruses, and protozoa. The most widely used microbial pesticide is *Bacillus thuringiensis* (Bt), which has a great potential to be fatal to different orders, such as Coleoptera, Lepidoptera, Diptera, Hymenoptera, Hemiptera, and Orthoptera, as well as phytopathogenic nematodes and terrestrial gastropods (Malovichko et al. 2019). Entomopathogenic fungi have also gained a remarkable relevance, and most products are based on *Beauveria* genus (Sala et al. 2019).

Bt is a gram-positive, sporulating, and facultative-aerobic bacterium. It is widely used as a biopesticide because, during its sporulation phase, it synthesizes a crystalline inclusion that contains δ-endotoxins or Cry proteins (Sanchis and Bourguet, 2008) (Figure 6).

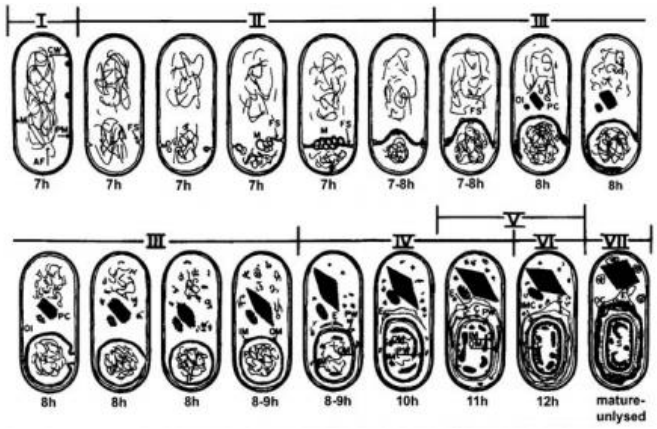

Figure 6: Sporulation and Cry protein formation process of *Bacillus thuringiensis.* Image taken from Federici et al. (2006)

These crystals must be ingested to be toxic (Figure 7). Its solubilization into the digestive tract causes the cleavage of interchain disulfide bonds and the release of Cry protoxins. Endogenous Bt proteases or other proteases from the host gut will process solubilized protoxins, obtaining activated toxins with different sizes. Most well-studied Cry protoxins are sequentially digested to a 65- to 55-kDa toxin core. Once they reach the midgut, the activated Cry toxins bind to molecules located on the brush border membrane of the midgut cells, starting a signal cascade that ends with the dead of the midgut cells (Adang et al., 2014).

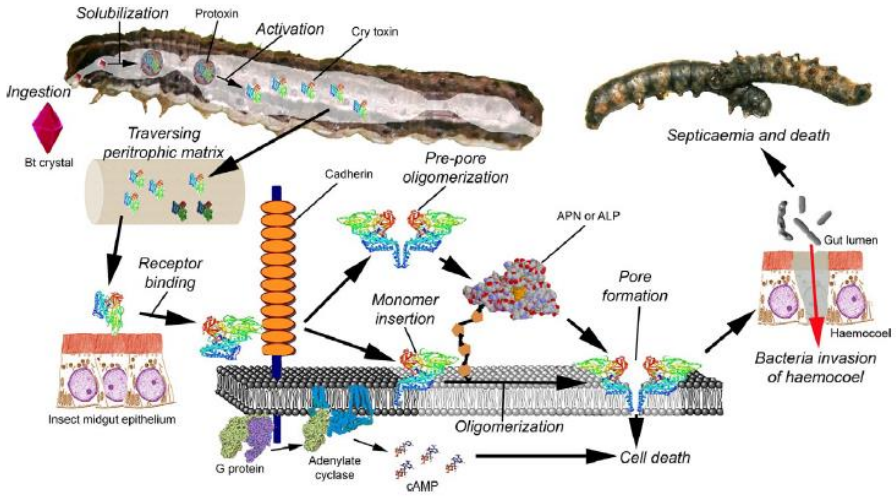

Figure 7: Bt biopesticide mechanism action. Image taken from Adang et al. (2014).

Currently, 90% of marketed biopesticides are derived from this entomopathogenic bacteria, with more than 200 products sold in the US market, and 60 products in the EU (Kumar and Singh, 2015). Among their qualities, they are considered safer, more specific to target pests, effective in small amounts, and rapidly decomposable without leaving toxic residues, compared to conventional chemical pesticides (Damalas and Koutroubas, 2018). Bt var *kurstaki* (Btk) and Bt var *israelensis* (Bti) are two subspecies used as biological control agents. Btk formulations have been used against bollworms, loopers, and other lepidopteran pests, as well as in citrus pests (Kumar et al., 2019). Bti is toxic to mosquitoes and black fly larvae, having some advantages compared to Btk, such as temperature tolerance, family specificity, and long storage stability at room temperature (Foda et al., 2010).

1.7. Recent studies of *Bacillus thuringiensis* production through SSF

Few studies have used SSF technology to produce Bt-derived biopesticides. Between them, the reported using of substrates such as soy waste (Ballardo et al., 2016), sugar beet pulp, and sesame meal (El-Bendary et al., 2017), and spent mushrooms (Wu et al., 2014), and using reactors with working volumes below 15-L. Zhang et al. (2013) have obtained a successful fermentation process using 35 kg of fermentation medium composed mainly of kitchen waste. An increase in final spore count was observed when scaling-up from 4 to 8 kg. However, the spore production slightly decreased when the process was scaled-up to 35 kg. This value rapidly decreased when using 40 kg of substrate (from 9.6×10^8 CFU g^{-1} to 6.4×10^6 CFU g^{-1}), where the main hypothetical causes were poor ventilation and poor heat removal. El-Bendary et al. (2019) analyzed the efficiency of different methods for recovery of the spore toxin complex from the

fermented solid. They observed a good recovery when the SSF liquid extract was centrifuged after a pH adjustment.

Rodríguez et al. (2019) studied the possibility of using digestate as a fermentation media for Btk cultivation and spore production, within the framework of the DECISIVE project. The success of the process relies on the determination of the maximum viable cell and spore concentration, as this is used as an indirect method of Cry protein estimation (Montiel et al., 2001). Lab-scale experiments, working with 100 g of substrate, demonstrated that Bt survived in these conditions. When working in a bench-scale system (10-L insulated bioreactors), a 2.2-fold increase of total cells and 89% of total sporulation was observed, even although thermophilic temperatures were reached (68.2ºC). To overcome temperature effects, the fermentation was performed in a 22-L, stirred, non-insulated bioreactor. Although temperatures were maintained around 27ºC, a decrease in the total cells was observed (from 7.4 to $5.9x10^9$ CFU g^{-1} DM), with a final spore concentration of $5.7x10^9$ spores g^{-1} DM. The process was scaled-up to a 100-L reactor, but a successful production was not achieved. As a general overview, this proof of concept revealed that the process was feasible, and although showing the ability of Bt to sporulate, Bt was not able to grow. Thus, the process to produce biopesticides must be studied and understood in-depth in order to enhance Bt ability to colonize the solid material and maximize its sporulation and biopesticide production. A standardized operational strategy must also be developed to guarantee the success of the operation at the pilot reactor.

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CHAPTER 2: RESEARCH

OBJECTIVES

The objectives of this thesis are related to the activities described in two work packages of the DECISIVE project grant agreement (WP4.2 and WP6.2). The general objective is the production of Bt-derived biopesticides from digestate by using SSF technology. This work will also tackle the strategies to scale the process up to the demonstration SSF reactor.

In order to achieve the main objective, several specific objectives were developed and are presented below.

- To optimize the SSF process conditions by means of identifying and selecting those operational parameters with a significant effect on the process.
- To develop a robust operation strategy ensuring the maximum biopesticide production at two different reactor scales (1.6 and 22-L). The developed strategy should tackle and overcome the main challenge of SSF scale-up: heat and mass transfer.
- To demonstrate the technical feasibility of operating this process in semi-continuous or fed-batch mode.
- To design and build a SSF demonstration plant of 290-L according to the specific requirements for the correct SSF performance.
- To scale-up the SSF technology from a 22 to a 290-L reactor and validate the developed operational protocol with the demonstration scale reactor.

CHAPTER 3: MATERIALS AND METHODS

In this chapter, the materials and methods used in this study are presented. This work has been carried out in the Department of Chemical, Biological and Environmental Engineering, located in the Engineering School of the Autonomous University of Barcelona. Industrial scale trials were performed in the solid waste treatment plant of Granollers. Microplastics identification and quantification from the solid organic samples was performed in collaboration with the Institute for Energy and Environmental Technology (IUTA) (Duisburg, Germany).

3.1. Substrates

For the process development and scale-up studies, digestate was provided by the solid waste treatment plant of Granollers (*Consorci per a la Gestió dels Residus del Vallès Oriental*, Barcelona, Spain). Prior to the SSF process, digestate was subjected to a hygenization process (70ºC for 1 hour), as it is a legal requirement specified in European Regulation No 142/2011. The hygienization process was performed in a laboratory oven (Binder). Due to the low thermal conductivity of solid matrices, a temperature ramp was applied to accelerate the process: the initial temperature was set at 120ºC for 1 hour and 30 min until the solid reached a temperature of 70ºC. The temperature was checked at different points of the trays. The oven set-point temperature was later decreased to 95ºC for 1 hour to maintain the temperature of the solid. Digestate characterization is shown in Table 2.

Table 2: Digestate characterization.

	Value (n = 10)
Moisture [%, w. b.]	29 ± 2
Organic matter [%, d. b.]	68.1 ± 0.7
pH	8.6 ± 0.3
DRI_{24} [g O_2 kg^{-1} DM h^{-1}]	1.7 ± 0.5
AT_4 [g O_2 kg^{-1} DM]	107 ± 26

When a few Kg were needed, biowaste was collected in a grocery store of Cerdanyola del Vallès (Barcelona, Spain). When a larger amount was required (25 Kg), biowaste from a weekly local market was collected at *Consorci per a la Gestió dels Residus del Vallès Oriental* (Barcelona, Spain). Biowaste was cut to a particle size of 1-5 cm for the laboratory and bench-scale experiments and shredded when used was for pilot plant trials. In both cases, it was also subjected to a hygienization process (70ºC for 1 hour). Biowaste characterization is shown in Table 3.

Table 3: Biowaste characterization.

	Value (n = 7)
Moisture [%, w. b.]	11 ± 3
Organic matter [%, d. b.]	91 ± 3
pH	4.6 ± 0.5
DRI_{24} [g O_2 kg^{-1} DM h^{-1}]	4.6 ± 0.7
AT_4 [g O_2 kg^{-1} DM]	266 ± 45

A bulking agent was added to provide porosity and structure to the mixture, similar to the composting process. At lab-scale (0.5 and 1.6-L), a standardized bulking agent (zig-zag cut toothpicks) was used, while wood chips (Acalora, Palets Pla d'Urgell) were used in 22 and 290-L reactors.

Figure 8: Digestate, shredded biowaste, and lab-scale bulking agent.

3.2. Microorganism

Bacillus thuringiensis var *kurstaki* (CECT 4497) and *Bacillus thuringiensis* var *israelensis* (CECT 5904) were obtained from *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain). To preserve both strains, they were grown in agar slants of Nutrient Agar medium (Oxoid), with a composition of 1 g L^{-1} of Lab-Lemco powder, 2 g L^{-1} of yeast extract, 5 g L^{-1} of sodium chloride and 15 g L^{-1} of agar, at 30ºC and 20 hours. Strains were cryopreserved at -80ºC in cryovials which contain cryopearls impregnated with Bt.

For inoculum preparation, 100 mL of sterile Nutrient Broth Nº 2 (Oxoid) were placed in a sterile 500 mL Erlenmeyer flask and were inoculated with a cryopearl. Cultures were incubated at 30ºC and at 130 rpm for 20 hours. After the incubation period, with an optical density (OD) of 2.5-3, the inoculum was centrifuged (3500 rpm, 10 min,

room temperature) and the supernatant was decanted. The pellet obtained from 100 mL of culture was resuspended with 3 mL of supernatant (depleted medium) (Figure 9). The resuspended pellet was recovered and diluted 1/10 with supernatant. The concentration of the processed inoculum was around $5x10^8$ CFU mL^{-1}. No spores were detected at this point. All this protocol was performed under sterile conditions, working in a laminar flow cabin with autoclaved material (121ºC for 30 min).

Figure 9: Bt culture and concentrated inoculum.

The inoculum preparation for the pilot plant performance consisted of two steps: pre-inoculum and inoculum. The pre-inoculum was prepared as previously described. Once the culture was in its exponential growth, 1 mL aliquot was used to inoculate 380 mL of fresh media placed in a 1-L sterile Erlenmeyer flask. A total of 14 Erlenmeyer (1-L) flasks were necessary to obtain the 5-L Bt culture required for pilot plant fermentation.

3.3. Experimental set-up

SSF reactors used in this study are classified as packed bed reactors, not thermally insulated, and forced aerated, with air blown forcefully through the bed. Its geometry is maintained in all reactor scales, with the height being twice the diameter. A metallic net is placed at the bottom to hold up the solid material and improve the air diffusion.

SSF reactors are connected to an airflow meter (Mass-Stream^{TM} D-6311-DR, Netherlands), which supplies and controls a specific airflow rate (Figure 10). Airflow first goes through a humidifier, to saturate the air of water, and it enters the reactor from the bottom. Exhausted air exits from the top of the reactor, passes through a water trap to finally go to an oxygen sensor (O_2-A_2 oxygen sensor, Alphasense, UK), to monitor the oxygen content of the outlet air, in a range of 0-20.9%.

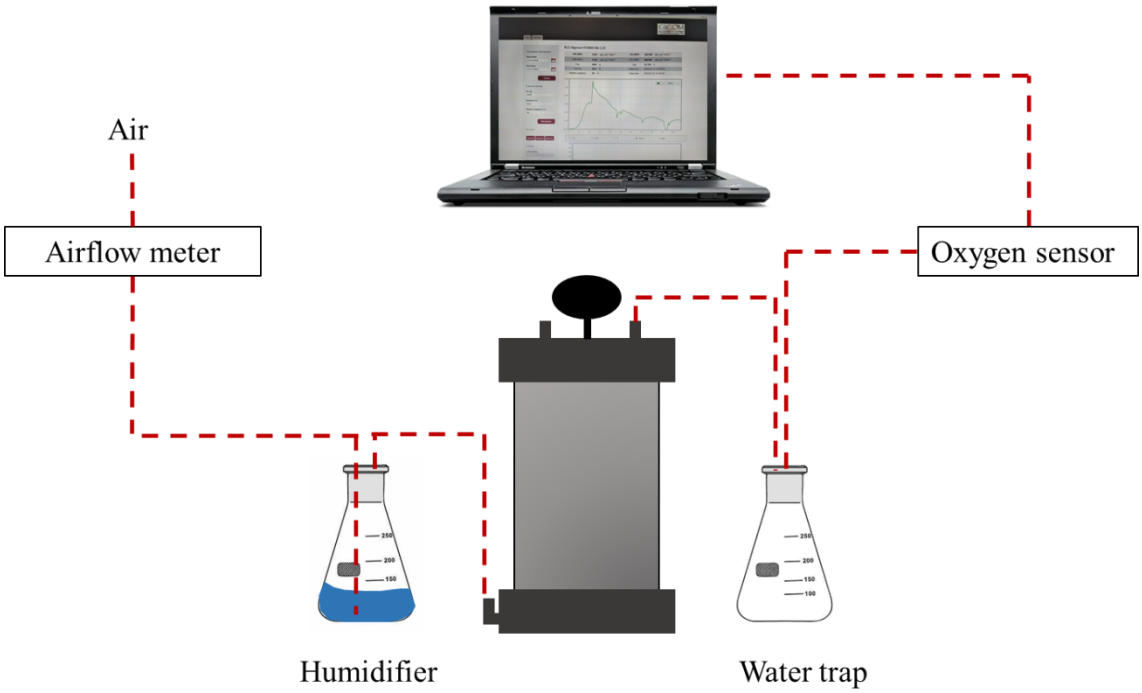

Figure 10: Airflow and oxygen monitoring setup.

3.3.1. Lab-scale reactors

0.5 and 1.6-L reactors are defined as lab-scale cylindrical bioreactors, made of PVC. 0.5-L reactors (Setup 1) are designed with a working volume of 380 mL (6 cm in diameter and 13.5 cm height), and two 70 mL chambers at both top and bottom of the packed bed. They were placed in a thermostatic water bath to work with constant fixed temperature, where the packed bed temperature will be the same as water temperature

(Figure 11). A maximum amount of 105 g of solid was used at a 0.5-L scale, mixing 95 g of substrate and 10 g of lab-scale bulking agent. A volume of 2.8 mL of inoculum was used for reactor inoculation.

Figure 11: 0.5-L reactors (Setup 1).

1.6-L reactors (Setup 2) have a working volume of 1.6-L (10 cm in diameter and 20 cm height), and two 120 mL chambers at the top and bottom. Experiments using 1.6-L reactors were performed at room temperature, with no temperature control, in order to study the effect of working with a dynamic temperature profile (Figure 12). A total of 470 g of solid was used, mixing 425 g of substrate and 45 g of lab-scale bulking agent, inoculated with 11.6 mL of inoculum.

Each reactor is connected to one mass airflow meter, which supplies and controls a specific airflow rate. Additionally, 1.6-L reactors are adapted to insert a temperature probe to monitor the temperature of the solid at the center of the packed bed (Pt-100 sensors, Sensotrans).

Figure 12: 1.6-L reactors (Setup 2).

In the course of the process, oxygen content, airflow, and temperature were continuously monitored. Monitorization was performed by a self-made acquisition and control system based on Arduino® and self-made software.

3.3.2. Bench-scale reactor

A 22-L stainless steel reactor (Setup 3) was provided with an automatic helical stirrer, where the engine is placed at the bottom of the reactor (Figure 13). In this system, the solid substrate is placed in a removable basket with 21.7-L of working volume, with small holes (2 mm diameter) in the bottom to favor the air diffusion. The basket is also provided with two deflectors to complement and improve the mixing of the solids. However, the presence of the helical stirrer interferes with the packed bed structure, as well as occupying a working volume. A temperature probe, which can measure the temperature at a height of 15 cm, can be inserted from the bottom of the vessel.

A total amount of 4 kg was used, with 1 kg of wood chips as a bulking agent, and 3 kg of substrate. The mixture was inoculated with 82 mL of final inoculum. Temperature,

oxygen content, airflow rate, and the specific oxygen uptake rate (sOUR) were monitored during the process.

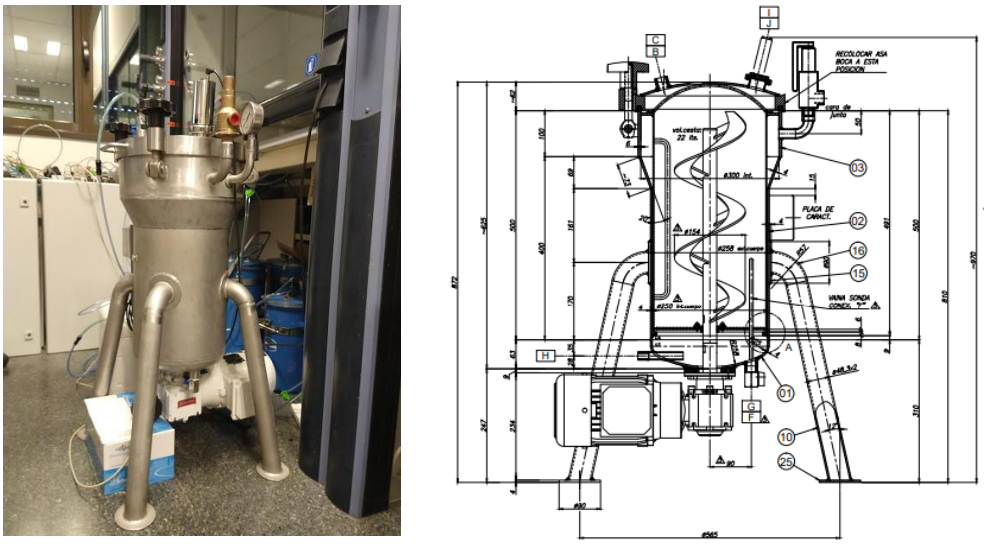

Figure 13: 22-L reactor (Setup 3).

3.3.3. DECISIVE pilot plant reactor

The pilot plant of the DECISIVE project (Setup 4) was designed in collaboration with Aeris, and located in a solid waste treatment plant (*Consorci per a la Gestió dels Residus del Vallès Oriental*, Barcelona, Spain). The characteristics of this reactor are described in detail in Chapter 8. Briefly, it consists of a 290-L vessel, equipped with a stirrer with a frequency inverter, and a temperature probe (Figure 14). The airflow rate is applied by a volumetric airflow meter (300-3100 L h^{-1}) or a rotameter (60-600 L h^{-1}). These devices measure, but do not control, the airflow, and although it can be manually adjusted with the attached valve, it is still affected by fluctuations in air pressure in the system. Thus, a slight fluctuation of the airflow rate value was observed. The airflow rate enters from the bottom of the reactor and diffuses between the pores of the metallic net that sustains the solid. The outlet gas exits from the top of the reactor, passes through a water trap, and ends up in the oxygen sensor box located in the electric panel. Values of

airflow rate, oxygen content, and temperature are continually monitored and displayed on the software screen. A trolley for the substrate feed and discharge, and a ladder to facilitate the access to the top of the reactor are also available. In this system, the reactor is fed from the feed hopper placed at the top of the reactor, and it is discharged by the bottom part, opening the bottom cover, and collecting the falling material onto the trolley.

The digestate and biowaste used were collected from the same waste treatment plant at Granollers. Prior to the fermentation, substrates were transported to UAB facilities to perform the hygienization process. A maximum of 70 kg is treated per batch, with 17.5 kg of wood chips, 32.8 kg of digestate, and 19.7 kg of biowaste. 1.4 L of final inoculum being needed. Thus, it was prepared in the UAB, and then transported to the pilot plant.

Figure 14: DECISIVE pilot plant reactor (Setup 4).

3.4. Monitoring parameters

3.4.1. Specific Oxygen Uptake Rate

Specific oxygen uptake rate (sOUR) was calculated on-line as an indirect method for biological activity follow-up. It was calculated according to Ponsá et al. (2010) (Eq 1):

$$sOUR = F * (0.209 - yO2) * \frac{P*32*60*1000^{a}}{R*T*DW*1000^{b}} \text{ [Eq 1]}$$

Where: sOUR is the specific oxygen uptake rate (mg O_2 g^{-1} DM h^{-1}); F, airflow rate (mL min^{-1}); yO_2, oxygen molar fraction in the exhaust air (mol O_2 mol^{-1}); P, 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; 1000^a, conversion from mL to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, temperature at which F is measured (K); DW, dry weight of solids in the reactor (g); 1000^b, conversion from g to mg.

3.4.2. Colony forming units and spore estimation

Determination of CFU and spores was performed as described in Ballardo et al. (2016). A total of 10 g of solid was mixed with 90 mL of Ringer solution and shaken at 180 rpm for 20 min. Serial dilutions were prepared with Ringer solution and plated onto Petri dishes containing Nutrient agar medium (Oxoid). For spore estimation, a thermal shock was needed to lysate viable cells. Thus, 20 mL of the previous extract were incubated at 80ºC for 10 min and then placed into ice. A sample was also serially diluted with a Ringer solution for plating on Petri dishes with a Nutrient agar medium. Petri dishes were incubated at 30ºC for 20 hours. After the incubation period, CFU and spores were counted in a range between 30 and 300 colonies, and the values of CFU g^{-1} DM and spore g^{-1} DM were calculated according to the following equation (Eq 2):

$$CFU\ g^{-1}\ DM = \frac{Nº\ colonies\ x\ D\ x\ 9}{0.05\ x\ DM} \qquad \text{[Eq 2]}$$

Where Nº of colonies are the CFU counted by Petri dish, in the range between 30 and 300; D is the dilution factor of the sample; 9 are the mL added to 1 g of solid; 0.05 are the mL plated; DM is the dry weight of 1 g of wet solid.

The value of CFU g^{-1} DM includes the total of viable cells (vegetative cells, cells which started the sporulation phase, and spores). Furthermore, the value of spores at a certain time of the process can be expressed as the ratio between the spores at a specific time and the total viable cells. This ratio is the sporulation ratio (Eq 3):

$$Sporulation\ ratio\ at\ time\ t\ (\%) = \frac{Spores\ g^{-1}DM}{CFU\ g^{-1}\ DM} \qquad \text{[Eq 3]}$$

3.4.3. Oxygen concentration and temperature

Oxygen concentration and temperature were important factors to monitor throughout the fermentation process. In this study, it was defined that limiting oxygen conditions were achieved when the oxygen content of the exhaust air was lower than 5%, while the fully aerated system was considered when the oxygen content of the outlet gas was above 15%.

On the other hand, the temperature was monitored at different points of the packed bed: bottom, middle, and top parts of the packed bed, as well as at the air inlet, air outlet, and at the external wall of the reactor (Figure 15). iButton Devices (Thermochron, UK) were used, recording data every 10-15 min. Data were exported for further analysis once the experiment was ended. As these temperature values were available only when data

was exported, a temperature probe was also inserted in the reactor, to monitor the temperature values in real-time.

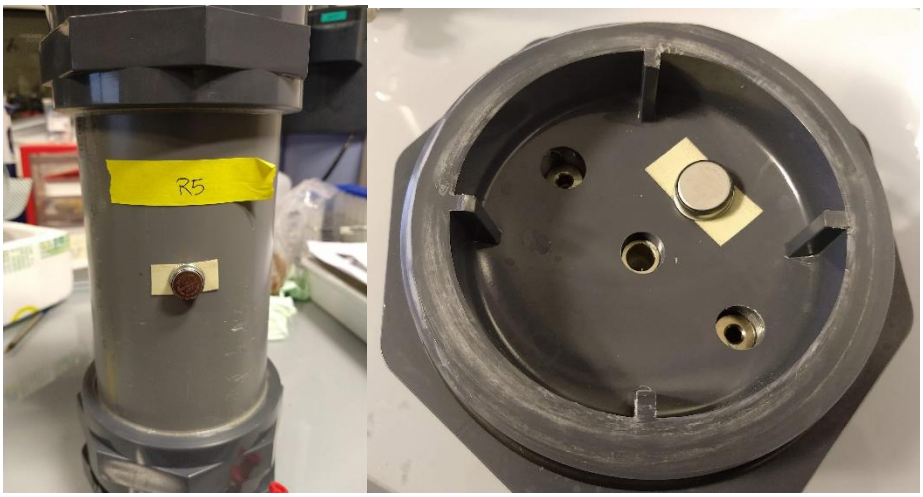

Figure 15: Sensors used to monitor temperature at different positions.

3.5. Design of Experiments

Design-Expert v11 (Stat-Ease, Inc, United States) was used as a tool to set the Design of Experiments (DoE). DoE refers to the process of planning, designing, and analysing the experiment in order that valid and objective conclusions can be drawn effectively and efficiently. It is necessary to integrate simple and powerful statistical methods into the experimental design methodology (Antony, 2014).

Box-Behnken designs always have three levels of each factor, and are purpose built to fit a quadratic model. The Box-Behnken design does not have runs at the extreme combinations of all the factors, but compensates by having better prediction precision in the center of the factor space. ANOVA test was used to the identify important terms in the model by means of p-values and coefficient values.

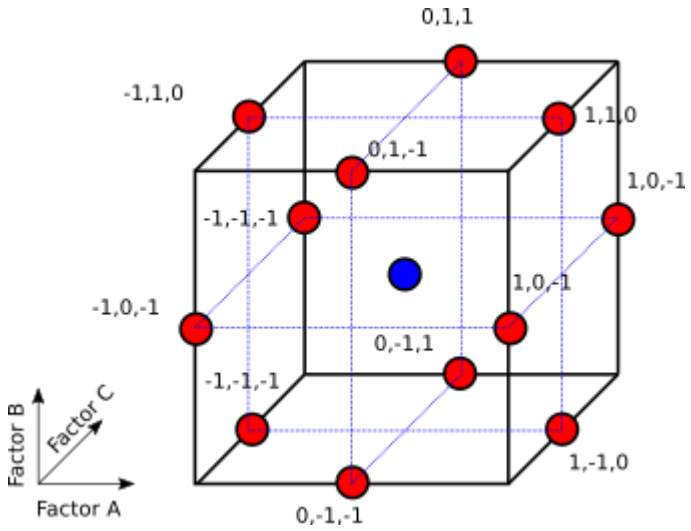

Figure 16: Box-Behnken design.

3.6. Analytical methods

3.6.1. Moisture and dry matter

To determine moisture content (M) and dry matter content (DM), a specific amount of solid sample (30-100 g) was weighed and placed in a previously weighed dry capsule, and then dried in an oven at 105ºC for 24 hours. After the drying period, the capsule is weighed, and the loss of weight is attributed to the quantity of water evaporated.

$$M(\%) = \frac{(Wi - Wf)}{(Wi - Wo)} x100 \text{ [Eq 4]}$$

$$DM(\%) = 100 - M(\%) \text{ [Eq 5]}$$

Where Wo is the weight of the dry capsule, Wi is the initial weight of the wet sample, and Wf is the final weight of the dry sample.

3.6.2. Organic matter

To determine the organic matter content (OM, equivalent to volatile solids), the dried solid was submitted to ignition conditions at 550ºC for 3 hours. Again, the loss of weight of the sample is attributed to the volatile solids. It is calculated as follows:

$$OM(\%) = \frac{(Wi-Wa)}{(Wi-Wo)} x100 \text{ [Eq 6]}$$

Where Wo is the weight of the dry capsule, Wi is the initial weight of the dry sample, and Wa is the final weight of remaining ash.

3.6.3. pH and conductivity

pH was determined by mixing the sample and distilled water in a ratio of 1:5 (w/v) and stirring for 30 min. pH and conductivity were then analyzed using an electronic pH-meter (pH50+ DHS, XS instruments, Italy) and an electronic conductivity meter (COND8, XS instruments, Italy).

3.7. Substrate mixing quality assessment

Bulk density, air-filled porosity, and effective mixing volume were the parameters analysed to evaluate the quality of substrates and bulking agent mixture. The air-filled porosity equation was taken from Richard et al. (2004).

$$BDw\ (Kg\ L^{-1}) = \frac{Ws}{Vs} \text{ [Eq 7]}$$

$$Air-filled\ porosity\ (\%) = 1 - BDw[\frac{(1-DM)}{DW} + \frac{(DMxOM)}{PDom} + \frac{DM(1-OM)}{PDash}] \text{ [Eq 8]}$$

$$Effective\ mixing\ volume\ (L) = \frac{Non-compacted\ mass}{BDw} \text{ [Eq 9]}$$

where BDw is wet bulk density, Ws is sample weight (Kg), Vs is sample volume (L), DW is water density (1000 kg L^{-1}), PDom is organic matter particle density (2500 Kg L^{-1}), and PDash is ash particle density (1600 Kg L^{-1}).

3.8. Determination of the biological stability and maturity

Two respiration indices were used to evaluate the biodegradability potential and the stability level of substrates and final fermented solids: dynamic respiration index (DRI_{24}), which is the average of instantaneous sOUR during the most intense 24 hours of activity, and AT_4, which is the total cumulated oxygen consumed for 4 days (Ponsá et al., 2010) (Eq 10).

$$AT4 = \int_{t1}^{t2} DRI\ dt \text{ [Eq 10]}$$

Where t1 is the lag phase, and t2 is the 96 hours after lag phase.

3.9. Seed germination test for toxicity evaluation

The objective of this test is to evaluate whether the fermented material is suitable as fertilizer by measuring its phytotoxicity. The test is based on obtaining a liquid extract of the solid and using it as seed germination media. Its quality will depend on the number of seeds germinated and the elongation of its roots. This methodology is extracted from Komilis and Tziouvaras (2009).

An aqueous extract of the fermented solid was performed by adding two parts of water per part of weight of the sample (v/w, on wet weight basis). This extract was centrifuged and passed 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 as follows:

$$Relative\ seed\ germination\ (\%) = \frac{Nº\ of\ seeds\ germinated\ in\ the\ solid\ extract}{No.of\ seeds\ germinated\ in\ the\ control} x100 \text{ [Eq 11]}$$

$$Relative\ root\ growth\ (\%) = \frac{Mean\ root\ lenght\ in\ the\ solid\ extract}{Mean\ root\ lenght\ in\ the\ control} x100 \text{ [Eq 12]}$$

$$Germination\ index = \frac{Relative\ seed\ germination\ x\ Relative\ root\ growth}{100} \text{ [Eq 13]}$$

3.10. Specific methods for toxin determination

3.10.1. Purification process

To determine the concentration of Cry protein, a purification process was performed as described in Mounsef et al. (2015). A liquid-solid extraction was needed, mixing the fermented solid with Ringer solution in a ratio of 1:9 (w/v) and shaken for 20 min at room temperature. The final suspension was then lyzed, using a sonicator (Vibracell VC50, Sonics&Materials, USA), to release the protein crystal into the extracellular medium. At this point, the protocol followed was focused on the separation of the crystal from the other proteins or cellular debris, and its solubilization.

Reagents

0.14M NaCl-0.01% Triton solution and 0.05N NaOH solution

Procedure

- Centrifuge lysate suspension at 10000 rpm for 10 min and discard the supernatant, recovering the pellet.
- Wash the pellet twice with a solution of 0.14M NaCl-0.01% Triton.
- Wash the pellet four times with cold water.
- Dilute the remaining pellet with a solution of 0.05N NaOH and shake it for 3 hours.
- Centrifuge the suspension at 10000 rpm for 10 min.

- Discard the pellet and keep the supernatant.

3.10.2. Protein quantification (Lowry)

Lowry assays were performed to determine the concentration of total protein from the purified samples, as described in Lowry et al. (1951). This method combines the reaction of copper ions with the peptide bonds in alkaline conditions, with the oxidation of aromatic protein residues. Thus, Cu^{2+} produced by the oxidation of peptide bonds reacts with the Folin-Ciocalteau reagent used in the assay, changing the colour of the reaction media. Protein concentration can be linearly correlated with absorbance using a calibration curve.

Reagents

- Solution A: 5.72 g of NaOH and 28.62 g of Na_2CO_3 in 1 L of distilled water.
- Solution B: 0.71 g of $CuSO_4 \cdot 5(H_20)$ in 50 mL of distilled water.
- Solution C: 1.43 g of $C_4H_4O_6Na_2 \cdot 2(H_2O)$ in 50 mL of distilled water.
- Lowry reagent: Solution A + Solution B + Solution C (100:1:1, v).
- Final solution: 54.5% (v) of mili Q water and 45.5% (v) of Folin-Ciocalteau reagent.

Procedure

1. Add 0.7 mL of Lowry reagent to 0.5 mL of the liquid sample.
2. Shake it (vortex) and keep it in dark conditions for 20 min.
3. Add 0.1 mL of final solution to the reaction media.
4. Shake it (vortex) and keep it in dark conditions for 30 min.
5. Measure the absorbance at λ=750 nm.

Calibration curve

The protein standard curve was obtained by a serial dilution of 2 mg ml^{-1} BSA protein standard (Table 4).

Table 4: Protein calibration curve standard preparation.

Nº	Standard (mL)	Distilled H_2O (mL)	Protein concentration (mg mL^{-1})
0	0	0.5	0
1	0.05	0.45	0.02
2	0.1	0.4	0.04
3	0.15	0.35	0.06
4	0.2	0.3	0.08
5	0.25	0.25	0.1
6	0.3	0.2	0.12
7	0.35	0.15	0.14
8	0.4	0.1	0.16
9	0.45	0.05	0.18
10	0.5	0	0.2

3.10.3. SDS-PAGE Electrophoresis

Mini-PROTEAN® TGX™ electrophoresis gels were used due to its versatile system for separating polypeptides by molecular weight (SDS-PAGE), between 12 and 200 kDa. An electric field is applied across the gel, causing protein migration from the negative electrode to the positive electrode. Depending on the molecular size, each biomolecule moves differently through the polyacrylamide gel, being easier for small molecules than for bigger ones.

Reagents

- 12% Mini-Protean® TGX™ Gel.
- 10x Tris/Glycine/SDS Buffer.
- Bio-safe Coomassie Stain.
- 2-Mercaptoethanol.
- 4x Laemmli Sample Buffer.
- Precision Plus Protein All Blue Prestained.

Procedure

1. Dilute the sample, if necessary
2. Sample buffer preparation: add 100 µL of 2-mercaptoethanol per 900 µL of 4x Laemmli sample buffer under the extraction hood.
3. Dilute 3 parts of the sample (30 µL) with 1 part of the sample buffer (10 µL).
4. Heat at 95ºC for 5 min.
5. Centrifuge samples for 1 min and store them with ice before using.
6. Running buffer preparation, by diluting 100 mL of 10x Tris/Glycine/SDS buffer with 900 mL of distilled water.
7. Load the sample (15 µL) onto the corresponding well. Use Precision Plus Protein All Blue as a marker. Apply 150 V to the electrophoresis system.
8. For gel staining, wash the gel with distilled water for 5 min. Repeat this 3 times. Then, discard all water, and add 50 mL of Coomassie per each gel. Shake it for 1 hour.
9. Lastly, wash the gel with distilled water for 30 min.

3.11. DNA extraction and 16S rRNA sequencing

DNA extraction was performed using a Soil DNA Isolation Plus Kit (Norgen), following the manufacturer instructions and samples were stored at -20ºC. Massive sequencing of 16S rRNA genes was performed using the Illumina MiSeq platform by the Genomic Service of UAB. Gene sequences were processed, and the reads were classified into multiple taxonomic levels.

3.12. Microplastics identification and quantification

Microplastics (MP) 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 – 600 °C at 10 °C min^{-1}, N_2 flow 50 mL min^{-1}. Terminating with a 3 minutes isotherm at 600 °C, N_2 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 in a cooled injection system (CIS4, Gerstel GmbH & Co KG, Germany), separated through a gas chromatograph (GC7890, Agilent, USA) with a (HP 5ms Ultra Inert 30m x 250 µm x 0.25 µm, Hewlett Packard, USA) column, and detected in the mass spectrometer (MSD 5973N, Agilent, USA).

The adsorber is first heated up in the TDU from 50 - 300 °C at a rate of 40 °C min^{-1} which terminates with a 300 °C 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 -120 °C to 270 °C 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 – 300 °C at a rate of 5 °C min^{-1}, which then ends with an isothermic step at 300 °C 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 230 °C, quad 150 °C, energy 70 eV, low mass 30, high mass 450).

3.13. References

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CHAPTER 4: EFFECT OF OPERATIONAL PARAMETERS AND SUBSTRATE CHARACTERISTICS ON Bt SPORE PRODUCTION

4.1. Overview

In the framework of the DECISIVE project, the production of Bt spores was first assessed by Cerda et al. (2019) and Rodríguez et al. (2019). Those proofs of concept performed at different reactor scales demonstrated the ability of Bt to sporulate and produce crystal proteins. Moreover, important findings were observed in terms of reactor operation, such as the highest spore concentration was reached after 72 hours of process and its value remained constant if the operation was prolonged. However, Bt growth stage must be improved for boosting spore production. This chapter focuses on a better understanding the biopesticide production process using the digestate as a substrate. For this purpose, a methodology based on the Design of Experiments was chosen to study the effects of most relevant operation variables on *Bacillus thuringiensis* spore production.

Part of this chapter was used to elaborate Deliverable 4.7: Design parameters to scale-up the SSF process to a demonstration plant. Technical, environmental and preliminary economical recommendations.

4.2. Selection of parameters

In this study, six different variables were analysed: substrate moisture and biodegradability, operating temperature, airflow, mixing, and inoculation ratio.

Airflow rate and mixing are two traditional operational parameters analyzed in aerobic fermentations. Airflow rate is a frequently evaluated parameter in many early-stage studies, not only for being an oxygen source for the SSF, but also for its effect on the metabolic heat and gas removal from the packed-bed (Martínez et al., 2017; Piedrahíta-Aguirre et al., 2014). However, high airflow rates can also alter the structure of the packed bed, changing the porosity, permeability, and water content, causing pressure gradients through the axial axis (Mitchell et al., 2006). In the present study, a

non-limiting airflow rate range was taken from our previous study (Mejias et al., 2017), where the effect of the airflow rate on the composting process was assessed using respirometric indices. Here, it described a flowrate threshold at 0.16 mL min^{-1} g^{-1}, below which could negatively affect the biological activity. In this first study, we wanted to observe any specific effect on Bt spore production when working with non-limiting airflow rates. Thus, airflow rate values of 0.16, 0.32, and 0.47 mL min^{-1} g^{-1} were evaluated.

Mixing was selected due to its effect on metabolic heat removal, incrementing convective heat, and mass transfer. However, mixing is not always possible because of the substrates or microorganisms used in the SSF (Mitchell et al., 2006). For example, when working with a fungi strain, mixing cycles destroy the mycelium structure, affecting fungi growth. However, in other studies using yeast as producers, intermittent mixing improved the mass transfer, obtaining a higher growth and metabolite production. Jiménez-Peñalver et al. (2016) reported a 31% increase in sophorolipid production when three manual mixings were applied in a 10-day batch.

Substrate composition is also of great importance for the SSF performance. Digestate biodegradability determines the amount of biodegradable organic matter that can be assimilated for microorganism growth. If a very stable digestate is used as a substrate, Bt growth could be negatively affected due to a lack of an easily assimilable carbon source (Odeniyi and Adeola, 2017). To increase substrate biodegradability, fruit and vegetable biowaste was used as a co-substrate, as it is a rich source of easily biodegradable organic matter. Ballardo et al. (2017) used biowaste as a sole substrate, and kitchen waste was used by Zhang et al. (2013). Furthermore, biowaste is the main waste to valorize in the DECISIVE management scheme, and it was considered more

appropriate to incorporate it as co-substrate than any other organic waste. For that reason, 0%, 25%, and 50% of biowaste (wet weight basis) was used as co-substrate.

In this context, the temperature is a parameter related to biodegradability, airflow rate, and mixing, which has a relevant effect on microorganism growth. The optimal temperature for Bt growth is reported as 30ºC (El-Bendary, 2006; Mounsef et al., 2015). However, because of the connection between SSF scale-up and metabolic heat retention, its effect on the process should be analyzed. Some authors have reported high temperatures as suitable for Bt spore production. In Ballardo et al. (2017), temperatures over 45ºC were maintained for 7.5 days, achieving a maximum value of 69ºC. After the thermophilic phase, viable cells and spores were observed. In Smitha et al. (2013), the incubation of the solid was performed at 37ºC, obtaining a successful Bt toxin recovery. Thermophilic temperatures were not expected at laboratory scale due to digestate biodegradability. Thus, a range of mesophilic temperatures was selected (30, 37, and 45ºC) for the DoE.

Moreover, substrate moisture content can also be a variable parameter, mostly depending on the dewatering yield. In the solid waste treatment plant of Granollers, biowaste treatment follows the anaerobic digestion treatment and further digestate composting. The concentration of the organic suspension fed into the mesophilic anaerobic digestors ranges from 43 to 57 g L^{-1} of total solids (Abad et al., 2019). Thus, a digestate dewatering process is needed for its further composting, while the liquid digestate is either externally or internally treated. In this first study, a range of moisture from 50-70% was assessed, as reported by other authors as adequate for Bt growth in SSF (Wu et al., 2014).

Inoculum size is another studied parameter in lab-scales studies (da Cunha et al., 2018; El-Bendary et al., 2017), aiming to obtain the maximum production using the

minimum inoculum. El-Bendary et al. (2017) observed a decrease in spores count (around one order of magnitude) when higher inoculum sizes were tested. Hence, the effect of inoculum size on spore production should be assessed. Moreover, an excess of inoculum use is an extra running cost, that could be avoided. In da Cunha et al. (2018), a range between $1x10^6$ and $1x10^7$ spores g^{-1} was used. In the present study, Bt vegetative cells were used as inoculum, and the inoculum range was extended from $1x10^6$ to $1x10^8$ CFU g^{-1} DM.

Summarizing, two designs of experiments were performed to evaluate the effect of these parameters and their synergies on Bt spore production. Specifically, two Box-Behnken designs were performed and evaluated, using the Design Expert® software, analysing three factors at three levels, with a triplicate run of the central point. Thus, each DoE consisted of 15 runs, testing 13 different combinations. Parameters evaluated in each DoE and the range of values selected are shown in Table 5.

Table 5: Range of parameters evaluated on the set of DoEs.

1^{st} DoE		2^{nd} DoE	
Airflow rate [mL min^{-1} g^{-1}]	0.16, 0.32, 0.47	Co-substrate added [%, wet weigh basis]	0, 25, 50
Moisture content [%]	54, 60, 68	Inoculum size [CFU g^{-1} DM]	10^6, 10^7, 10^8
Temperature [ºC]	30, 37, 44	Mixing [total times]	0, 2, 4

4.3. Experimental methodology

The DoE experiments were carried out for 3 days in the Setup 1. Digestate from different batches was used in the two sets of experiments. In DoE 1, reactors were placed in thermal baths at different temperatures and connected to the aeration system at a fixed airflow rate value. Digestate moisture was adjusted by adding water to the substrate. The combination of the different set parameters is shown in Table 6.

Table 6: Temperature, moisture, and airflow rate combinations tested in DoE 1.

Run	Temperature [ºC]	Initial moisture [%]	Airflow rate [mL min^{-1} g^{-1}]
R1	44	68	0.32
R2	37	68	0.47
R3	37	68	0.16
R4	30	54	0.32
R5	30	60	0.16
R6	44	60	0.16
R7	37	54	0.16
R8*	37	60	0.32
R9	30	60	0.47
R10	44	54	0.32
R11*	37	60	0.32
R12	44	60	0.47
R13	30	68	0.32
R14*	37	60	0.32
R15	37	54	0.47

* corresponds to central point condition.

In DoE 2, the substrate biodegradability, inoculation ratio, and mixing were assessed. The fermentation was performed at 30ºC with an airflow rate of 0.21 mL min^{-1} g^{-1}, considering results obtained from DoE1. Digestate biodegradability was increased by adding biowaste as co-substrate, while the inoculation ratio was modified by inoculating the solid substrate with the same volume, but using different inoculum concentration

stocks. Regarding the agitation regime; the mixing effect was assessed by performing a static fermentation or manually mixing the packed bed content every 24 or 48 hours. The tested combinations of these three parameters are shown in Table 7.

Table 7: Biodegradability, inoculation ratio, and mixing combinations tested in DoE 2.

Run	Biodegradability [weight of biowaste, %]	Inoculation ratio [$CFUg^{-1}$ DM]	Mixings [total times]
R1	50	10^7	0
R2	0	10^7	4
R3	50	10^7	4
R4	0	10^8	2
R5	0	10^6	2
R6*	25	10^7	2
R7*	25	10^7	2
R8*	25	10^7	2
R9	50	10^8	2
R10	25	10^6	0
R11	0	10^7	0
R12	25	10^6	4
R13	25	10^8	4
R14	50	10^6	2
R15	25	10^8	0

* corresponds to central point condition.

The final spore production was set as DoE's objective function, as an indirect method of toxin quantification. Results were statistically analysed using Design Expert software.

4.4. Results

4.4.1. The effect of the airflow rate, moisture content, and temperature on the Bt spore production (DoE 1)

Taking the preliminary work performed by Cerda et al. (2019) and Rodríguez et al (2019) as a reference, this experiment allowed us to understand the requirements for the proper Bt growth and sporulation. Initial and final CFU, and final spore concentration are shown in Figure 17. As observed, null final viable cells and spore counts were obtained in runs R1, R10, and R12, coinciding with those runs performed at a constant temperature of 44ºC. In fermentations performed at 30 and 37ºC, the final spore production ranged from 10^7 to 10^8 spores g^{-1} DM. However, as in Cerda et al. (2019), the final spore concentration was lower than the initial viable cells inoculated in all conditions tested, indicating that in these conditions, Bt was not able to grow.

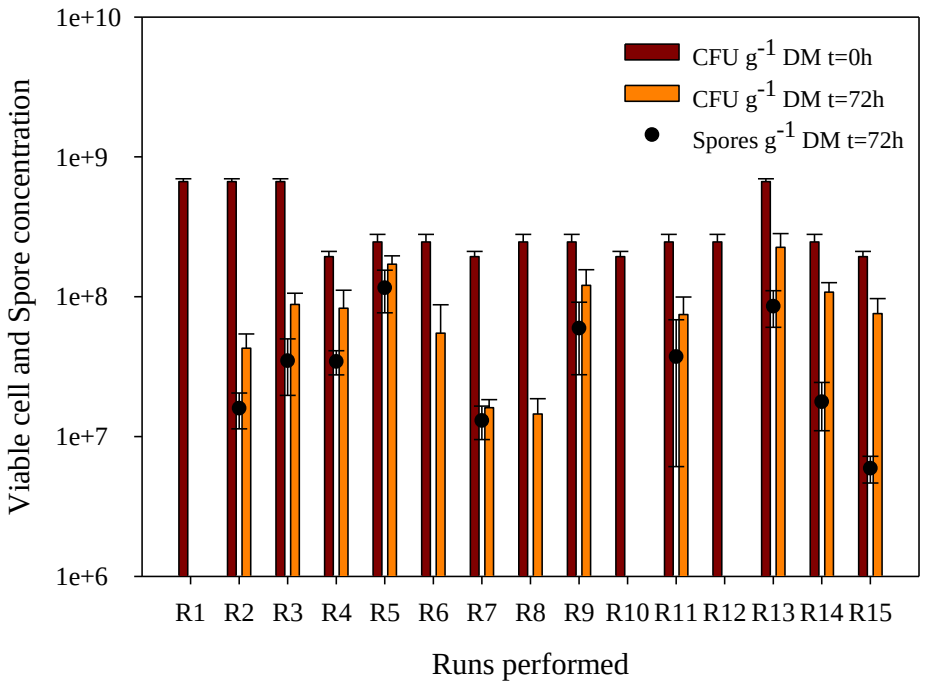

Figure 17: Comparison between initial CFU and final CFU and spore's concentration applying combinations tested in DoE 1.

Regarding the total biological activity, sOUR max values ranged between 1.4 and 3.1 g O_2 kg^{-1} DM h^{-1}. sOUR profiles can also be used as an indirect method for products or metabolites production. In Cerda et al. (2017), a correlation was found between the sOUR peak and the maximum enzymatic activity. Thus, the time with maximum biological activity corresponded with the maximum cellulase production. However, Cerda et al. (2019) reported maximum spore production at 72 hours, while the maximum sOUR was achived within the first 24 hours. Nonetheless, it would be interesting to find out if higher values of biological activity led to higher spore values. Any strong correlation was found between spore production and maximum sOUR. However, it was observed that, when performing the SSF at different temperatures, higher biological activity was monitored in those runs performed at 44ºC by linear multivariable regression (R^2 of 0.67) as shown in Table 8. Thus, the increase in biological activity did not occur due Bt growth, but for the temperature effect on other microorganisms present in the non-sterile but hygienized substrate. It may be considered that the hygienisation of substrate does not eliminate all the microorganisms present, and inoculated Bt must compete with those autochthonous microorganisms.

Table 8: The effect of temperature, moisture, and airflow rate on the biological activity (sOUR max).

Source	Coefficient	p-value
Regression	-	0.005
Lack of fit	-	0.58
Constant term	1.37	-
Temperature	0.08	0.001
Moisture	-0.03	0.08
Airflow rate	0.76	0.36

Analysing the spore production results with the Design Expert software, the best fit was using a linear model (p-vale of 0.007, lack of fit of 0.97, and R^2 of 0.55) (Table 9). A quadratic model was not suitable for describing the response surface, as the p-value was higher than 0.05.

Table 9: Anova statistical test for DoE 1, considering a linear model on the spore production at 72 hours.

Source	Coefficient	p-value
Model	-	0.007
Lack of fit	-	0.98
Constant term	5.00	-
Temperature	-3.91	0.001
Moisture	0.16	0.86
Airflow	-0.12	0.89

Temperature was the only parameter with a significant effect on the process, with a p-value below 0.05. Results showed that performing the fermentation process with stable temperatures over 30ºC negatively affects the process. On the other hand, airflow rate and moisture content did not significantly affect spore production in the tested range (Table 9). This means a modification of moisture content or a change in airflow rate setpoint did not show a significant increase in spore production. As stated in Mejias et al. (2017) this allows working with low airflow rates, obtaining an economical saving, as the airflow rate is related to energy operational costs. In consequence, environmental impacts are also reduced. Catalán et al. (2017) reported that high-energy consumption systems in SSF processes lead to higher environmental impacts due to indirect GHG emissions in terms of life cycle assessment (LCA).

For DoE 2 performance, an airflow rate of 0.21 mL min^{-1} g^{-1} was selected, as a low value within a non-limiting airflow rate range (Mejias et al., 2017). In terms of water

content, moisture would not be adjusted, whenever digestate moisture fell between 50 and 70%. Lastly, the water bath temperature was set at 30ºC.

4.4.2. The effect of the biodegradability, inoculation ratio, and mixing on the Bt spore production (DoE 2)

Figure 18 shows the initial and final CFU, as well as the final spore concentration. Some promising results were observed in this second DoE. In runs R1, R3, R9, and R14, final CFU and spore concentration were equal or higher than initial CFU, meaning that Bt was able to colonize the solid and sporulate. These mentioned runs corresponded to those with 50% of biowaste as co-substrate. On the other hand, runs performed without co-substrate (R2, R4, R5, and R11) suffered a decrease in CFU between two and three orders of magnitude compared with initial values, and with poor or null spore production.

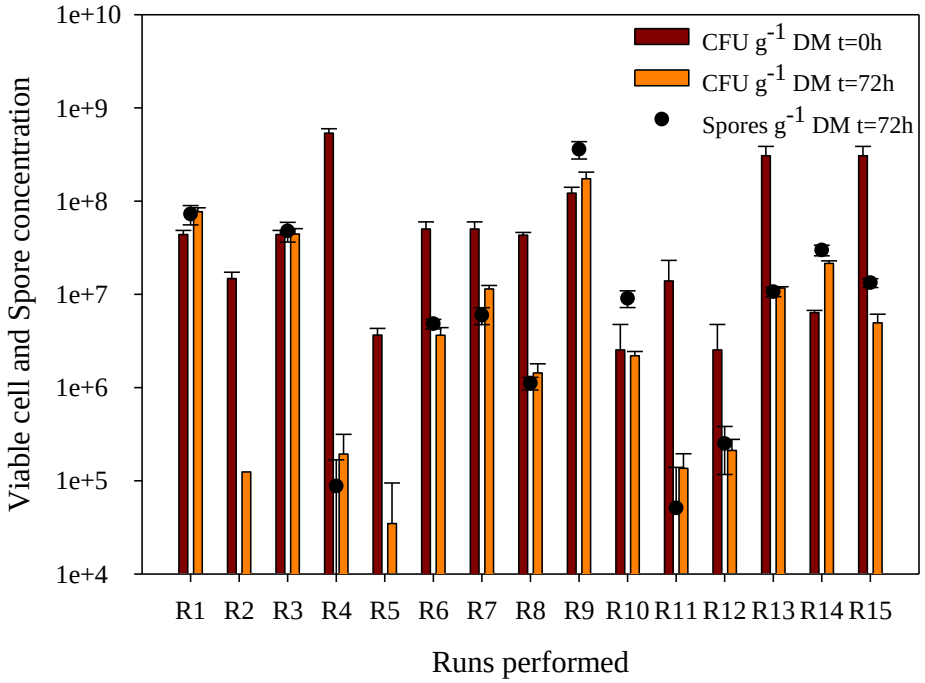

Figure 18: Comparison between initial CFU and final CFU and spore concentration applying combinations tested in DoE 2.

In this second DoE, a quadratic model was suitable for describing the surface response, with a model p-value of 0.0064. Coefficients and p-values of the quadratic terms are shown in Table 10. The quadratic model adjusted R^2 was 0.88.

Table 10: Anova statistical test for DoE 2, considering a quadratic model on the spore production at 72 hours.

Source	Coefficient	p-value
Model	-	0.006
Lack of fit	-	0.115
Constant term	8.61	-
Mixing	-0.61	0.149
Biodegradability	2.45	0.001
Inoculum	0.98	0.028
Mixing x Biodegradability	1.13	0.053
Mixing x Inoculum	0.52	0.237
Biodegradability x Inoculum	-0.70	0.130
Mixing^2	-0.03	0.954
Biodegradability^2	-1.14	0.030
Inoculum^2	-1.96	0.280

Analysing Table 10, it was observed that biodegradability, inoculum ratio, and biodegradability^2, were the significant terms of the quadratic model. The response surface showed how maximum spore production could be achieved when performing the SSF using biowaste as co-substrate (1:1 ratio), and inoculating the substrate mixture with 10^7 CFU g^{-1} DM. Mixing and the other interaction parameters were not statistically significant.

Results confirmed that biodegradability was one of the key parameters of the process, as a change of substrate biodegradability implied a large variation of the final

spore production. Specifically, adding 50% (w/w) of biowaste with digestate improved the final spore concentration by 4 orders of magnitude, in comparison to those runs without co-substrate. Consequently, it could be hypothesized that the development of Bt was limited in SSF with digestate as a sole substrate, due to a lack of easily biodegradable carbon source and other nutrients. These findings were of significant relevance, since the success of the SSF process would strongly depend on the biodegradability of substrates.

Even though mixing was not selected as a key parameter, better results were obtained when manual mixing was applied. Nevertheless, mixing can affect other parameters, such as matrix compaction or shear force impact, affecting microorganism growth, and being restricted by nutrient diffusion (Chen, 2013).

Lastly, in terms of inoculum requirements, an important inoculum saving was achieved compared with previous works. Rodríguez et al. (2019) worked with initial values around $2x10^9$ CFU g^{-1} DM, which exceeds the inoculum threshold found in DoE 2. Thus, this inoculum reduction benefits spore production, as well as the economy of the process.

In summary, temperature, substrate biodegradability, and inoculation ratio were selected as key parameters with a significant role in Bt growth and spore production. Best results were obtained when 50 % of biowaste was used as co-substrate, inoculated with $1\text{-}5x10^7$ CFU g^{-1} DM, and working at a temperature of 30ºC.

4.4.3. Implication of temperature and substrate biodegradability on the scalability of the process

In a composting process, the temperature fluctuates in response to microbial activity, which consumes organic matter and oxygen, and generates heat (Gajalakshmi and Abbasi, 2008). In the first mesophilic stage (10-45ºC), microorganisms break down

easily biodegradable molecules. Usually, in a short time (2 or 3 days), the temperature rises to 50-60ºC. Barrena et al. (2006) estimated the metabolic heat generated was according to the biodegradable organic matter of the samples used. Hence, the connection between the material biodegradability and the temperatures reached in the process is not negligible. Besides, the self-insulation properties or low thermal conductivity of these organic wastes when the composting or SSF in performed at industrial scales makes the metabolic heat removal difficult (Haug, 1993).

In the studied process, using biowaste as co-substrate involves an increase in the content of easily biodegradable organic matter. In consequence, temperatures higher than 30ºC can be expected (Arora et al., 2018; Cooney,1968). This effect can be observed in Ballardo et al. (2016) and Rodríguez et al. (2019). Using the same bioreactor configuration (10-L adiabatic reactor), thermophilic temperatures were maintained for 7.5 days when using biowaste as substate. However, when using digestate, the thermophilic phase was reduced to 2 days. Therefore, scale-up studies would take into account to the temperature dynamics at different positions of the packed bed, and mitigate its effects on spore production. The key point, in terms of this dynamic temperature profile, would be to determine the adequate temperature for Bt growth, and afterward, the suitable temperature for promoting its sporulation. If an operation strategy is developed to maintain these conditions, a successful scale-up could be expected.

4.5. Conclusions

Temperature, inoculation ratio, and substrate biodegradability were selected as key parameters of the process, which affected the final spore production when their value was modified in the range tested. While some protocols can be developed to maintain the inoculation ratio and the substrate composition within a desirable range, the difficulty of

the process would depend on the temperature response during the scale-up.

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CHAPTER 5: DEFINITION AND DEVELOPMENT OF THE OPERATIONAL BASIS OF THE PROCESS

5.1. Overview

In this section, the effect of adding biowaste as co-substrate on the temperature dynamics and spore production was studied at lab-scale (1.6-L). The reproducibility of the process was also assessed. Since digestate and biowaste from different batches would be used for running the pilot reactor, it was necessary to determine if a constant spore production could be obtained despite the inherent lot variability. Thus, digestate and biowaste from different sampling campaigns were used. Due to the linkage of biodegradability and temperature dynamics in the SSF, the possibility of supplementing digestate with a mixture of ions for boosting Bt growth was also evaluated.

To complete the study, the effect of oxygen on spore production was also studied. Oxygen content has stood out as an important variable affecting the *Bacillus* sporulation rate. Karim et al. (1993) demonstrated a rapid formation of *Bacillus sphaericus* free spores when working at high dissolved oxygen in submerged fermentations. Otherwise, an important decrease of spore formation rate was observed working with oxygen levels lower than 5%, promoting an increase of vegetative cells. Accordingly, when a non-sterile substrate is used, and Bt being an anaerobe facultative bacterium, a limiting oxygen environment during the first hours of the process should be a competitive advantage versus autochthonous microorganisms. It is important to remark that autochthonous microorganisms are a combination of different aerobic, anaerobic, and facultative anaerobic microbial populations.

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5.2. Experimental methodology

The process development was studied in the Setup 2 (see chapter 3). A first experiment was performed evaluating five different substrates mixtures (% biowaste-% digestate): 0-100; 12.5-87.5; 25-75; 37.5-62.5; 50-50 (wet weigh basis). As scale-up criteria, the same superficial air velocity (0.006 m min^{-1}) was maintained between scales (Pitol et al., 2016), setting the airflow rate at 0.12 mL min^{-1} g^{-1}.

Secondly, we studied the alternative of supplementing digestate with a solution of mixed ions to improve the adequacy of the digestate as a substrate. Zhang et al. (2013) observed a lack of K^+, Mg^{2+}, and Fe^{2+} in kitchen waste, which describes as needed ingredients for Bt growth. To validate the results obtained in the first experiment and explore this new possibility, three different conditions in terms of substrate composition were tested, using digestate, a mixture of digestate and biowaste, and using digestate supplemented with a solution of mixed ions. This solution was composed of a 29% $MgSO_4 \cdot 7H_2O$, 29% $FeSO_4 \cdot 7H_2O$, and 42% KH_2PO_4. 10 mL of this solution were added to the 470 g of solid matrix.

Lastly, a multi-categorical design was constructed to study the effect of the oxygen content and the percentage of biowaste on Bt growth and sporulation. Each factor was treated as a categorical variable into two levels (low or high). Low biowaste mixtures contained 42.5 g of biowaste and 382.5 g of digestate (90% digestate-10% biowaste), while high biowaste mixtures composition was 159.4 g of biowaste and 265.6 g of digestate (62.5% digestate-37.5% biowaste, wet weight basis). An airflow rate of 0.28 mL min^{-1} g^{-1} was set to reach a high oxygen, while an airflow rate of 0.03 mL min^{-1} g^{-1} was set to get a low oxygen content. Reactors with a higher fraction of biowaste were run in duplicates. So, a total of 6 reactors were operated and sampled after 72 hours of fermentation.

5.3. Results

5.3.1. Interaction between the substrate biodegradability and temperature on the spore production at 1.6-L reactors

The characterization of the substrates used is shown in Table 11. Initial digestate moisture content ranged from 29 to 38%, whereas pH values were maintained between 8.3-8.5. As observed, the addition of biowaste produced a reduction of pH and initial dry matter. In El-Bendary et al. (2017), a different range of initial pH was tested (from 6.5 to 8.5), observing better results when the initial pH was set at 6.5. Thus, it was expected that this decrease in pH would favor the process. The option of decreasing the pH value with an acid solution was not contemplated because of the high buffer capacity of the digestate. Preliminary studies showed that a large amount of an acid solution was needed to decrease the pH, obtaining a semi-solid solution not suitable for SSF performance (Mejias et al., 2018).

The addition of biowaste also implied an increase of DRI values that can be directly related to an increase of easily biodegradable organic matter and a higher diversity microbial load. DRI values were mostly doubled when using a mixture of 50% of digestate and 50% of biowaste (from 3.8 to 4.4 g O_2 kg^{-1} DM h^{-1}) compared to when digestate was the only substrate (from 1.2 to 2.3 g O_2 kg^{-1} DM h^{-1}).

Table 11: Characterization of substrate mixtures used in R1.6-L.

	Experiment A			Experiment B			Experiment C		
Biowaste [%]	Initial pH	Initial dry matter [%]	DRI [g O_2 kg^{-1} DM h^{-1}]	Initial pH	Initial dry matter [%]	DRI [g O_2 kg^{-1} DM h^{-1}]	Initial pH	Initial dry matter [%]	DRI [g O_2 kg^{-1} DM h^{-1}]
0	8.38	34±1	1.22±0.01	8.63	38±1	1.42±0.04	8.54	29.1±0.5	2.3±0.3
12.5	8.01	31±1	2.9±0.9	8.51	32.3±0.6	-	8.42	27.6±0.5	-
25	7.84	29±2	2.6±0.2	8.24	30.6±0.2	-	8.16	27.0±0.2	-
37.5	8.87	27.5±0.5	2.9±0.3	8.11	30.4±0.8	-	8.13	25.3±0.7	-
50	7.33	25.6±0.1	3.8±0.5	7.82	28.6±0.5	3.9±0.2	7.68	23.8±0.9	4.4±0.4

Figure 19 shows the evolution of sOUR throughout the fermentation run. In experiment A, higher sOUR peaks were reached, slightly shifted in time, as higher biowaste was added to the mixture. Almeira et al. (2015) attributed the longer lag phase of fresher substrates to the lack of acclimated biomass. Moreover, no differences in terms of sOUR profiles were observed between runs with 0 and 12.5% of biowaste, as well as those with 25 and 37.5%. However, in experiments B and C, runs with 50% of biowaste behaved significantly different compared to experiment A in terms of oxygen consumption profiles. Maximum sOUR values did not exceed 3 g O_2 kg^{-1} DM h^{-1} and the sOUR peak did not occur within the first 24 hours of the process.

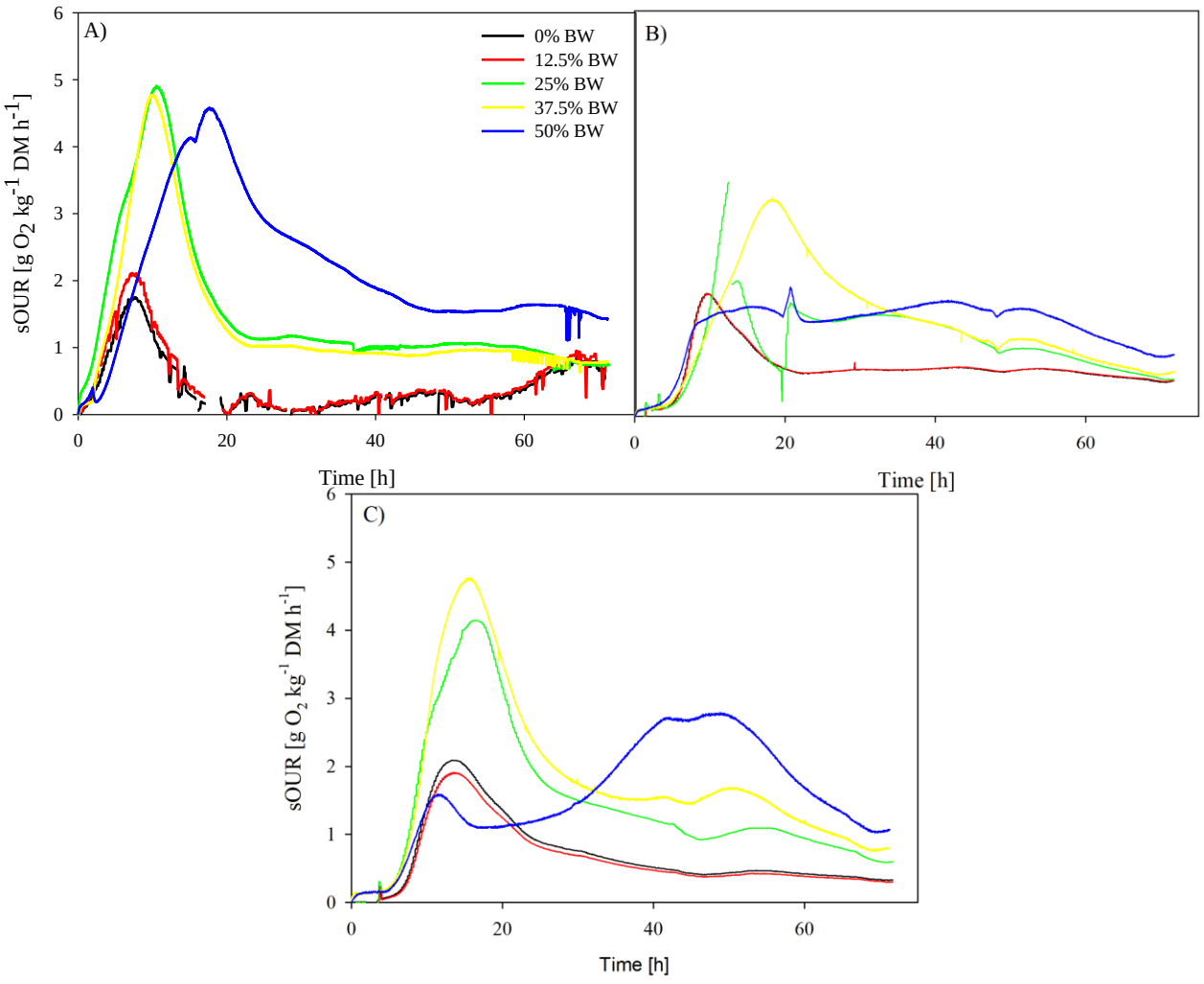


Figure 19: sOUR profiles of experiments A, B, and C, depending on the biowaste added to the mixture.

In terms of temperature dynamics in the packed bed, temperature gradients below 3ºC were observed along the axial axis (bottom, middle, and top). In Castro et al. (2015), a reactor with a working volume of 1.8-L was used to produce hydrolases from babassu cake using *Aspergillus awamori.* Temperature gradients of 18ºC were monitored along the axial axis, showing that heat transfer by forced aeration was not efficient for removing the metabolic heat in their process.

As shown in the box plot temperature representation (Figure 20), most of the recorded temperature values fluctuated between 24 and 32ºC, with a punctual temperature increment ranging from 29 to 37ºC. Room temperature had an important role since temperature fluctuation in packed beds also depended on whether the climate was running. No strong correlation was found between maximum temperature achieved and maximum sOUR.

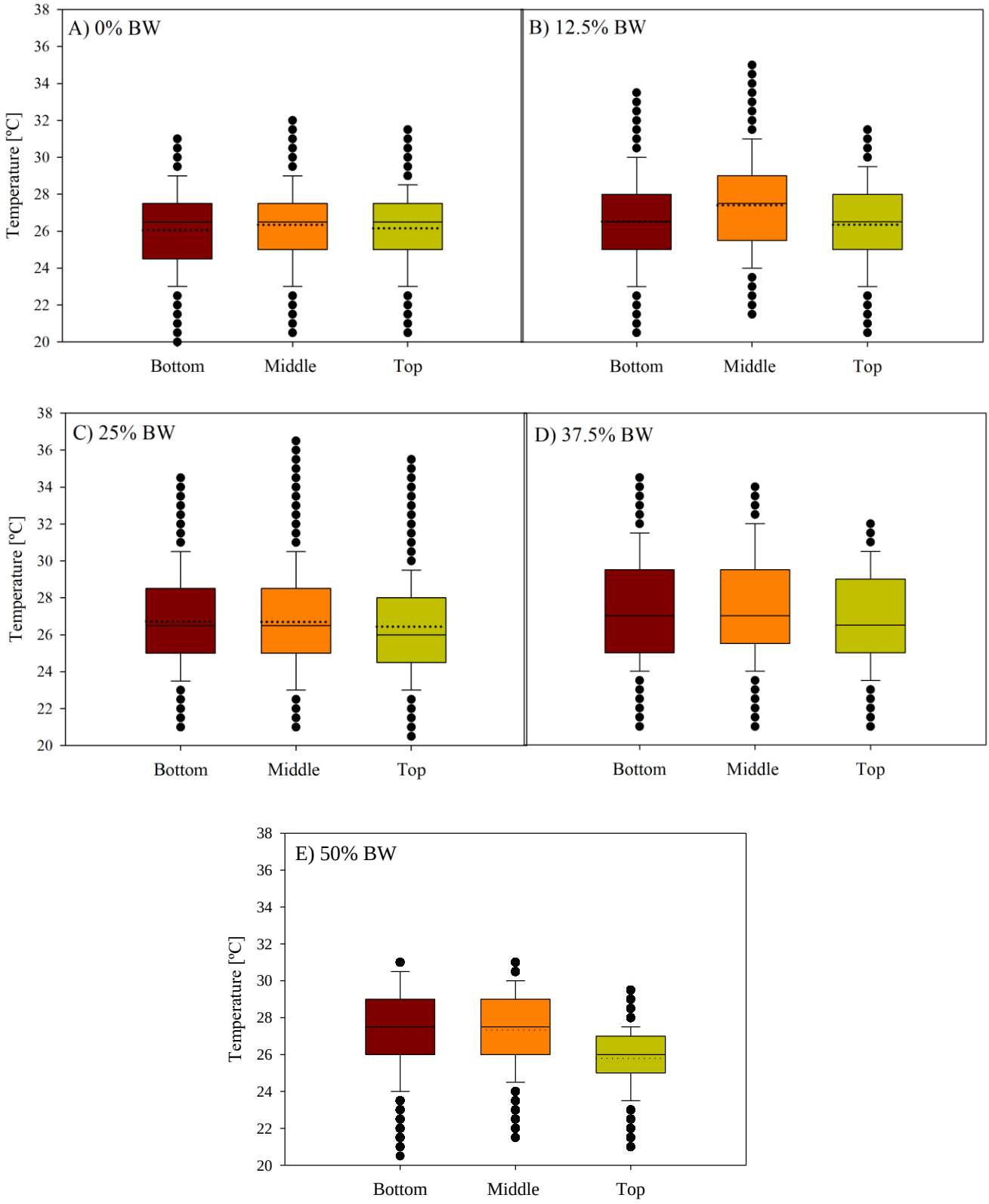
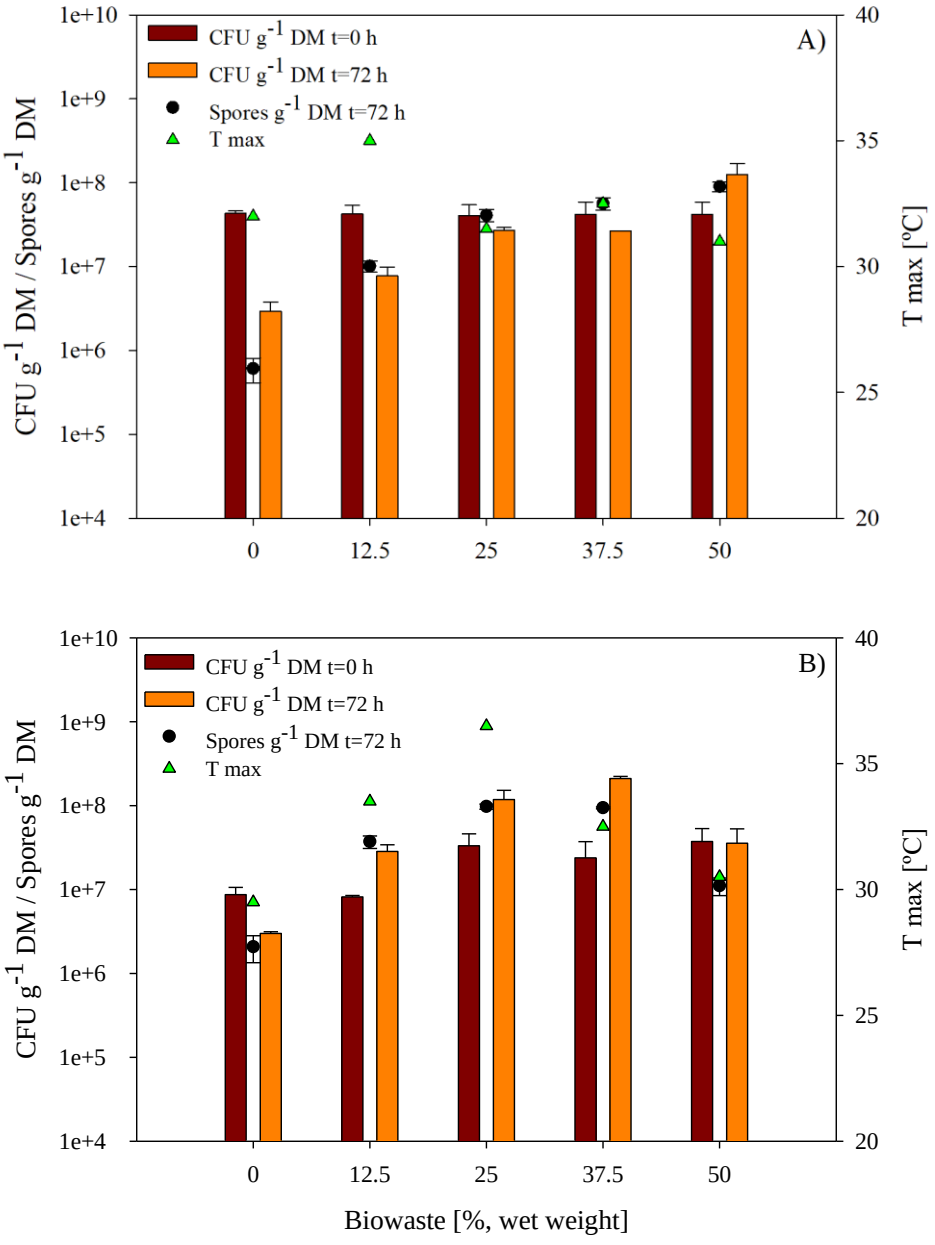


Figure 20: Box plot representation of temperatures recorded at three different positions (bottom, middle, and top) from the three experiments in function on the biowaste added.

While constant temperature exceeding 30ºC harmed the spore production in Setup 1, this was not observed in Setup 2, when working with a dynamic temperature profile (Figure 21). Again, runs with no co-substrate showed a spore decrease of one order of magnitude from the initial CFU concentration, reaffirming the results of DoE's experiments. Spore concentration was increased progressively as more biowaste was used as co-substrate. However, this linear trend was broken when using 50% of biowaste in experiments B and C, obtaining less spore concentration than the initial CFU concentration, being a non-profitable process.



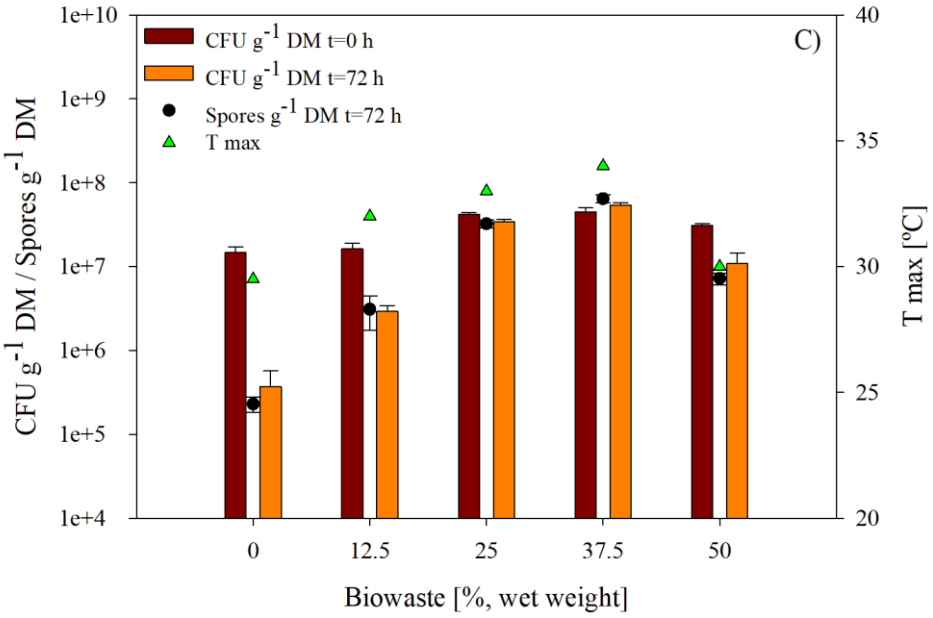


Figure 21: Initial CFU, final CFU, and final spore concentration depending on the biowaste percentage added to the mixture in the experiments A, B, and C.

In terms of reproducibility of spore production, significant differences were observed between experiments. In this sense, the use of the coefficient of variation (CV) was useful to estimate the most promising scenario. Abraham et al. (2017) studied the reproducibility of proteases production from nitrogen-rich wastes through SSF systems from experiments performed over 2.5 years. A CV of 4% was obtained for maximum protease activity, demonstrating that SSF can be reproducible at bench-scale.

As observed in Table 12, the CV enabled the dispersion of different magnitude values to be studied, whereas the value of the standard deviation was not adequate for this purpose, due to its order of magnitude. Hence, the scenario with a lower CV was that of using a 37.5% of biowaste, and also being the one with a higher spore production.

Table 12: Spore production mean, standard deviation, and coefficient of variation of the three experiments performed. Same letters indicate statistically similar means at $p<0.05$ (comparison is made applying the Tukey's pairwise comparison test).

Biowaste [%]	Mean	Standard deviation	Coefficient of variation [%]
0	$1.1x10^6$ (A)	$9.5x10^5$	89
12.5	$1.7x10^7$ (A)	$1.6x10^7$	95
25	$5.7x10^7$ (BC)	$3.1x10^7$	55
37.5	$7.2x10^7$ (C)	$1.8x10^7$	25
50	$3.6x10^7$ (AB)	$4.1x10^7$	113

Therefore, the use of co-substrate was reaffirmed as necessary to achieve a profitable Bt spore production. Furthermore, the value of 37.5% of biowaste was selected for further experiments, due to its higher spore production and its lower CV. It was also demonstrated that the process was not reproducible in terms of absolute production, but a final range of spores could be expected. However, the use of this co-substrate would imply extra work in terms of substrate conditioning. Hence, a last attempt to use the digestate as a sole source of carbon and nitrogen was considered.

5.3.2. The effect of digestate supplementation with a mixed ions solution

El-Bendary (2006) and Zhang et al. (2013) reported the importance of metal ions (K^+, Mg^{2+}, and Fe^{2+}) to obtain a more suitable medium for Bt growth. So, the idea of supplementing digestate with a solution of mixed ions was considered to avoid the need for using biowaste as co-substrate. Fermentation controls with non-supplemented digestate, as well as an SSF using a co-substrate (37.5% of biowaste) were also performed

in order to validate results from the previous experiments. Initial dry matter and pH conditions are presented in Table 13.

Table 13: Initial dry matter and pH of the three conditions tested.

	Digestate	Ion supplemented digestate	Digestate-biowaste
Initial DM	36.3±0.9	28±1	30±2
Initial pH	8.58	7.36	8.05

Figure 22 shows the sOUR profiles. A sOUR peak of 4.18 g O_2 kg^{-1} DM h^{-1} was reached when using biowaste. Digestate supplemented with mixed ions showed lower values of oxygen consumption (sOURmax of 0.98 g O_2 kg^{-1} DM h^{-1}) compared to the reactor with only digestate as substrate (sOURmax of 1.69 g O_2 kg^{-1} DM h^{-1}). Temperatures ranged from 20 to 30ºC, with a noticeable temperature drop at night, coinciding with turning off the heating system.

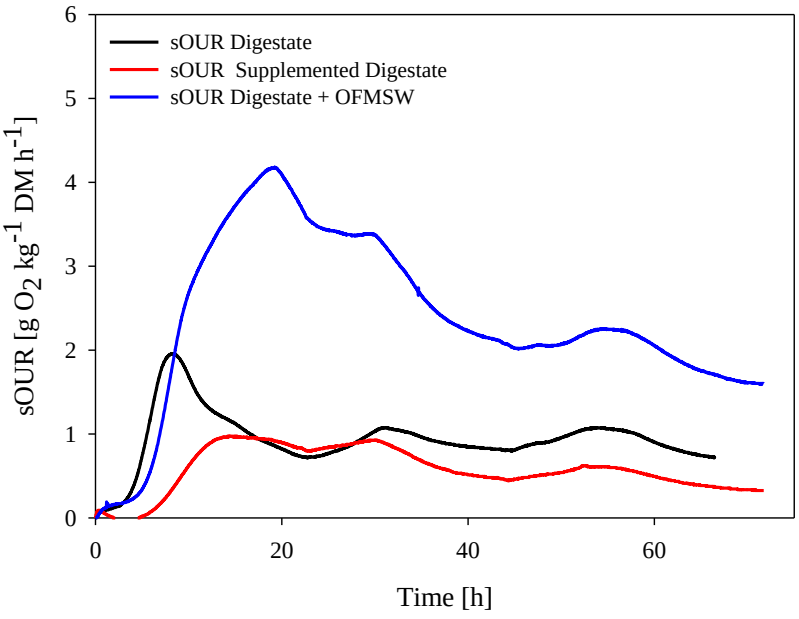


Figure 22: sOUR profiles during SSF of the three conditions tested.

In terms of Bt growth and sporulation (Figure 23), a significant decrease in total cells was observed when the solid matrix was composed of digestate, with a final sporulation ratio of 89%. Meanwhile, when the digestate was supplemented with a mixture of metallic ions, the total cell concentration was maintained, with a 100% sporulation ratio at the end of the process. However, the best scenario again was the SSF using co-substrate, with a significant increase in total cell concentration. Even if the sporulation ratio was low (42%), it was the scenario with highest spore concentration. That is, supplementing digestate with ions improved the total sporulation of vegetative cells, but it was not enough compared to Bt growth and final spore production when using the co-substrate.

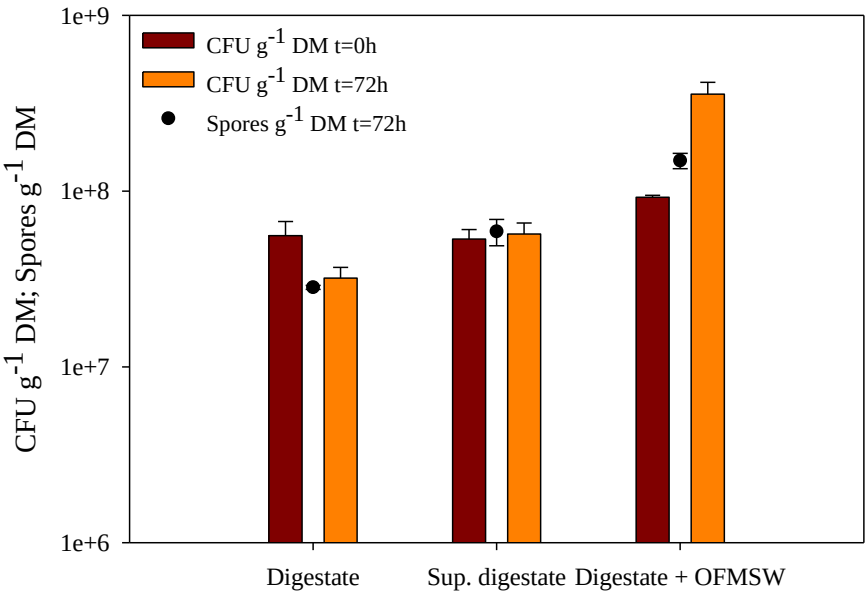


Figure 23: Initial and final CFU, and final spore production of the three conditions tested.

Ozkan et al. (2003) stated that Mn^{2+} was the most critical element for the biosynthesis of some Cry proteins at 10^{-6} M concentration. However, Fe^{2+}, Zn^{2+}, and Cu^{2+}

could negatively affect their synthesis. Zn and Cu inclusions also inhibited growth and sporulation at concentrations higher than 10^{-7} M. Moreover, the positive effect of biowaste as a co-substrate was validated again, establishing this mixture as the most appropriate for the process and the scale-up studies.

5.3.3. Setting the basis for the pilot reactor operation: the oxygen level and its effect on the spore production

The oxygen content during the fermentation was the last parameter studied to define the operational strategy. For the results seen so far, the SSF operation needs to promote Bt growth and sporulation in a non-sterile environment. Thus, a strategy focused on boosting its growth can be considered as the limiting step of the process. Bt is a facultative anaerobic bacterium (Sanchis and Bourguet, 2008), therefore it is able to survive in an anoxic or anaerobic environment for some time. Moreover, Karim et al. (1993) determined the importance of the oxygen level for the spore formation rate, observing a delay of its formation rate when oxygen levels lower than 5% were set in submerged fermentation, promoting an increase in vegetative cells.

In SSF systems, working with materials of different biodegradability, and maintaining the same airflow rate, leads to different oxygen levels. This effect was already observed in our system. Figure 24 shows the final spore concentration depending on the percentage of biowaste and the minimum oxygen level reached in the SSF. It showed a higher spore concentration when lower oxygen levels were achieved, due to its effect on Bt growth.

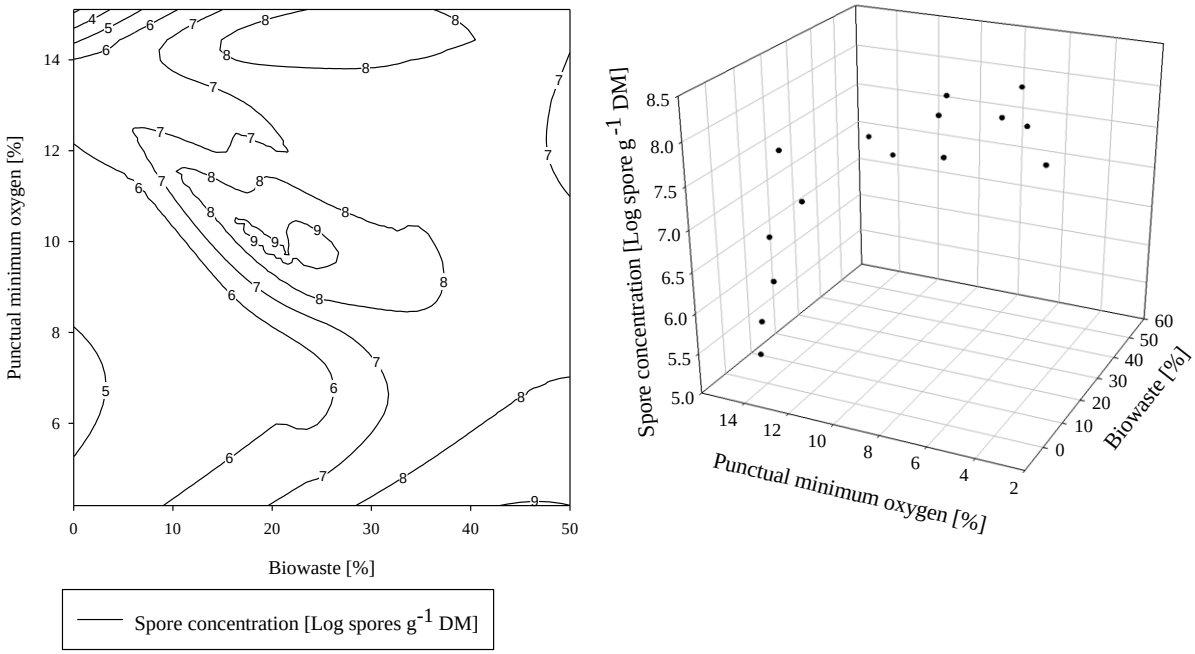


Figure 24: Relationship between the final spore production, the minimum oxygen level achieved and the biowaste added to the mixture of the experiment from Section 5.3.1.

However, some authors observed that low aeration rates caused the failure of microorganism survival or sporulation in submerged fermentation (Foda et al., 1985). Thus, the effect of biowaste as co-substrate and the aeration regime, affecting the oxygen availability within the reactor, requires further study in the SSF system in order to maximize final spore production. Table 14 shows the final viable cell, spore count, and spore yield according to the amount of biowaste used in the mixture (low: 10% or high: 37.5%) and the oxygen content (%) in the reactor (low: <10% or high: >10%).

Table 14: Final viable cell and spore counts for varying fractions of co-substrate and oxygen content during the fermentation process. Mean initial CFU g^{-1} DM was 3.1±1.3 ($x10^7$).

Run	Biowaste [%, wet weigh basis]	Minimum oxygen achieved [%]	CFU g^{-1} DM ($x10^8$)	Spore g^{-1} DM ($x10^8$)	Spores produced per initial CFU yield
1	10	17.1	0.41±0.02	0.19±0.03	0.6
2	37.5	5.2	3.1±0.4	1.3±0.2	3
3	37.5	17.3	2.8±0.8	1.3±0.1	2.9
4	10	5.0	0.76±0.06	0.8±0.1	2.7
5	37.5	5.5	5±1	3.3±0.3	7.3
6	37.5	16.7	2.5±0.1	1.3±0.1	0.6

Lower viable cell and spore counts were observed when a lower percentage of biowaste was used in the solid matrix (Run 1 and Run 4), confirming previous results. However, a clear effect of the oxygen levels can be observed. Using 10% of biowaste and applying an operation strategy leading to low oxygen levels, a 4.2-fold increase of spore concentration was observed compared to when supplying a non-limiting airflow rate. Otherwise, when a higher fraction of co-substrate was used (37.5%), higher total cell growth was detected when lower airflow rate was applied, achieving a yield between 3 and 7.3 spores produced per initial CFU. This higher cell growth would eventually lead to a higher spore production when providing enough time. For now, in the studied fermentation time, both aeration strategies led to similar spore yields.

From an operational point of view, the oxygen-limiting conditions were only maintained for a few minutes, but seemed to have an important effect, especially during

the firsts 24 hours. The increase of vegetative cells during the limiting oxygen period made the difference to the spore production. As observed in Figure 25, a 5.9 and 7.1-fold increase was observed when a high percentage of biowaste and higher airflow rates were used. However, when only the airflow rate was modified by decreasing its value, a 7.4 and 24.5-fold increase was achieved. That is, a fast and higher vegetative cell development led to a greater spore production.

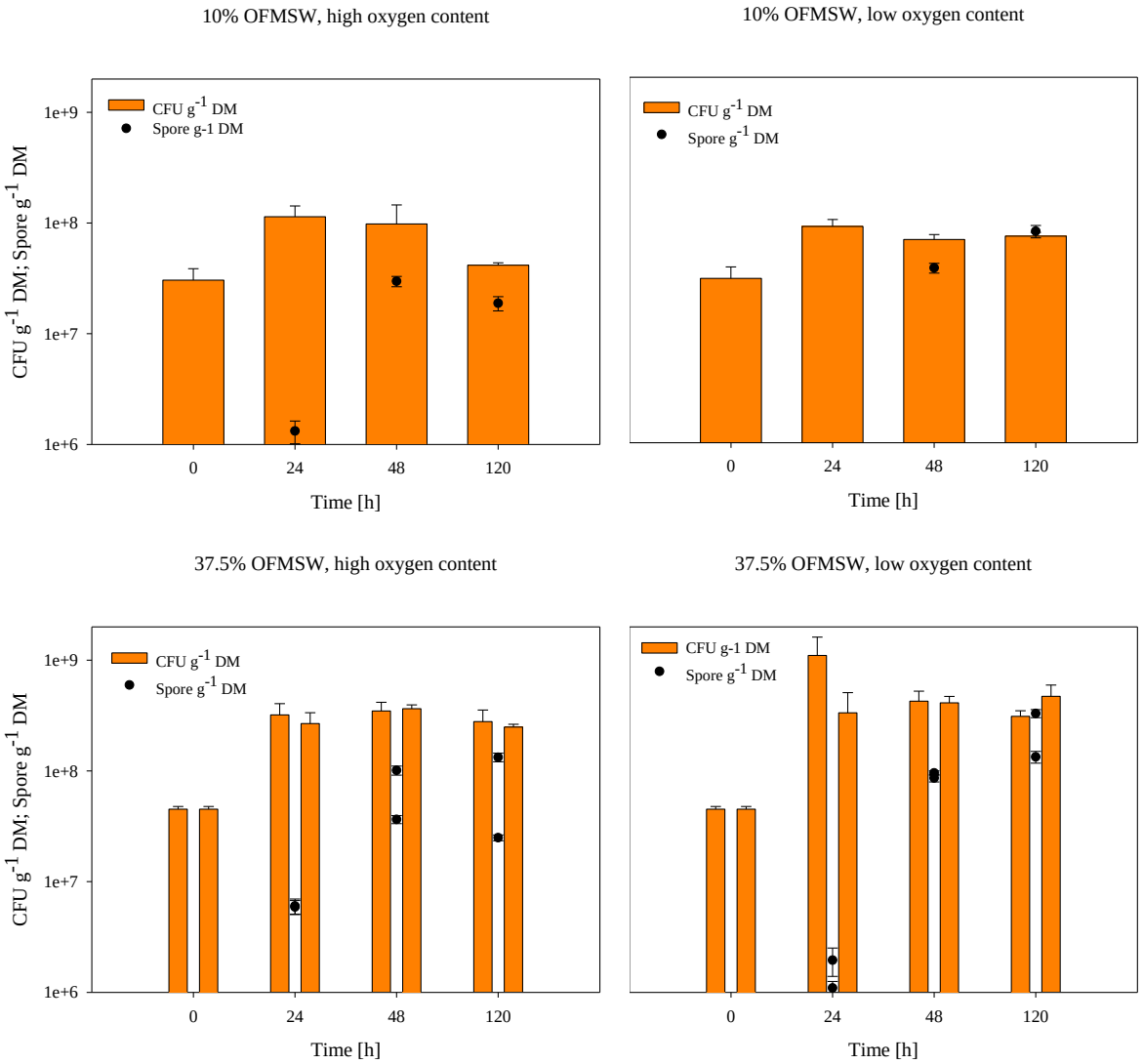


Figure 25: CFU and spore concentration along time varying the substrate composition and the aeration regime.

In Sarrafzadeh and Navarro (2006), the effect of the dissolved oxygen on the sporulation process was studied in fed-batch cultures. By testing four different aeration conditions (interrupted, limited, non-limited, and saturated) it was observed that maximum sporulation ratio was achieved when the aeration regime was stopped after 24 hours of culture. Ghribi et al. (2007) observed how high oxygen saturation levels led to high cell densities but lower toxin synthesis. However, when applying lower aeration regimes, cells had higher capacities of δ-endotoxins synthesis. Applying the adequate dissolved oxygen control, it was possible to partially overcome the carbon catabolite repression, which affected the final δ-endotoxin concentration.

In our process, low oxygen levels seemed to promote the Bt growth in a non-sterile environment. This limiting oxygen condition was achieved in the first 24 hours of the process and was then progressively increased until total aerobic conditions. The increase in spore concentration suggested that it was not affected by an aerobic environment. Nevertheless, the SSF process of 3 days performed with limited airflow rate is not enough to degrade all biodegradable organic matter and stabilize the solid. Hence, the option of increasing the aeration regime after the anoxic phase was considered for the 22-L reactor operation, in order to accelerate the degradation of the remaining organic matter and its stabilization.

5.4. Conclusions

A successful operational strategy was achieved in 1.6-L reactors. It consisted of using a mixture of 62.5% of digestate and 37.5% of biowaste as co-substrate. Furthermore, an aeration strategy consisting of a first micro-aeration stage showed to promote Bt growth, incrementing the spore production potential. Following this strategy,

final spore counts higher than $1x10^8$ spores g^{-1} DM were obtained. This strategy would be further validated in non-adiabatic 22-L reactors.

5.5. References

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CHAPTER 6: OPERATIONAL STRATEGY VALIDATION AT 22-L

6.1. Overview

This chapter focuses on the validation of the operational protocol developed at lab-scale. The 22-L reactor used is a prototype of the DECISIVE pilot reactor, where a maximum of 4 kg of the studied mixture could be loaded. As the amount of substrate to be used increased significantly, the bulking agent was changed from toothpicks to wood chips. Consequently, the substrate mixture must be optimized to maximize its quality.

In this chapter, the downstream and characterization of Cry proteins are also attempted. In SSF systems, the complexity and heterogeneity of substrates hinder the purification of certain products. This difficulty could be increased when working with non-sterile materials, representing an operation cost up to 50% of the total running cost (Bhatt and Shilpa, 2019; Rudakiya, 2019). This fact remarks the need of maximizing the product concentration at the end of the operation to assure the process viability.

6.2. Experimental methodology

In previous experiments at lab-scale, the initial mixture for the SSF was prepared manually. However, this could be laborious for the pilot plant operation. Additionally, the bulking agent was changed to wood chips, and the ratio of substrate:bulking agent to achieve a good quality mixture must be set. Therefore, a Box-Behnken design was proposed to determine the best combination of bulking agent ratio, mixing velocity and mixing time (Table 15). Fifteen mixtures were evaluated, analysing the mixture quality through image analysis, bulk density, air-filled porosity, and effective mixing volume. This last value was calculated considering the non-compacted mass and the bulk density. A summary of the performed runs is shown in Table 16.

Table 15: Ranges of bulking agent, mixing velocity, and mixing time evaluated to obtain a good quality mixture.

Bulking agent (%w)	Mixing velocity (rpm)	Mixing time (min)
15	2	5
25	5	17.5
35	8	30

Table 16: Combinations of bulking agent, mixing velocity, and mixing time to assess the mixture properties.

Run	Bulking agent (% w)	Mixing velocity (rpm)	Mixing time (min)
R1	25	5	17.5
R2	35	2	17.5
R3	25	2	5
R4	25	5	17.5
R5	35	5	30
R6	25	2	30
R7	35	5	5
R8	15	5	5
R9	15	5	30
R10	25	5	17.5
R11	25	8	30
R12	15	2	17.5
R13	35	8	17.5
R14	25	8	5
R15	15	8	17.5

The reactor (Setup 3) was fed by alternating 3 layers of digestate and 2 layers of bulking agent and the specific values of agitation rate and mixing time were applied in each run. Digestate was the only substrate used in this experiment, as it is the worst

conditions in terms of porosity. The final bulk density, effective mixing volume, air-filled porosity and mixture integration and homogeneity were determined.

Regarding the image analysis, the software Mixture Quality Beta v.0.6.7 was used (http://gicom.cat/MXQlty_BETAv0.6.7.0/index.html#). A photo of the mixture was processed and converted to a black and white image to analyse a map of 800x600 pixels. A number was assigned to each pixel according to its colour, ranging from 0 (corresponding to black) to 255 (corresponding to white) and the data array obtained was statistically analysed (frequency and normal distribution).

6.3. Results

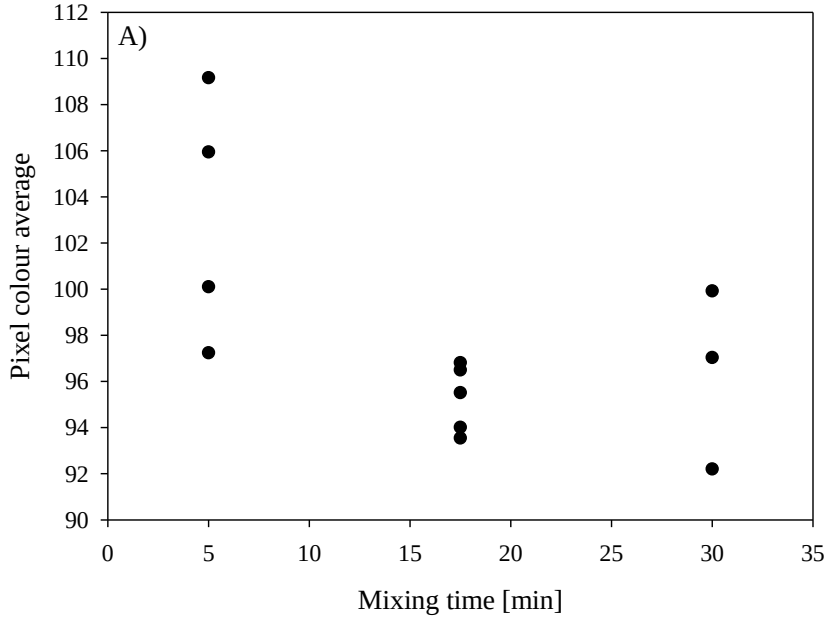
6.3.1. Optimization of digestate and bulking agent mixture

The selection of an optimum mixture aimed to tackle and prevent problems related to packed bed compaction. In this sense, low bulk density values are targeted, in response to a satisfactory integration of digestate and bulking agent. Moreover, compaction problems can appear when using denser mixtures, mainly caused by a smaller particle size (Ruggieri, 2008). Bulk density is related to air-filled porosity (AFP). Berthe et al. (2007) observed that AFP initial values between 41 and 70% were not limiting to the biological process. Secondly, in some conditions, a compacted mass can remain under the stirrer after mixing, caused by shear forces. This volume must be reduced, maximizing the effective mixing volume. Lastly, a homogeneous mixture is desired. From the image analysis, those mixtures with a lower value of average colour number and deviation corresponded to a good integration of digestate with bulking agent. This value was given by the software used.

The agitation system failed when the mixtures with 35% (w) of bulking agent were evaluated. The high ratio of wood chips provoked a high mechanical resistance and the

power of the agitator was not enough. For this reason, this percentage of bulking agent was discarded.

Focusing on the mixtures with 15 and 25% of bulking agent, final mixtures had a low bulk density (from 0.22 to 0.32 kg L^{-1}) and a satisfactory porosity (from 63 to 73%). Data obtained from non-compacted mass and bulk density were introduced to Design Expert software and analysed separately. Unexpectedly, the interaction between mixing time and agitation velocity had not a significant effect in the final properties of the mixture. The agitation speed did not affect the mixture properties in the narrow range studied. However, while bulking agent had a significant effect on bulk density, the agitation time affected the compaction of the substrates through the vessel walls. So, mixing time was detected as a key parameter with double effect, since more agitation time resulted to better integrated materials and more homogeneity (Figure 26A), but it also implied a higher loss of effective mixing volume due to material compaction in the deflectors and the bottom of the reactor (Figure 26B).



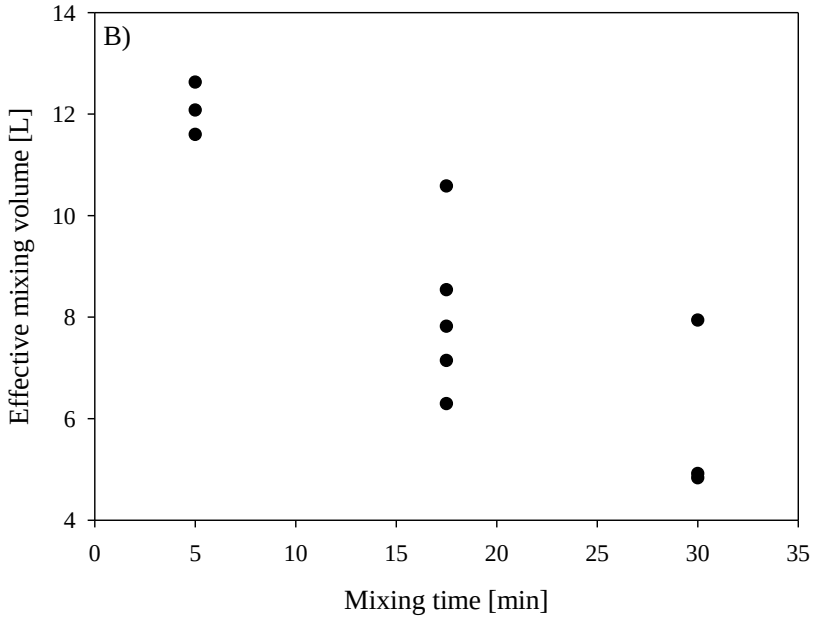


Figure 26: Final pixel colour (A) and effective mixing volume (B) after a certain mixing time.

Table 17: Anova statistical test for final bulk density considering a lineal model. R^2=0.83

Source	Coefficient	p-value
Model	-	0.0005
Lack of fit	-	0.9
Constant term	0.2	-
Bulking agent	-0.07	0.0001
Agitation time	-0.02	0.06
Agitation speed	-0.01	0.2

Table 18: Anova statistical test for final non-compacted mass considering a lineal model. R^2=0.79.

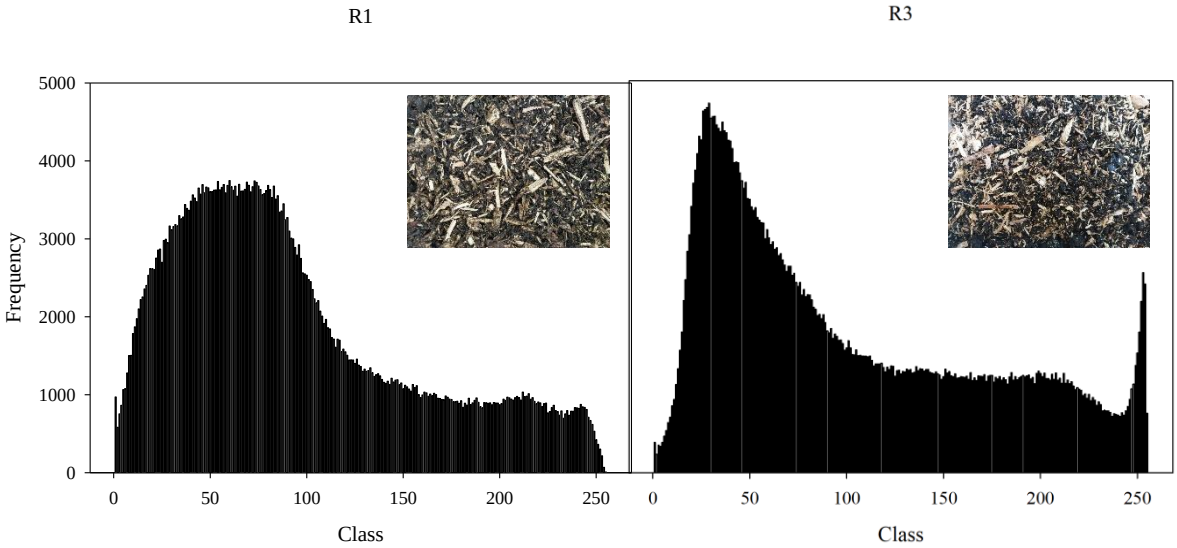
Source	Coefficient	p-value
Model	-	0.003
Lack of fit	-	0.6
Constant term	54	-
Bulking agent	-13	0.1
Agitation time	-25	0.0008
Agitation speed	-9	0.09

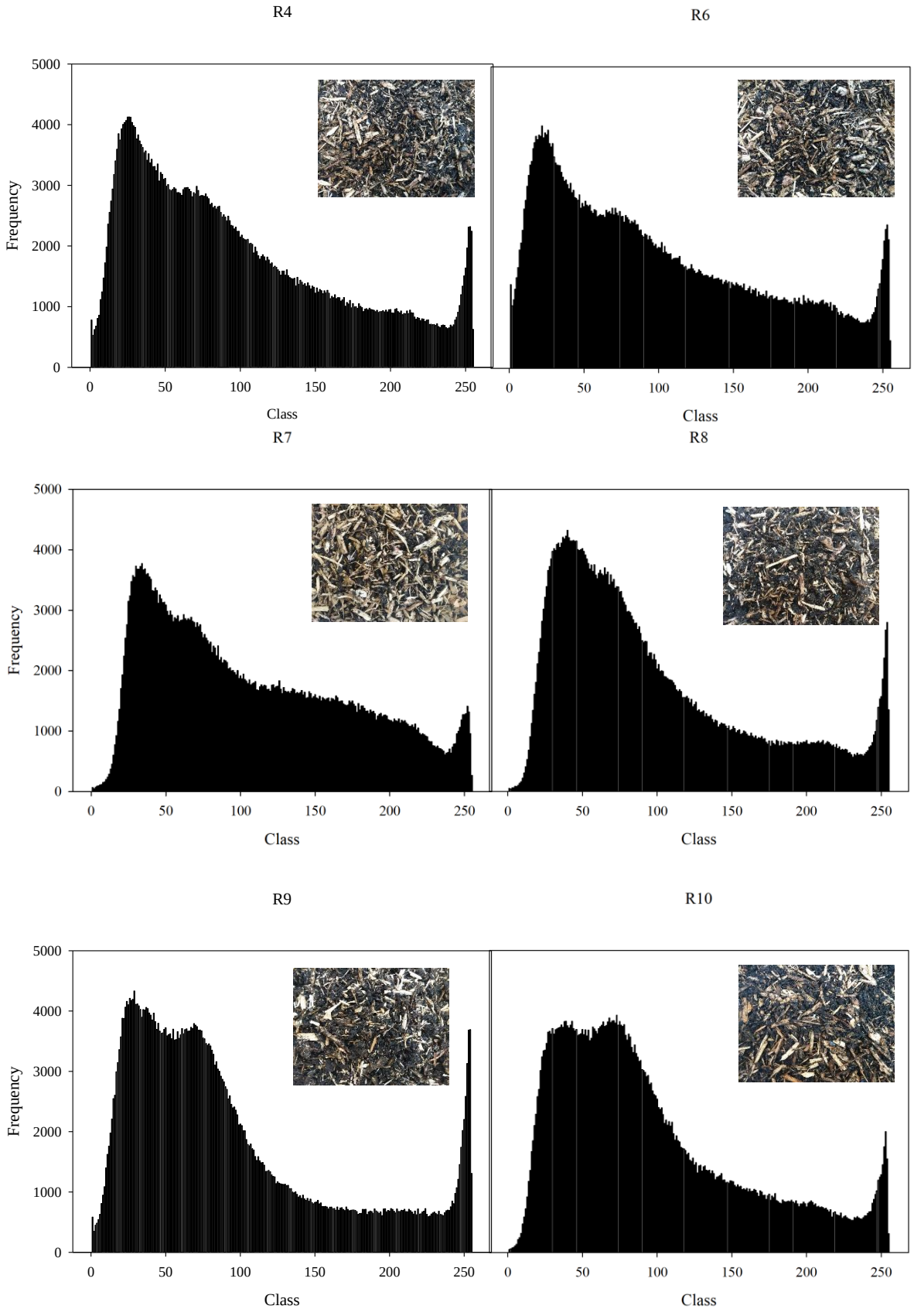
Table 19: Results obtained from the digestate-BA mixing test.

Run	% BA (w)	Mixing time (min)	Agitation rate (rpm)	Image analysis average	Image analysis deviation	Bulk density (kg L^{-1})	Porosity (%)	Effective mixing volume (L)
1	25	17.5	5	93.54	61.75	0.236	0.704	6.3
2	35	17.5	2	-	-	-	-	-
3	25	5	2	100.1	69.94	0.293	0.633	11.6
4	25	17.5	5	96.81	67.44	0.232	0.709	7.8
5	35	30	5	-	-	-	-	-
6	25	30	2	99.92	70.49	0.232	0.709	7.9
7	35	5	5	109.16	65.33	0.179	0.731	-
8	15	5	5	97.24	64.76	0.321	0.674	12.1
9	15	30	5	92.2	67.23	0.307	0.688	4.8
10	25	17.5	5	95.51	61.56	0.283	0.645	8.5
11	25	30	8	97.03	58.47	0.224	0.719	4.9
12	15	17.5	2	94.01	67.02	0.318	0.677	10.6
13	35	17.5	8	-	-	-	-	-
14	25	5	8	105.94	71.7	0.259	0.676	12.6
15	15	17.5	8	96.49	68.06	0.297	0.699	7.1

Mixture Quality Beta v.0.6.7 was developed as an innovative image analysis for mixing quality and integration of both materials. The aim for developing this software was the need to quantify, compare, and statistically analyse the visual appearance of the different mixtures. When resizing the image to 800x600 pixels, a 480000 data file was created. This data was organized and represented as histograms, showing the frequency of each colour, from black (0) to white (255). Our hypothesis was that a bad quality mixture would show an important frequency of values related to white, as a consequence of a poor integration of digestate with the wood chips. Figure 27 presents the histograms obtained. It can be stated how Runs 8, 9, and 12 presented a major frequency of classes related to white colours. At the same time, these runs contained the lower amount of bulking agent (15%). Thus, using this agitation system, when a low proportion of bulking agent was added to the mixture, it did not integrate as desired with digestate regardless the time of mixing. Hence, a low-quality mixture was obtained, except when increasing agitation rate to 8 rpm (run 15) where a better integration of the mixture was obtained.

Nevertheless, data from the other colours between black and white were difficult to understand, without being able to draw clear conclusions. This software was very useful since a first image assessment could be performed, however, more indices to express the quality of the mixture can be further developed and studied.

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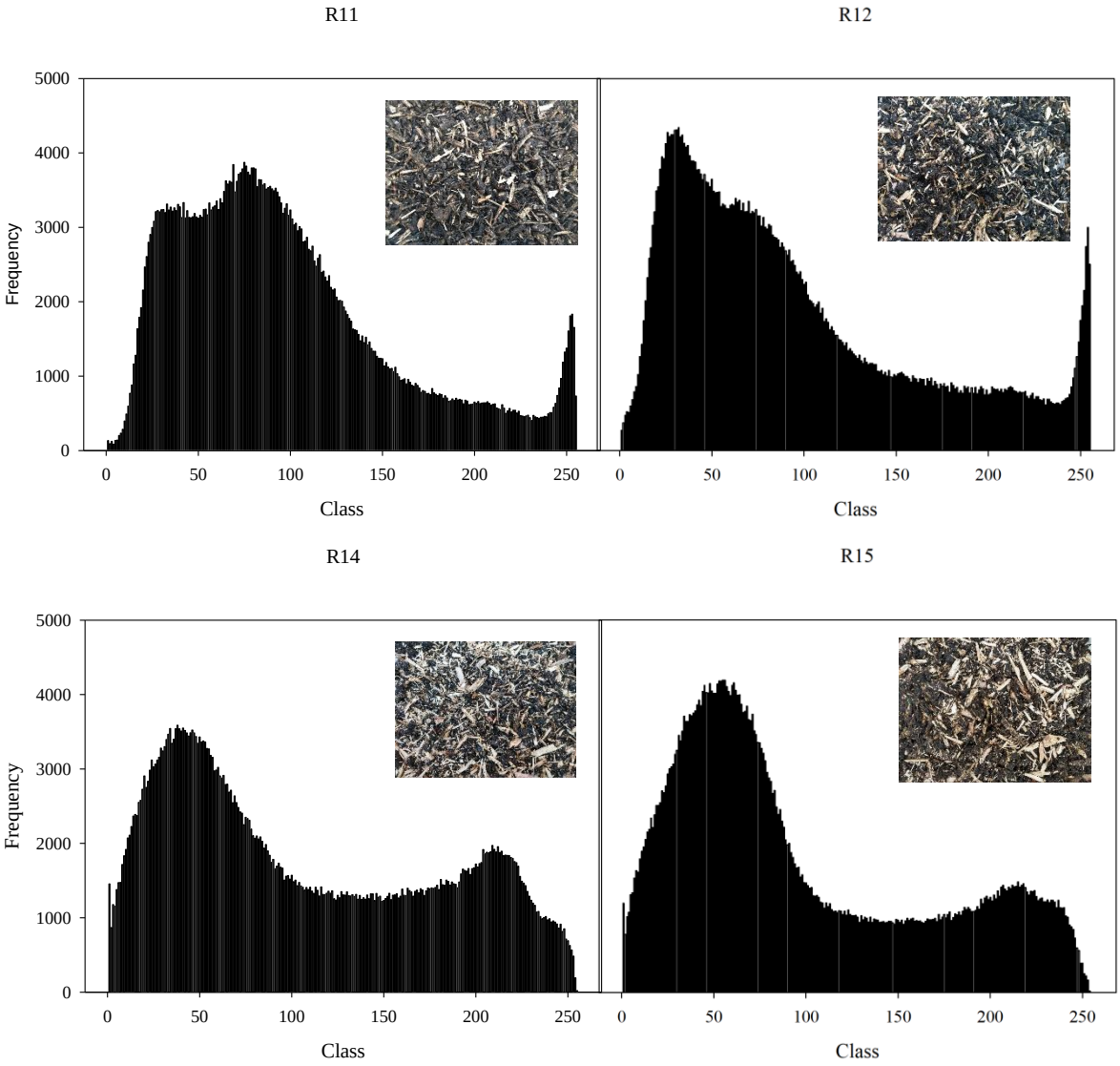

Figure 27: Histograms obtained from image analysis data. Pictures of the mixtures analysed are shown.

6.3.2. Strategy validation performance working in batch mode

The aeration strategy was validated in Setup 3. A first batch was carried out as a control experiment. In that, high airflow rates were supplied to the reactor to ensure oxygen levels above 10%. The maximum sOUR achieved was 3.6 g O_2 kg^{-1} DM h^{-1} and oxygen content was always higher than 10%. Temperatures ranged from 20 to 27 ºC, never reaching values higher than 30ºC. After 48 hours, viable cells did not increase

significantly (from $2.63x10^7$ to $2.83x10^7$ CFU g^{-1} DM), with a yield of 0.35 spores produced per initial CFU. So, although co-substrate was used in this batch, no relevant production was obtained in terms of Bt growth, and consequently, poor production of spores was achieved. These results were in line with the observed by Rodríguez et al. (2019) using a similar configuration reactor with different operation conditions (airflow rate set at 1000 ml min^{-1} with a stirring regime of 120 rpm for 15 min every 24 hours) where no growth of Bt was observed, as viable cells decreased until reaching a stable value.

A second batch was done by applying the aeration strategy but using digestate as a sole substrate. In Figure 28 it is shown the process evolution in terms of biological activity (sOUR), oxygen levels, airflow rate applied, temperature dynamics, and the Bt cell and spore concentration. In the first stage, the airflow rate was set at 0.1 mL min^{-1} g^{-1} to maintain the superficial air velocity scale-up criteria, and minimum oxygen levels of 2.8% were reached at 21 hours. In this period, the packed bed reached a maximum temperature of 25ºC. The airflow rate was doubled afterward (from 0.1 to 0.22 ml min^{-1} g^{-1}) and samplings were done at 48, 72, 96, and 120 hours. Viable cell concentration was mostly maintained during all the process, and spore concentration slightly increased until reaching a value of $3.34x10^7$ spores g^{-1} DM (1.3 spores produced per initial CFU). These results were very relevant as it was the first time that total sporulation of the starting inoculum was monitored using digestate as a sole substrate. However, it is necessary to increase Bt growth for increasing the spore production potential and achieving a more efficient process.

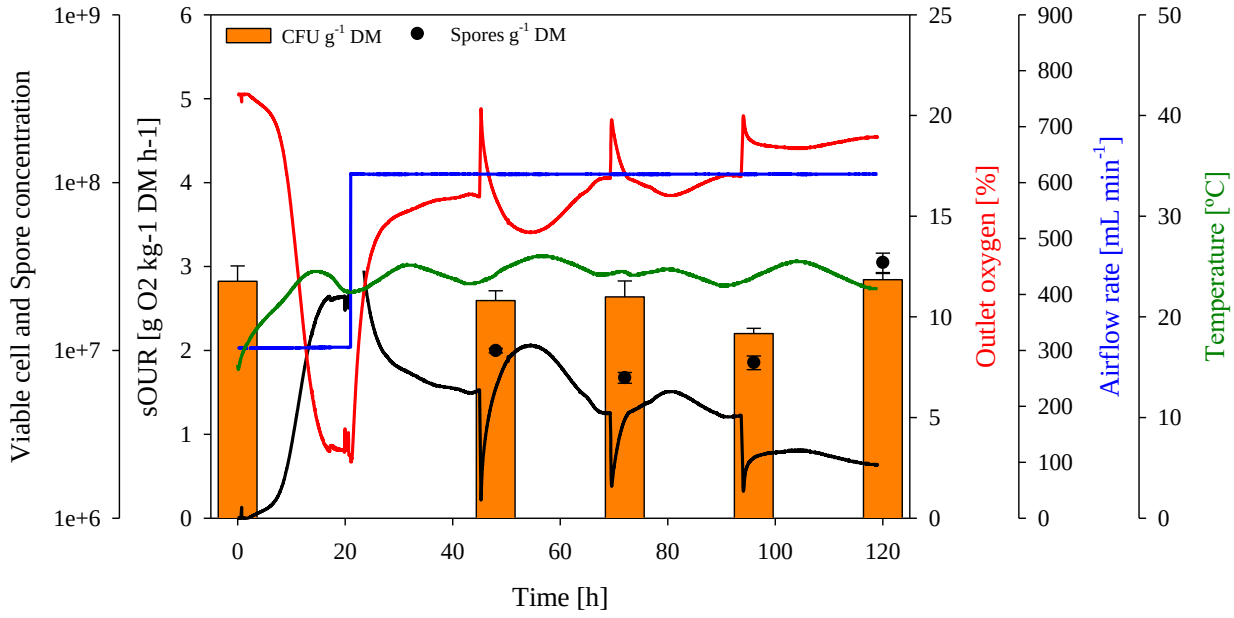

Figure 28: Monitored parameters evolution (sOUR, outlet oxygen, airflow rate, temperature and viable cell and spore concentration) when the aeration strategy was applied, using digestate as sole substrate.

Therefore, the operation was repeated following all steps indicated in the operation protocol proposed: the use of biowaste as co-substrate and the two-stage aeration regime. As expected, during the microaeration stage, low oxygen content decreased until values under 5%, reaching the minimum value of 0.3% at 21 hours (Figure 29).

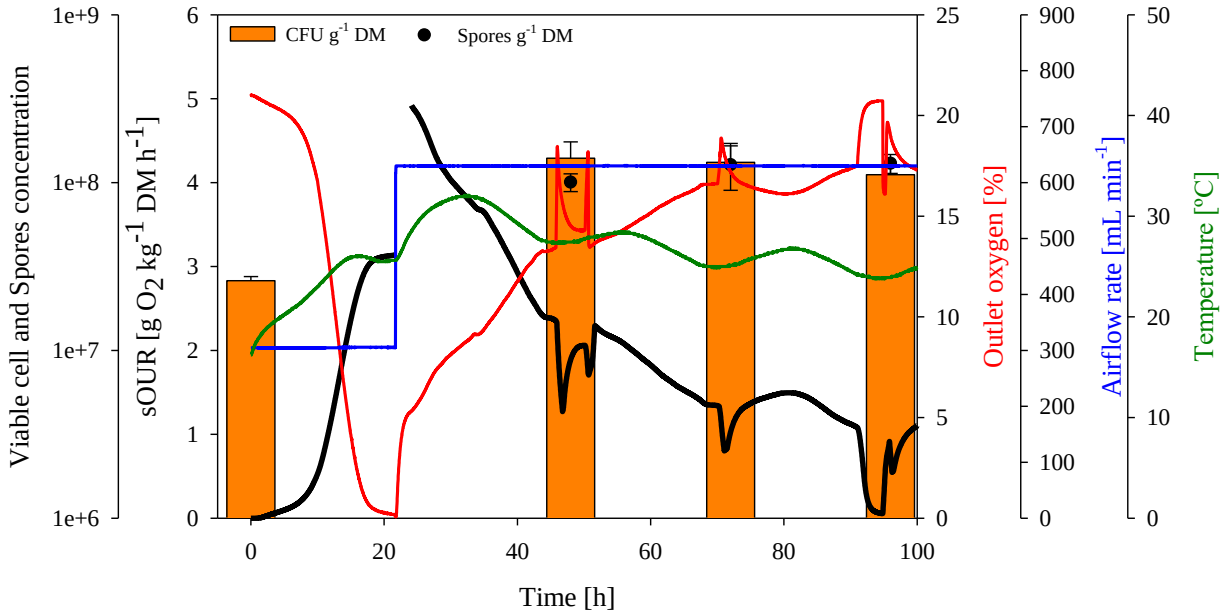

Figure 29: Monitored parameters evolution (sOUR, outlet oxygen, airflow rate, temperature and viable cell and spore concentration) when the aeration strategy was applied and biowaste added as co-substrate.

The airflow rate was doubled to proceed to the second full-aerobe stage. The temperature peak was monitored (31ºC) when a non-limiting oxygen environment was provided, as aerobic reactions produce more metabolic heat than anoxic reactions. However, the maximum temperature was lower compared to experiments performed in the Setup 2. This could be due to the presence of a stirrer in the centre of the reactor, affecting the structure of packed-bed, and providing a different temperature dynamic. The success of the strategy was demonstrated once more. A 4.2-fold increase of viable cells was reported (from $2.6x10^7$ to $1.1x10^8$ CFU g^{-1} DM). The concentration of Bt spores reached a maximum of $1.3x10^8$ spores g^{-1} DM, representing a ratio of 5 spores produced per initial CFU, after 4 days of operation.

In order to fully stabilize the solid and study the spore conservation, this batch was run for 20 days. The final fermented solid was analysed in terms of respiration

indices, obtaining a DRI value of 0.42 ± 0.09 g O_2 kg^{-1} DM h^{-1} and AT4 of 19 ± 11 g O_2 kg^{-1} DM, corresponding to a biologically stabilized material (Barrena et al., 2011). Additionally, the concentration of spore was well conserved. A slightly increase of viable cells was reported (from $1.1x10^8$ to $1.6x10^8$ CFU g^{-1} DM) and the spore concentration was almost maintained (from $1.3x10^8$ to $1.02x10^8$ spores g^{-1} DM). Hence, the stability and conservation capacity of spores in a fermented solid media was demonstrated. However, an assessment of crystal protein stability and its biopesticide properties is further required. Ballardo et al. (2020) operated an SSF process in a 50-L insulated reactor to obtain a biologically stable material enriched with Bt. After a short SSF process, the Bt-enriched material was used as a solid inoculum for a home composting process, using fruit and vegetables leftovers, and pruning waste as bulking agent. The composting process ran for 35 days, and the spore concentration was maintained in the range of 10^6 spores g^{-1} DM. Biological stability was also assessed by respirometry tests, showing DRI values less than 1 g O_2 kg^{-1} OM h^{-1}.

Finally, a fourth batch was carried out with the aim of obtaining more data at 24 hours, following the entire strategy proposed. As desired, the proper oxygen levels were obtained. Figure 30 shows the viable cell and spore concentration at 24 and 48 hours. At the time of minimum oxygen (3.2%), the total cell concentration value was determined at $5.3x10^7$ CFU g^{-1} DM, representing a 6.4-fold increase from the initial CFU values. At 48 hours of fermentation, total cell counts slightly decreased (from $5.3x10^7$ to $2.7x10^7$ CFU g^{-1} DM) and spore concentration increased up to $1.7x10^7$ spores g^{-1} DM. It represents a yield of 2.1 spores produced per initial CFU.

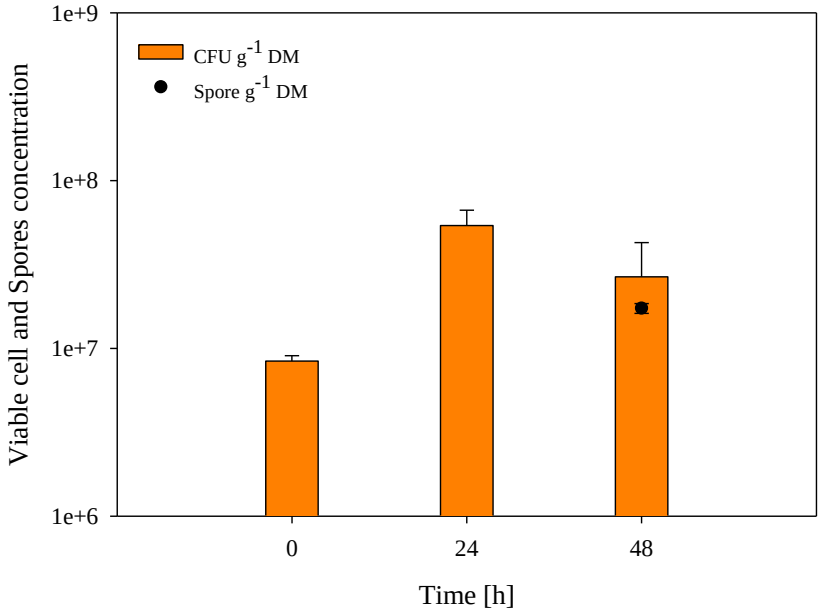

Figure 30: Viable cell and spore concentration at 0, 24 and 48 hours following the complete strategy.

That is, a first 24-hour stage of oxygen limitations demonstrate its positive effect on Bt growth, being a competing advantage against autochthonous microorganisms when working in non-sterile conditions. In that time, a significant increase in viable cell was monitored, and no spores were determined. The duration of the first stage was set at 24 hours because of Bt kinetics, when the maximum biomass concentration is reached (Lima-Pérez et al., 2019). Moreover, in the fourth batch performed at 22-L reactor, the maximum total cell concentration was observed at 24 hours. The second stage (full aerated system) showed a decrease in total cell concentration and promotion of sporulation rate. Hence, the anoxic stage could be considered as a critical path, when the maximum Bt growth should be obtained, in order to get the maximum spore potential. This behavior was also observed by Mounsef et al. (2015), on studying the effect of different K_La values on Bt growth, spore, and endotoxins production in submerged fermentation. The tested range of K_La showed an oxygen limitation condition in the first

15 hours of culture that did not affect Bt growth. While cell concentration increased during the oxygen limitation conditions, the spore concentration started to increase when dissolved oxygen values were increasing up to 100%.

6.3.3. Product downstream and characterization

Gel electrophoresis was performed as a first step in toxin characterization. The purified extract analyzed had a protein concentration of 1 mg mL^{-1}. A commercial Bt extract was also analyzed. The Bt commercial powder was diluted with water, as described in the recommendations for use, but also diluted with 0.05N NaOH, to simulate the alkaline environment of larval gut. This alkaline solution solubilized the crystal protein and released Cry proteins to the media (Adang et al., 2014). Figure 31 shows the gel electrophoresis obtained. A clear band around 100 kDa was observed in the fermented solid samples (lanes 2 to 4), which was also present in commercial Bt samples diluted with water (lanes 5, 6, and 7), and diluted with 0.05N NaOH (lanes 8, 9, and 10). The unique band observed in lane 8 (commercial Bt diluted with NaOH) can be also observed in lane 2 (fermented solid). Zhang et al. (2013) reported bands of 130 and 60 kDa working with kitchen waste as the main substrate, and Chang et al. (2007) observed a band of 135 kDa using sewage sludge as fermentation media. Bands with different molecular weight are found because Cry proteins are released as protoxin forms, which are activated to toxin form by the action of exogenous or endogenous proteases (Rukmini et al., 2000).

The limitation of this analysis was the low concentration of Cry proteins in the extracts, being out of range of the calibration curve of the molecular weight size marker to quantify the total amount (ng) of a specific protein band. Several trials were performed in an attempt to increase the total protein concentration by testing different solid:liquid

extraction ratios. However, this resulted in more background noise when running the electrophoresis, being also impossible to determine the ng of a specific band.

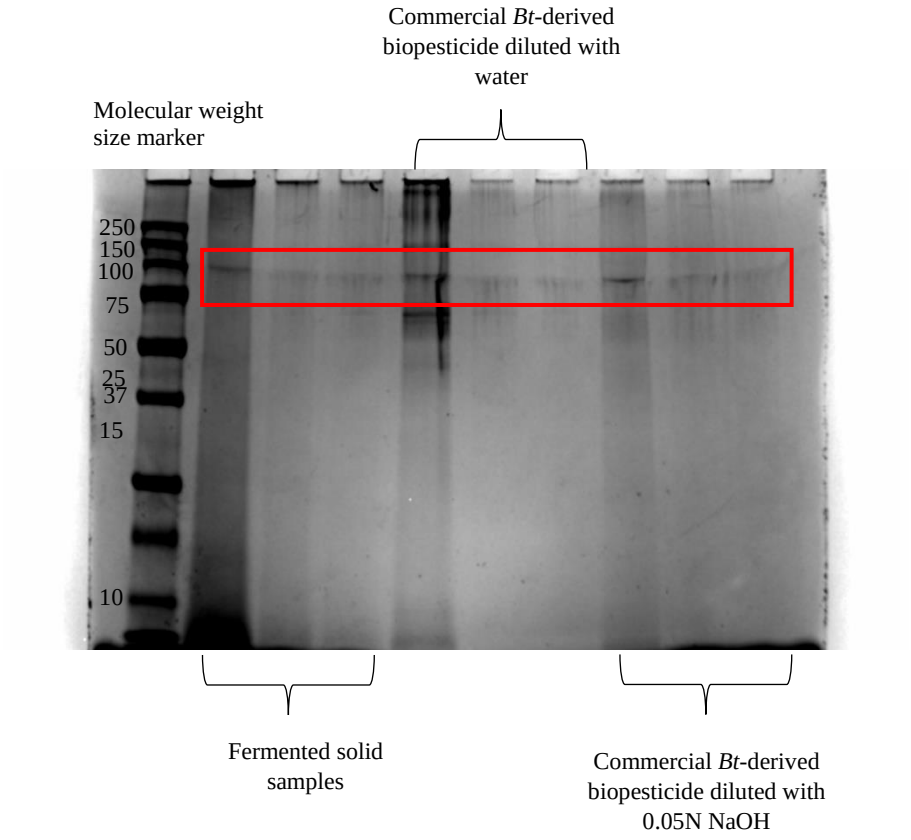

Figure 31: Electrophoresis gel. Comparison of bands obtained from fermented solid and commercial Bt-derived biopesticides.

Images obtained with SEM confirmed the presence of crystals in the fermented solid. Variable crystal morphology has been previously reported. While Bti crystallizes the produced endotoxins and hemolytic proteins in a spherical form, Btk crystallizes a combination of other endotoxins in a bi-pyramidal and cuboidal form (Nair et al., 2018). Figure 32 clearly shows the presence of bi-pyramidal crystals sized between 800 nm and 1µm at the end of the process. Both images are from the same sample, after the sonication process of the fermented solid.

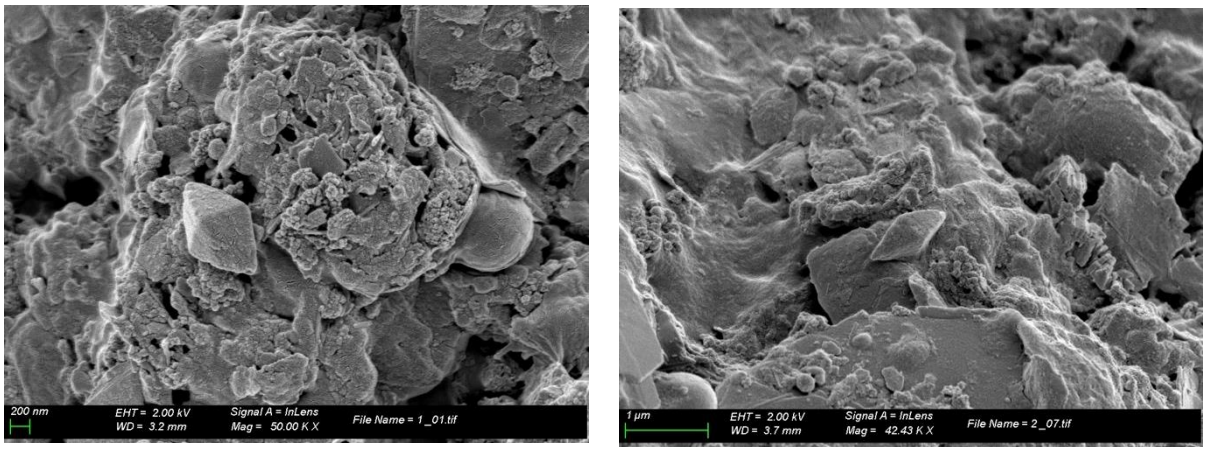

Figure 32: SEM images of Btk crystals at the end of the SSF process.

6.4. Conclusions

In this chapter, the ratio of bulking agent to be used for the pilot plant operation was studied, setting its value at 25%, as using a higher amount of wood chips could cause agitation problems. Besides, agitation time was detected as an important parameter to obtain a satisfactory mixture and avoid compaction. However, deflectors must be avoided as their presence implied a loss of the mixture's effective volume.

Additionally, the developed strategy was successfully validated in a 22-L reactor. This operational protocol was submitted for an European (EP19382861.3) and UK (1914324.7) patent on date October 4th, 2019.

A first attempt for Cry proteins purification was performed, managing to observe crystal proteins with SEM images, and Cry proteins using gel electrophoresis. Their presence also indicated the adequacy of the strategy developed for its production.

6.5. References

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CHAPTER 7: TOWARDS A PROSPECTING PROCESS AND UNDERSTANDING THE POSSIBILITIES OF RUNNING THE PROCESS IN A SEMI-CONTINUOUS MODE

7.1. Overview

In this part of the thesis, an analysis was carried out on the role of the hygienization step in the developed strategy. The demonstration site, located in Lyon, considered performing the hygienization process before the mAD. That leads us to question its role at the microbial and operational level. The hygienization step can be considered as a thermal treatment, and bacterial population can suffer a change due to its exposure to high temperatures. Furthermore, two different operation modes were studied in addition to batch: fed-batch, and sequential batch operation. The amount of substrate to be prepared and the feeding regime may differ, depending on the operation mode. When working in batch mode, a quantity of substrate equal to capacity of the bioreactor needs to be conditioned and inoculated. However, storage is sometimes required, causing chemical changes in the organic matter. Hence, the upstream equipment should be large enough to guarantee the treatment and inoculation of the substrate needed to start the batch process. This upstream capacity can be reduced when working in continuous mode, as smaller, but more frequent, substrate quantities are fed into the reactor (Mitchell et al., 2006). In this sense, fed-batch operation also allows the upstream capacity to be reduced, as the amount of substrate fed is split into different times. Additionally, this could cause less media compaction, as the material is mixed after each feeding, recovering the proper packed bed structure (Martínez-Avila, 2018). Other advantages are related to inoculum saving, since part of the fermented solid enriched with specialized biomass can be used as inoculum for the next (Cerda et al., 2017; Ballardo et al., 2017).

Liu et al. (2019) compared erythritol production from oil crop wastes when SSF was operated in batch or sequential batch mode in 5-L flasks. This second operation consisted of a succession of repeated batches, using 10% of the fermented solids as inoculum for the next cycle. This operation ran for 7 cycles. The authors observed how

the lag phase was shortened after cycle 1, and high erythritol production (182.1 mg per gram of dry solid) was maintained until the fourth cycle. Hence, this strategy avoided the need for fresh inoculum preparation, representing savings in time and economic resources. A similar study was performed by Astolfi et al. (2011), comparing the inulase production working in batch or fed-batch mode. Sugarcane bagasse, corn steep liquor, soybean meal, and sugarcane molasses were used as a substrate, in a 2-kg of dry substrate capacity reactor. Moreover, inlet air temperature and water saturation were varied to remove waste metabolic heat and improve inulase production. Process time was set at 24 hours, and for the fed-batch performance, 25% of the fresh substrate was fed at 0, 6, 12, and 18 hours. The highest inulase activities (586 ± 63 U per gram of dry solid) were achieved working in fed-batch mode with wet inlet air at 30ºC, corresponding to a 1.3-fold increase compared to the maximum inulase activity obtained in batch mode. This strategy has also been successfully applied in the production of carbohydrases (Cerda et al., 2016, 2017).

The production of rose-like aroma compounds in fed-batch and sequential batch operation was assessed in Martínez-Avila et al. (2019) at two reactor scales (1.6 and 22-L). In the fed-batch operation, 33% of substrate was added at t0, t1, and t2, with t0 being the initial time of the process, t1 the maximum microbiological activity monitored using respiration indices, and t2, the maximum microbiological activity after t1. The sequential batch operation was performed by replacing 78% of the fermented material with new fresh substrate. Both strategies showed improvements over the batch process. The fed-batch mode showed an improvement in production of 11.6% in the 1.6-L reactor and 12.5% in the 22-L. The production was also increased by 16.9% and 2.4% when a sequential batch operation was performed. Again, savings related to inoculum, air, and time were highlighted.

In the DECISIVE project, the possibility of performing a semi-continuous operation should be considered. If not, extra equipment and processes will be required for liquid inoculum preparation in the demonstration sites, preparing new fresh inoculum before each batch. The idea was to operate the SSF the most autonomously as possible. The semi-continuous operation would lead to a significant saving in fresh inoculum. Hence, this option was studied. The fed-batch operation was also investigated and compared.

Part of this work was carried out in collaboration with Mònica Estrada during her Master's Thesis "Evaluating different alternatives to develop a strategy for *Bacillus thuringiensis* derived biopesticide production from digestate through solid-state fermentation".

7.2. Results

7.2.1. The influence of the hygienization process on Bt and the development of other microbial communities over the SSF process.

The influence of the oxygen levels and biowaste on the growth and sporulation of Bt was demonstrated. Nevertheless, the relevance of the substrates hygenization step over the production process should also be studied. This evaluation was performed in the Setup 1, by using hygenized and non-hygenized substrates and applying the aeration strategy developed. Each condition was run in triplicate.

When substrates were not hygienized (Figure 33A), a rapid oxygen consumption was observed until limiting conditions (7%). This resulted in a peak of sOUR at 5 hours. However, the oxygen increased rapidly before the airflow rate was intensified, achieving aerobic conditions until the end of the fermentation. Hence, the targeted oxygen concentration was not achieved, as it was not possible to maintain the limiting oxygen conditions during the first stage. In contrast, when substrates were hygienized (Figure 33B), it took longer to reach the limiting oxygen condition compared to the non-

hygienized runs, although it was mostly maintained until the transition to the second aeration stage in the three replicates. These differences in terms of oxygen consumption could be due to the thermal effect on the bacterial population. That is, the thermal treatment affects the microbial diversity of substrates, reducing the potential competitors for Bt growth. Hence, the microbial activity observed during the limited oxygen phase could be attributed to Bt growth.

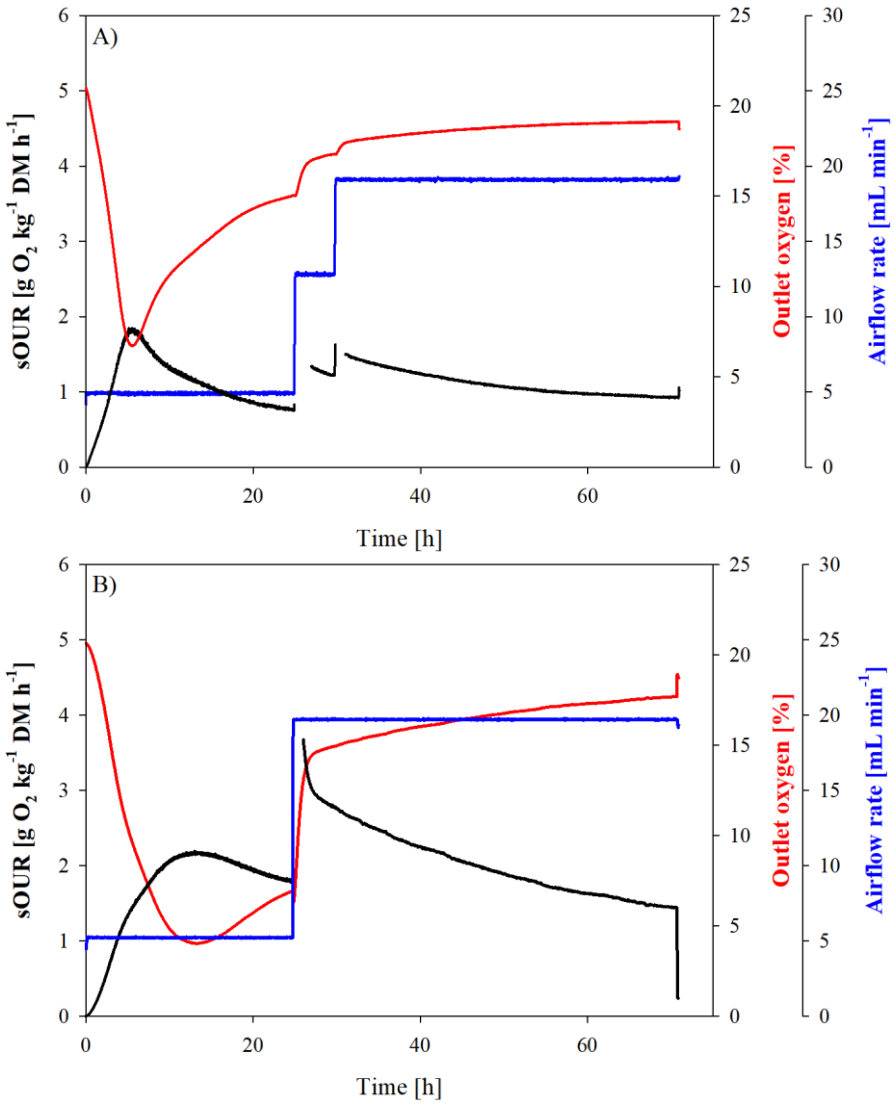

Figure 33: Evolution of SOUR, airflow rate and outlet oxygen throughout the fermentation process in 0.5-L reactors with non-hygienized (A) and hygienized (B)

substrate. All these parameters are an average of three replicates, with a standard deviation lower than 5% between replicates.

As regards total cell and spore concentration, the initial value in both conditions was around $3.8x10^6$ CFU g^{-1} DM (Figure 34). At the end of the process, no viable cells were able to be counted in non-hygienized reactors because of the abundant presence of other microorganisms. A sporulation ratio of around 90% of the inoculated CFU was achieved. In hygienized reactors, total cell concentration reached values between 3.8 and $9.2x10^7$ CFU g^{-1} DM after 72 hours of fermentation, which represented a 23-fold increase compared to the initial CFU concentration. The growth of Bt resulted in a higher spore production, reaching values up to $8.6x10^7$ spores g^{-1} DM. In Cerda et al. (2019), a maximum of 3-fold increase in viable cell count was monitored when using sterile digestate, working in the same Setup system and ensuring aerobic conditions during the entire process.

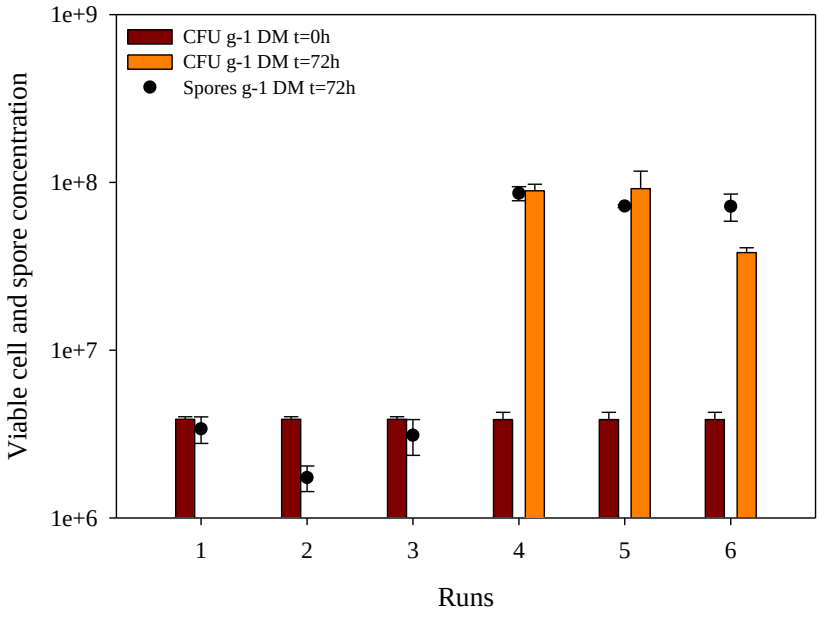

Figure 34: Viable cell and spore concentration at t=0h and t=72h of hygienized (runs 4-6) and non-hygienized reactors (runs 1-3).

These results revealed how the combination of both hygienization and the two oxygen stages promoted the spore production. On the one hand, the hygienization process reduces the microbial diversity of digestate and biowaste, and decreasing the autochthonous competitors. On the other hand, as seen in other experiments, the limiting oxygen conditions favor the development of Bt over the remaining microorganisms.

Microbial communities were studied to assess the presence of pathogens in the substrate used, evaluating the changes of the main bacterial populations throughout the hygienization and fermentation process. Results are presented in Figures 35 and 36. The triplicates of the final fermented material were very similar, indicating that the fermentation process performed similarly, which was a very positive result.

No regulated pathogens (*E. coli* and *Salmonella*) were found in hygienized material. However, *Salmonella* was present in the non-hygienized substrate, and *E. coli* in the fermented material when this thermal treatment was not performed. This confirmed the need for hygienization to eliminate these pathogenic microorganisms. Nevertheless, some changes in microorganism populations were observed due to this pretreatment. A 13% reduction by means of number of sequences found in hygienized samples indicated that some microorganisms did not survive after the thermal treatment, while some others decreased their relative abundance. This was the case of *Enterococcaceae* bacteria family, especially *Vagococcus* genus, where the relative abundance suffered a 273-fold decrease.

In contrast, some other families able to withstand thermophilic temperatures were favoured by this treatment. This was the case of the *Planococcaceae* and *Bacillaceae* families. In the *Bacillaceae* family, most of the group are aerobic or facultative anaerobe, and some of them can grow and sporulate at 70ºC (Schleifer, 2009). The *Planococcaceae* family, which includes *Sporosarcina, Solibacillus* or *Ureibacillus* genus, have an optimum growth temperature range between 42 and 65ºC (Shivaji et al., 2014).

Microorganism populations also suffered a change throughout the fermentation process. This was the case with *Desulfobacteraceae*, a strict anaerobe bacteria family, coming from the anaerobic digestion process. However, it was not observed after the aerobic SSF process. Other families, like *Flavobacteriaceae* family, increased their relative abundance after the fermentation process, due to its capacity for macromolecules digestion, such as proteins and polysaccharides, in aerobe conditions (McBride, 2014). It is reported that *Flavobacterium* genus contributes to the degradation of food products since the total mineralization of the organic matter (Towner, 2006).

Btk was not detected in non-hygienized or hygienized fermented material. This could be explained from three hypothesis. The first one is related to the primers used for sequencing. They might be not specific enough to detect this species. Secondly, the concentration of Btk in the solid could be under the detection limit of the analysis technique, due to the presence of a large number of other species. Lastly, as most of the viable Btk cells sporulated at the end of the fermentation, the DNA extraction kit may not have been efficient for spore lysis, and Btk DNA could not be released into the medium.

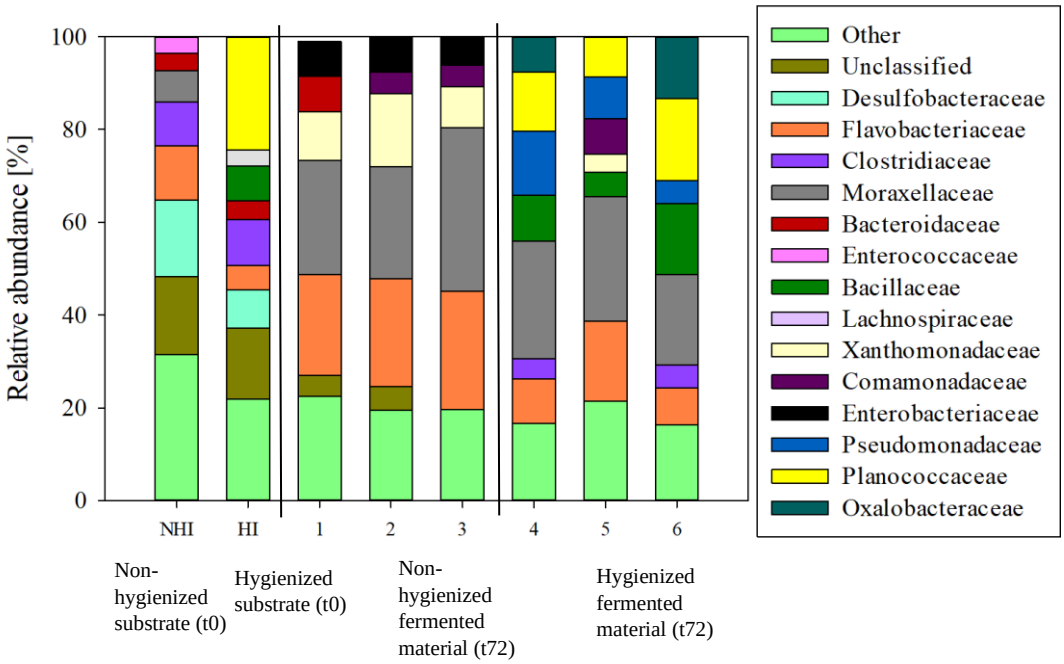

Figure 35: Variation of the bacterial families over the hygienization step and SSF process. The "Other" category is the sum of all classifications with less than 3.5% of relative abundance. The "Unclassified" category is the sum of the microorganisms that are not yet classified into any family group.

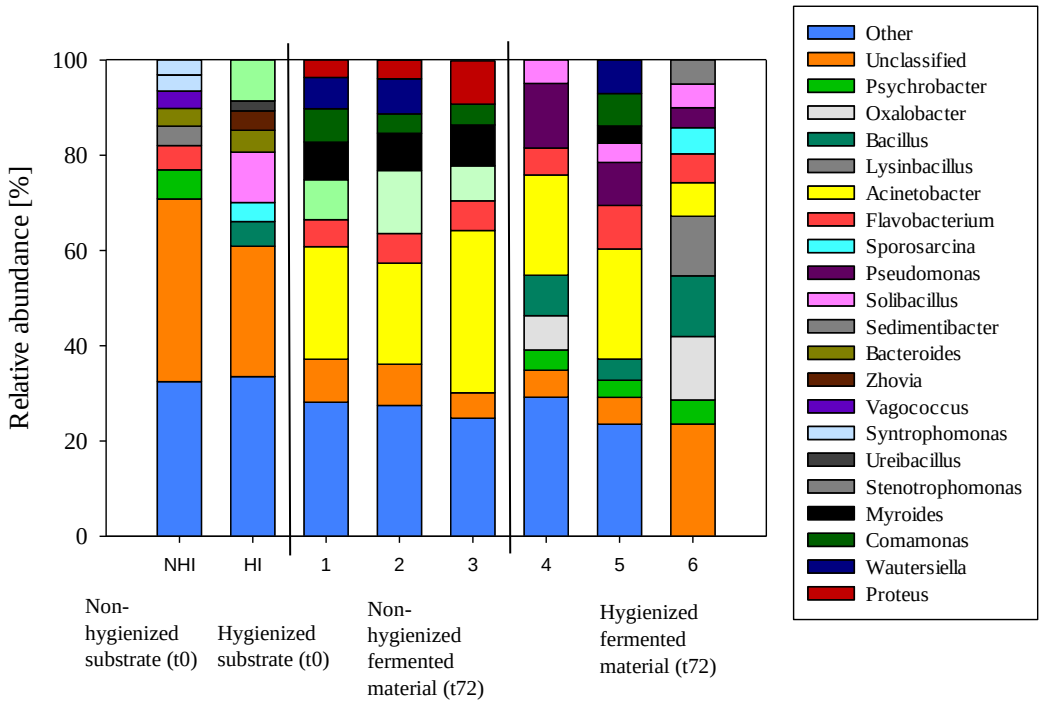

Figure 36: Variation of bacterial genus over the hygienization step and the SSF process. The “Other” category is the sum of all classifications with less than 3.5% of relative abundance. The “Unclassified” category is the sum of the microorganisms that are not yet classified into any genus group.

7.2.2. Fed-batch and sequential batch operation

The fed-batch strategy was studied as an alternative to batch operation in Setup 3. This strategy consists of adding extra nutrients at different times to extend the growth phase. To the best of our knowledge, fed-batch strategy for bacterial biopesticides production has been only studied in submerged fermentation. Vu et al. (2010) observed a 3.3-fold increase in endotoxin concentration when two intermittent feedings were done during the first 24 hours, compared to batch mode. However, when the substrate was added in three intermittent feedings, a significant decrease in spore and endotoxin concentration occurred. In our process, the total 4 kg of substrate was split proportionally into three feedings at 0, 24, and 48 hours. The inoculum requirement was reduced to 33%, as just 33% of the total material was inoculated at the beginning of the operation. At the time of feeding, the fermented material was generously mixed with the new substrate, to ensure homogeneity. The evolution of the process can be observed in Figure 37.

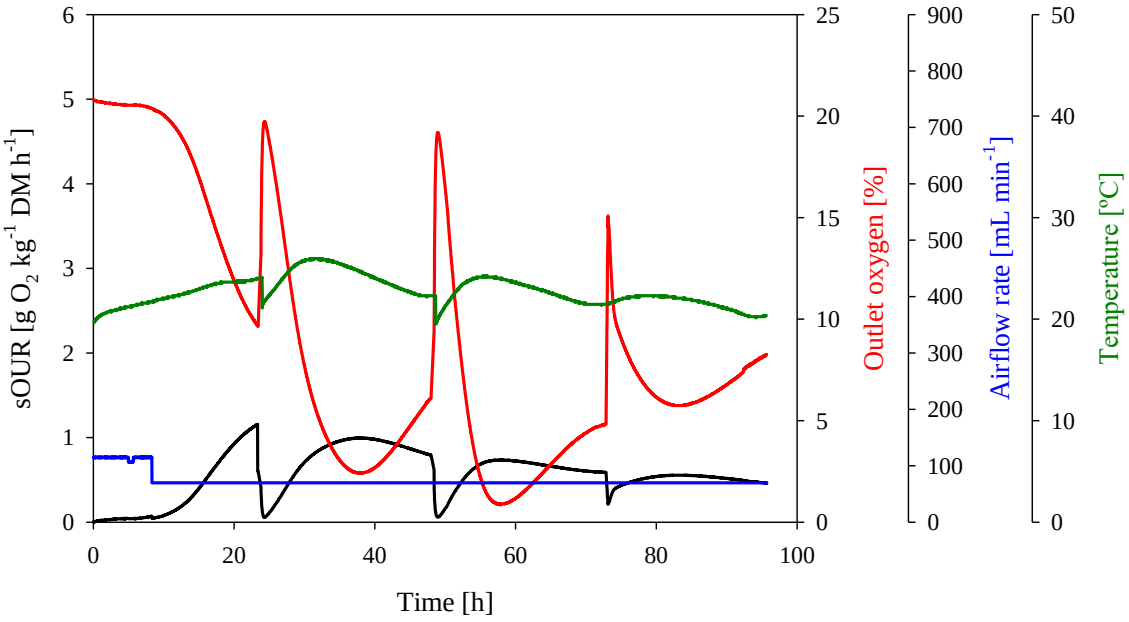

Figure 37: Monitored parameters (sOUR, outlet oxygen, airflow rate, and temperature) when operating in fed-batch mode in 22-L reactor.

An important delay was observed in oxygen consumption at the beginning of the process. Hence, the airflow rate was decreased after 8 hours of operation to promote the desired limiting oxygen condition. In consequence, a delay was also observed in obtaining the anoxic condition. At 24 hours, oxygen concentration was 10%, and the second feeding was performed. As the fresh substrate added contained fresh biodegradable organic matter and the airflow rate was maintained at low values, a second peak of oxygen consumption was observed at 37 hours, reaching oxygen levels of 2.4%. This trend was observed again after the third feeding, reaching oxygen concentrations of 1% at 56 hours. Full aerobic conditions were only obtained when the reactor was opened for feeding, and temperature was kept between 20 and 26ºC.

In terms of Bt development (Figure 38), a 44-fold increase in viable cells was observed after 24 hours of operation. However, the CFU concentration and its total amount (calculated as CFU concentration per total dry mass) decreased after each feeding,

with a reduction of two orders of magnitude being observed in CFU concentration, and a decrease of one order of magnitude in total CFU value from 24 to 96 hours. Hence, although fresh substrate was continuously added and the anoxic condition was almost maintained throughout the process, an extension of the growth phase was not observed, as the maximum viable cell count was monitored at 24 hours. On the contrary, its concentration was diluted after each feeding. Thus, even though the fed-batch strategy was thought to be suitable for extending the Bt growth phase and increase the spore production potential, it did not perform as expected.

Sporulation was also monitored from 48 hours until the end of the process. The spore concentration remained low compared to the batch performance. Its value did not exceed $5x10^6$ spores g^{-1} DM, with a sporulation ratio of 57% at 96 hours. These results were in line with Jackson (2017), who mentioned that the addition of extra nutrient could interfere with the nutrient depletion, and affect the sporulation process. Therefore, even though the fed-batch strategy represented an inoculum saving, the addition of extra biodegradable organic matter could negatively affect the spore production, by producing one order of magnitude less spores.

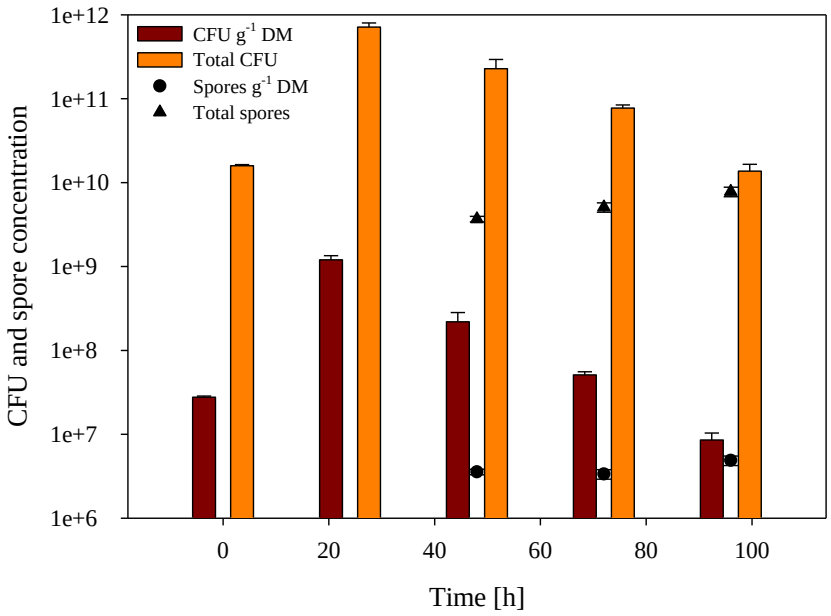

Figure 38: Viable cell and spore concentration, and total viable cell and spores determined throughout the fed-batch operation in a 22-L reactor. Total CFU and total spores stand for its concentration multiplied for the total dry mass of the packed bed.

A final attempt was made to achieve a semi-continuous operation with a reduction of liquid inoculum requirements. Working in a sequential batch mode, part of the fermented solid is used as an enriched inoculum for the following batch. The success of this operation relies on setting a proper retention time, to achieve a biomass acclimatization and specialization, and avoid the accumulation of inhibitory compounds (Cerda et al., 2017). This operation mode has scarcely been explored in SSF, as only few studies have been found, all performed with few grams of substrate, except the work by Cerda (2017).

A sequential batch operation was first performed, setting the retention time at 48 hours. After, 90% of the total fermented solids were replaced by new fresh substrate. The aeration strategy was executed over the first cycle and the airflow rate was maintained at

high levels throughout the second and third batch. Figure 39A shows the evolution of biological activity, oxygen levels, and temperature with the airflow rate applied. The first batch was successfully executed, as oxygen levels below 4%, along with a 15.5-fold increase of total cells was observed at 48 hours, with a spore concentration of $6.22x10^{7}$ spores g^{-1} DM (Figure 39B). The sporulation phase was shorter, and consequently the final spore determination represented 24% of sporulation ratio at the end of the first batch. As noted in the sOUR profiles, the lag phase of cycles 2 and 3 were reduced, similar to that observed in other studies (Cerda et al., 2017; Gao et al., 2020). Moreover, the biological activity was increased after every cycle due to biomass selection and adaptation (Jurado et al., 2015). However, the biomass selected did not correspond to Bt, as a significant decrease in total cell and spores was observed after the second and third batch. After the second batch, the diversity of other microorganisms in the samples made it impossible to determine the total cell concentration. Furthermore, a spore concentration dilution of one order of magnitude was observed. This dilution effect coincides with the solids exchange ratio applied. Specifically, 10% of the fermented material was used as inoculum, and the concentration of spores at the end of the second batch was the 6% of the spore concentration at the end of the first batch. At the end of the third batch, no Bt cells nor spores were observed.

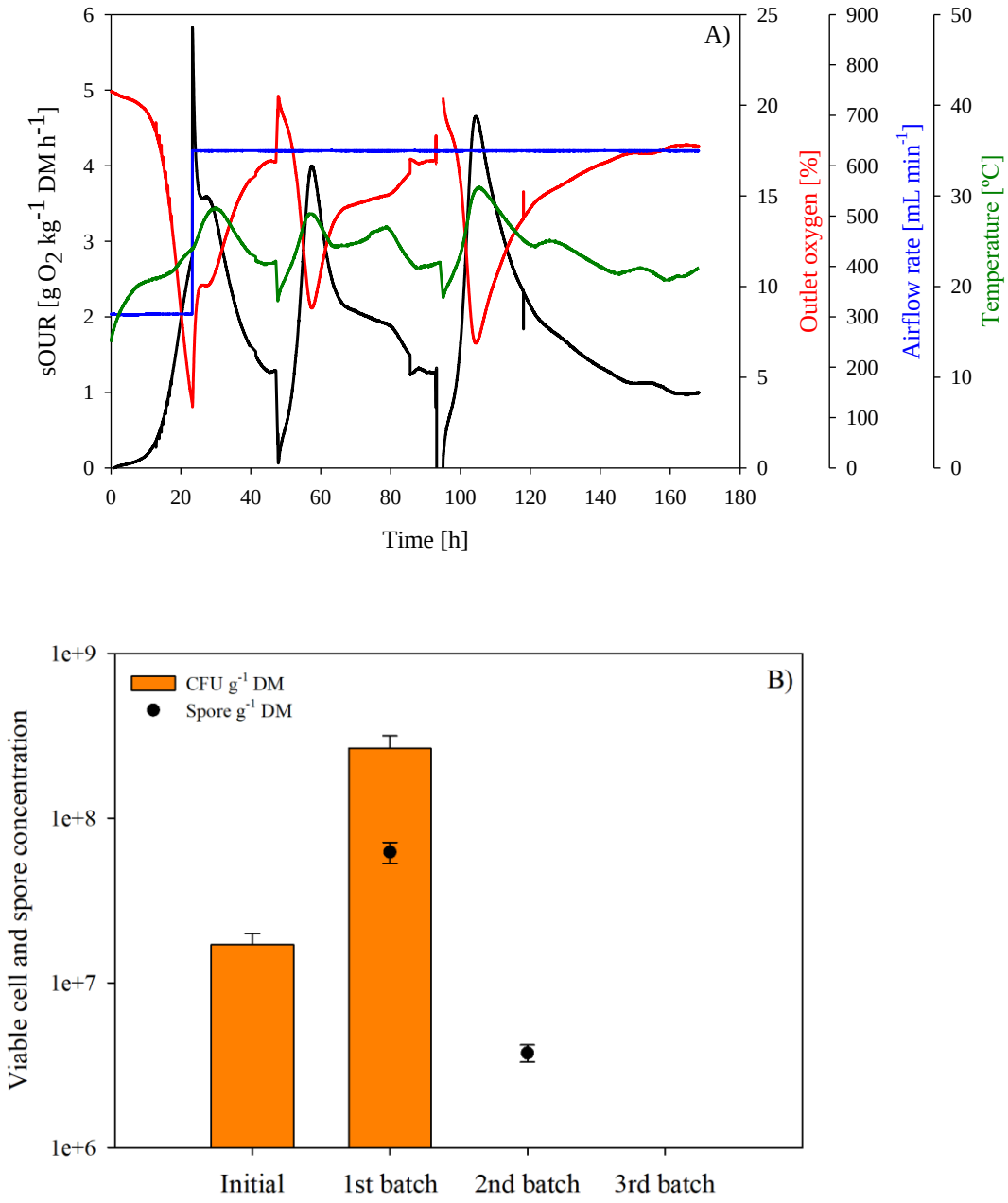

Figure 39: Monitored parameters (A) and viable cell and spore concentration (B) when operating an SBR with a solid replacement of 90% every 48 hours in a 22-L reactor.

A new sequential batch operation was carried out with some modifications: increase in the retention time to 72 hours, with an exchange ratio of 50% (wet weigh basis) and applying the aeration strategy in every batch (Figure 40).

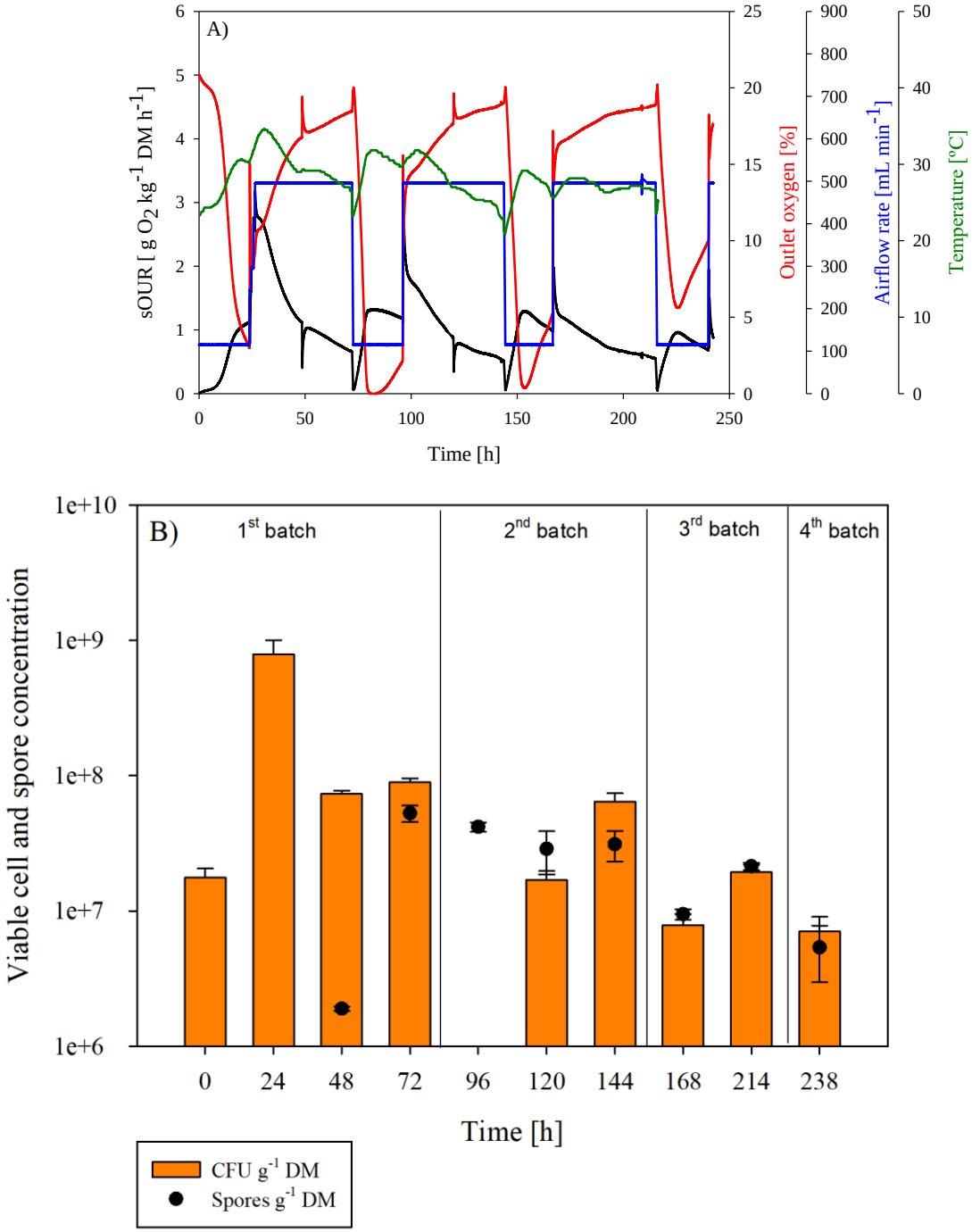

Figure 40: Monitored parameters (A) and viable cell and spore concentration (B) when operating an SBR with a solid replacement of 50% every 72 hours, performing the aeration strategy in a 22-L reactor.

In this second strategy, the operation performed as expected, in terms of oxygen levels, temperature and microbial activity. However, in the fourth batch, the oxygen concentration started to increase up to aerobic conditions during the first stage. A 45-fold increase in total cells was monitored at 24 hours, a decrease being observed at 48 and 72 hours, together with the increase of the spore concentration, reaching maximum values of $5.3x10^7$ spores g^{-1} DM at the end of the first batch. This value was almost maintained in the second batch, achieving a positive result. If the concentration remained constant, even with an exchange ratio of 50%, it meant that Bt was able to slightly grow and sporulate. However, spore concentration decreased in the third and the fourth batches. That is to say, as long as this sequential operation was continued, the dilution of the spore concentration was higher after each batch. The dilution rate was reduced, mostly due to the decrease of the solid exchange ratio (from 90 to 50%). Hence, the germination of spores to achieve a feasible operation was not observed.

The important point in this experiment was to observe the inefficiency of spore germination. The spore form is not a stable state, as if they are appropriately stimulated, these spores could initiate germination leading to new vegetative growth (Vu et al., 2009). New vegetative cells would enter into the sporulation process again, and in consequence, produce more Cry proteins. The strategy applied in the sequential operation did not provide the necessary environment for germination to occur, with a gradual decrease being observed in the vegetative cell and spore concentration.

The germination of *Bacillus thuringiensis* spores was studied by Bassi et al. (2016) using food models. Spores of many bacteria are slow to react to germinants. Factors triggering spore germination in food are amino acids, sugars, pH, sub-lethal thermal treatments, water activity, and storage conditions (Moir and Smith, 1990). Bassi et al. (2016) observed the start of the germination process 40 min after the heat activation

was performed on the samples, and under high water activity and nutrients availability conditions. Thus, the key point for a successful semi-continuous operation was the enhancement of spore germination.

7.2.3. Preliminary studies on Bt spore germination

As the spore germination was not observed in the solid matrix, the goal of this study was to determine whether the germination of spores would occur under more suitable condition, when a liquid extract from the fermented material was mixed with different volumes of fresh liquid medium (Oxoid) and incubated for 24 hours at 30ºC (Table 20).

Table 20: Bt germination ratios.

Condition	Extract (mL)	Medium (mL)	CFU after incubation/CFU before incubation	CFU after incubation/Spores before incubation	Sporulation in the liquid medium (%)
1	75	25	2.1	3.5	35
2	50	50	1.4	2.4	72
3	50	100	18	29.9	6
4	25	100	4.8	11.3	25
5	10	100	14	32.9	2
6	5	100	29.6	69.4	3

After the incubation period, a higher increase in vegetative cells was observed in conditions 3, 5, and 6. The increase in the ratios shown in Table 20 suggests that some of the spores germinated during the incubation and turned into new vegetative cells.

Specifically, a higher spore germination was observed in conditions 5 and 6, reaching a CFU after incubation/Spores before incubation ratio of 32.9 and 69.4, respectively. Additionally, it is important to select the conditions with lower sporulation ratios. As the product of interest is formed during the sporulation phase, this process should not occur during the incubation period. Conditions 5 and 6 showed the lowest sporulation ratio (2 and 3%, respectively). Therefore, these conditions were the best experimental condition tested to obtain new vegetative cells. However, final CFU concentration was one order of magnitude lower (6.6 and $7.3x10^7$ CFU mL^{-1}) compared to the CFU concentration of the prepared inoculum ($2\text{-}5x10^8$ CFU mL^{-1}). Thus, if using these extracts for inoculating the solid, a higher volume should be added to the mixture, increasing the moisture, and decreasing the mixture porosity. It could also be concentrated by centrifugation. Additionally, these conditions were the most diluted conditions, using more volume of fresh media. Hence, a balance between the germinated spores and the volume of fresh media should be studied in economic terms.

7.2.4. Production of *Bacillus thuringiensis* var *israelensis* following the developed operational strategy

To assure the robustness of the developed protocol, the operational strategy was reproduced using *Bacillus thuringiensis* var *israelensis* (Bti) as inoculum at Setup 3. Bti is highly active against disease vector mosquitoes, due to its high insecticidal activity and the lack of resistance to this Bt subspecies (Bravo et al., 2019). Specifically, its action is against earlier aquatic larval stages. Researchers have been studying and optimizing fermentation media to maximize its production. Zhuang et al. (2011) studied the production of Bti and *Bacillus sphaericus* (Bs) using sewage sludge, observing an

excellent adaptation of these microorganisms to sewage sludge, with an increment of two orders of magnitude of viable cells.

A batch was performed following the directions of the operation protocol. As can be seen in Figure 41, the batch ran successfully, as the desired oxygen content was achieved on both aeration stages and temperature ranged from 20 to 40ºC. In terms of the microbial development, a 24.5-fold increase in total Bti viable cell was achieved, reaching a final concentration of $3.4x10^8$ CFU g^{-1} DM after 96 hours of operation. At this point, 100% of sporulation was estimated, obtaining a spore concentration of $4x10^8$ spores g^{-1} DM.

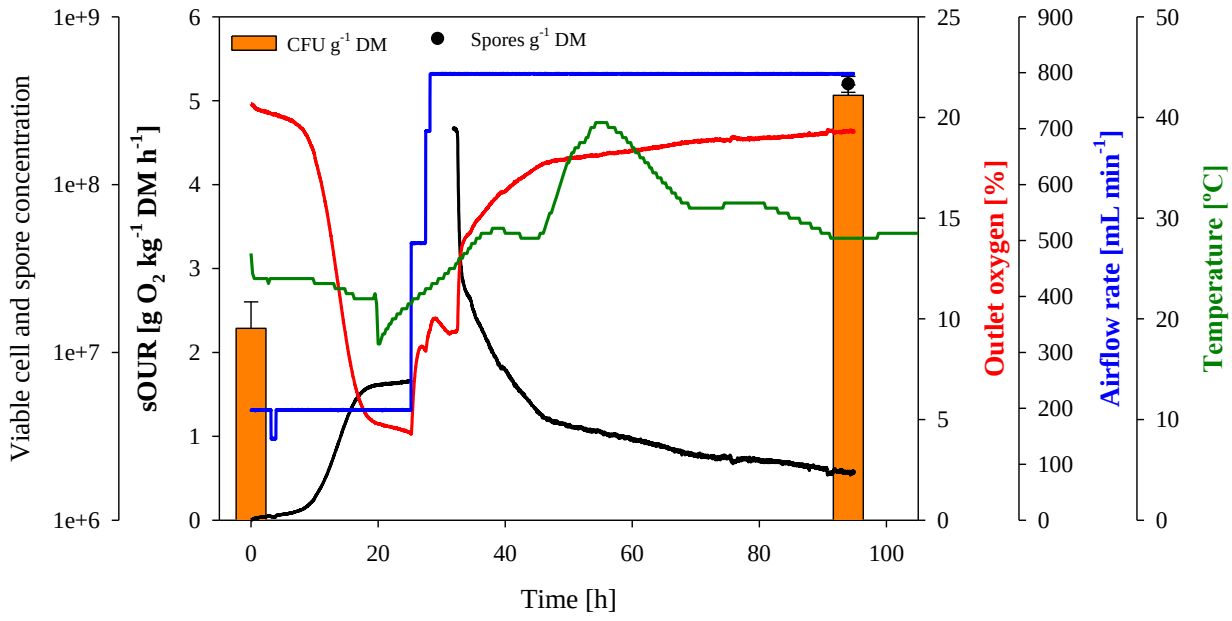

Figure 41: Monitored parameters (sOUR, outlet oxygen, airflow rate, temperature, viable cell and spore concentration) in the 22-L reactor, validating the operational strategy using Bti as inoculum.

7.3. Conclusions

The effect of the hygienization process and the oxygen levels on the Bkt growth and sporulation was demonstrated working in batch mode. A promising spore production was obtained even when digestate and biowaste from different batches and two different bacterial strains were used, highlighting the potential of the developed protocol to be reproduced at higher scales. This strategy has shown to be effective when using Bti as inoculum. However, the success of this strategy working in fed-batch or SBR mode was not achieved at this scale. Spore germination from fermented solids with liquid media after SSF is feasible, and could represent savings in inoculum production.

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CHAPTER 8: SSF PILOT PLANT DESIGN

8.1. Description of the instrumentation and equipment

This section describes the pilot plant designed and built for the SSF demonstration site. The reactor design was a collaborative work between GICOM and Aeris, as part of the knowledge transfer from the university to the company. The reactor is mainly composed by a 290-L vessel (1 m high and 0.61 m in diameter) with specific equipment incorporated, such as a helical stirrer with frequency inverter, air flowmeter, temperature probe, and an oxygen sensor (Figure 42). Deflectors were excluded because of the compaction problems observed when the stirrer was used in the 22-L reactor. It also includes an electrical panel with a programmable controller and a touch screen, where data from oxygen, temperature, airflow rate and sOUR are available.

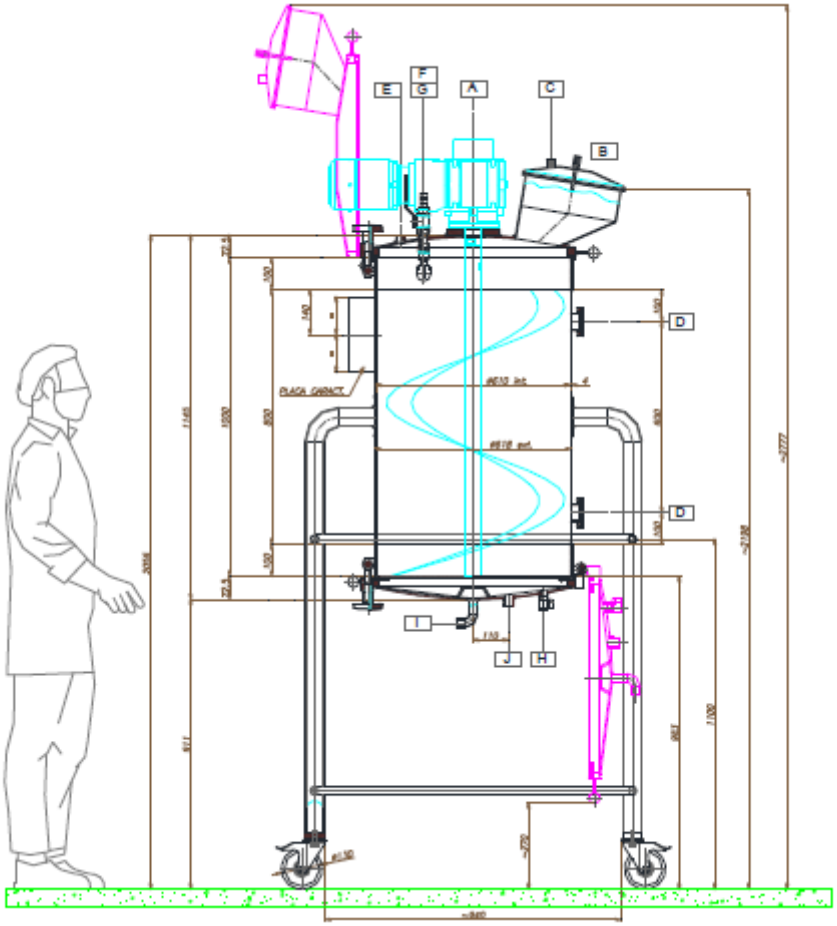


Figure 42: DECISIVE SSF pilot reactor.

As an important amount of material would be used, the reactor was designed to be fed from the top, using a manhole located approximately at 2.2 m of height, and discharged from the bottom, by opening the bottom cover. Hence, a ladder with a platform and a trolley were also incorporated.

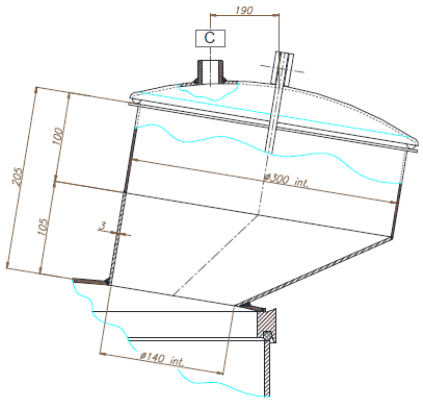


Figure 43: Reactor manhole for feeding.

The most relevant elements inserted in the pilot plant were:

Sample extraction: Three sample extraction ports were incorporated, allowing sampling at three different positions of the packed bed, and thus, having more representative information about the production gradients along the axial axis. They have a diameter of 50 mm and were located at 0.2, 0.5, and 0.8 m of the 1-m vessel.

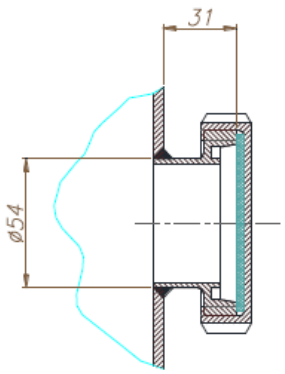


Figure 44: Sample extraction port.

Safety valve: A safety valve for gas release, in case of reactor overpressure, was incorporated in the upper cover as a safety requirement. An increase in pressure could occur if the gas outlet clogged or accidentally closed. If pressure is above 0.5 bars this safety valve will open.

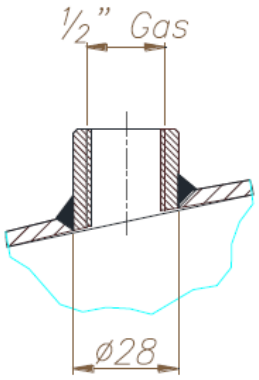


Figure 45: Safety valve.

Water supply connection: This connection was located in the upper cover of the reactor with the aim of using it for reactor cleaning or for adding the liquid solvent to perform the product extraction inside the vessel.

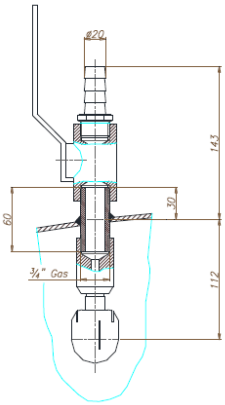


Figure 46: Water supply connection.

Drain connection: Related to the water supply connection, a drain connection was located on the bottom cover to facilitate the water drainage.

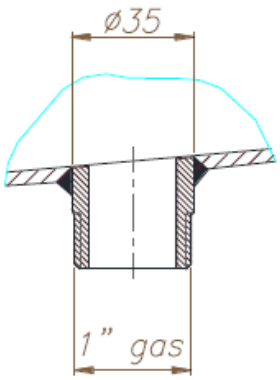


Figure 47: Drain connection.

Inlet/outlet air connections: These connections were located based on the Setups used at the laboratory. Hence, the inlet air connection was designed for the bottom part, while the outlet air connection was located on the upper part of the vessel.

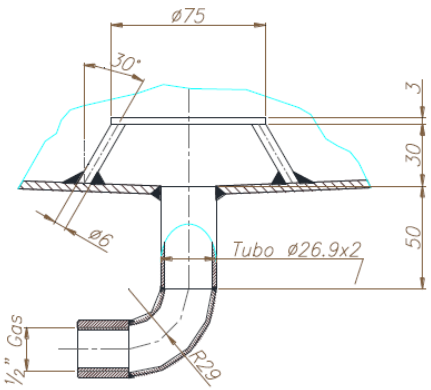


Figure 48: Inlet air connection.

Temperature probe: As temperature gradients over the packed bed is one of the key parameters of the process and has to be monitored, the defining of a representable location for inserting the temperature probe was difficult. However, due to the presence, and the diameter of the helical stirrer, the location of this probe on the side of the reactor could harm it. Thus, the probe was inserted at the bottom of the reactor. The temperature probe was a Pyro-Alloy TR-NNKp-250, with a measurement range between 0 and 100ºC and a length of 25 cm. It was connected to the PLC to register the temperature values. iButton

Devices (Thermochrom, UK) were also used to complement the temperature measurement.

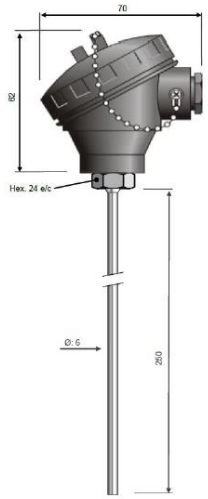


Figure 49: Temperature probe.

Grid: A tramex support grid was installed in the lower part of the reactor. This grid prevents solid material from entering and eventually blocking the air inlet. The grid, with a pore size of 2 mm, will also serve to facilitate the distribution of air through the reactor.

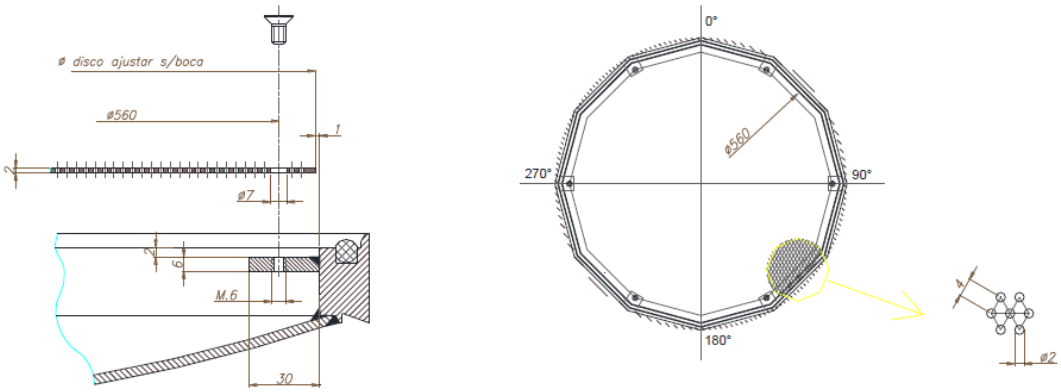


Figure 50: Grid.

Stirrer: The solid material could be mixed by means of a mechanical stirrer with an axis of 50 mm of diameter and a RIBBON impeller of 550 mm. This specific stirrer was

selected according its similarities to the stirrer used in the 22-L reactor trials. The agitator has a 1 kW engine and a frequency inverter, which enables the agitation speed to be regulated.

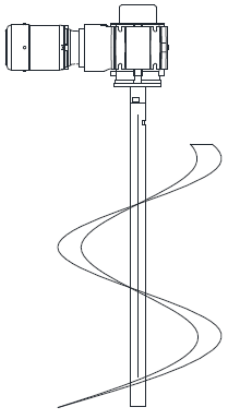


Figure 51: Helical stirrer.

Air flowmeter: The volumetric air flowmeter installed was a Tecfluid M21, with a measurement range between 310 and 3100 NL/h. It has an analog output to connect it to the PLC and register the measured values. The airflow rate was changed by means of a manual valve. Before the air flowmeter, a pressure regulator with filter was installed to ensure that the air flowmeter works at the device working pressure (1 bar).

Figure 52: Volumetric airflow meter.

Oxygen sensor: An oxygen sensor (O2-A2, Alphasense, UK), was installed to measure the oxygen concentration of the outlet air. The sensor was located in a closed box under the electric panel and was connected to the PLC to register the oxygen values.

Figure 53: Oxygen sensor.

Electrical panel: This panel was equipped with electrical protectors, the frequency inverter to regulate the stirrer speed, and the PLC. Additionally, the panel has a touchscreen to start/stop the data register, start/stope the agitation and its velocity, a visualization of the process parameters and to download the values of the process to an USB device in an Excel-like format.

Figure 54: Touch screen.

8.2. Composting and mixing tests

Firstly, trials were performed to define the amount of substrate to be fed and to establish the mixing procedure. As an important amount of substate and bulking agent must be mixed, the stirring system installed could be used to facilitate the operation. The idea was to feed the reactor with several layers of bulking agent, digestate, and biowaste, and to turn on the stirrer for a certain time, expecting the complete mixing and integration of those three components. Once the mixing was finished, a fermentation without Bt inoculation, similar to composting, was started in order to observe and analyse the process dynamics, especially the evolution of temperature and oxygen levels depending on the airflow rate applied. Furthermore, in these composting tests, the real input biowaste to be treated in the treatment plant facility was used with the aim of integrating the SSF unit into the current biowaste treatment scheme. However, this biowaste did not have sufficient quality, and an important amount of impurities were present, such as plastics, paper, cans, and textile waste (Figure 55). Digestate and biowaste were not hygienized in this first test.

Figure 55: Biowaste used as received at the plant.

In these composting trials, a total amount of 70 kg of non-hygienized material was fed into the reactor, with 32.8 kg being digestate, 19.7 kg of biowaste and 17.5 kg of bulking agent. A head space of around 25% was left in order to prevent the possible material compaction at the top cover of the vessel at the time of mixing. These materials were fed in three bulking agent layers and three substrate layers, and stirred at 35 rpm for 10 min. Apparently, the mixture was appropriate in the upper parts of the reactor.

The non-inoculated SSF tests also served to determine the airflow rates needed to achieve the desired oxygen conditions at this demonstrative scale for the forthcoming SSF tests (Figure 56).

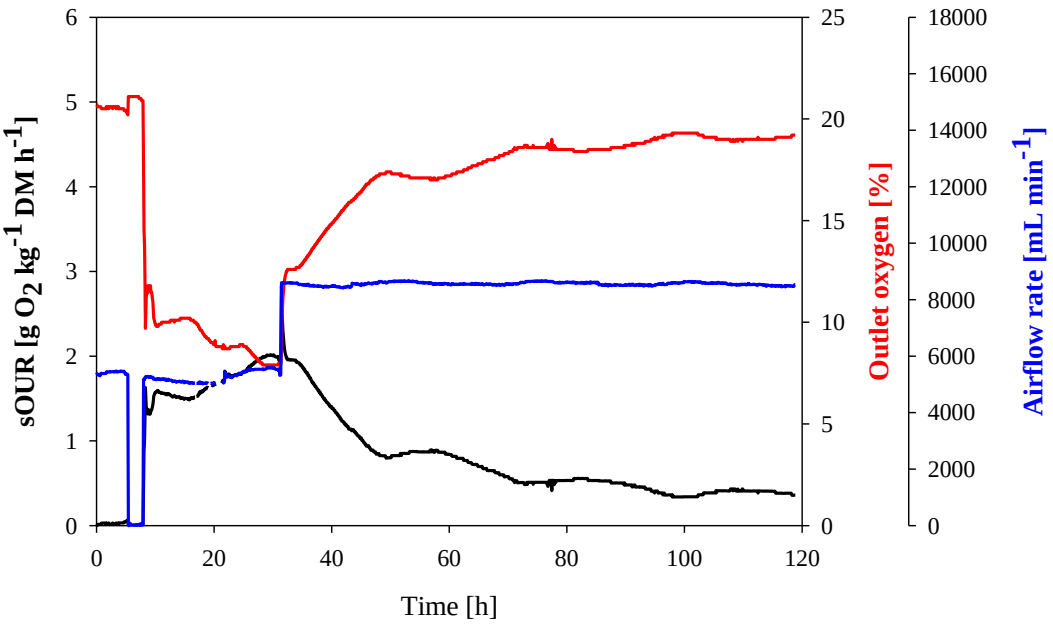


Figure 56: Monitoring parameters evolution throughout the composting process at the DECISIVE pilot reactor.

Initial airflow rate was set at 5400 ml min^{-1} (324 L h^{-1}, 0.1 mL min^{-1} g^{-1}), which is the minimum airflow rate that could be applied using the installed air flowmeter. It was important to note the fluctuation of the airflow rate, especially during the first microaeration stage. Minimum oxygen concentration monitored was 7.9% at 31 hours. In the second stage, the airflow rate was increased to 8400 mL min^{-1} (500 L h^{-1}), which was enough to reach aerobic conditions, with oxygen levels above 15%. However, the aeration applied was not enough for removing the metabolic heat produced from the solid biological activity, leading to thermophilic temperatures.

Three temperature sensors were placed at different positions of the packed bed. As observed in Figure 57, the temperature values over the process were very different depending on the position (Temp. 1, 2, and 3). Temperatures over 45 ºC were monitored in 2 of these 3 temperature sensors during the microaeration stage. A temperature peak was also monitored after the increase of the airflow rate in one position, reaching the maximum temperature of the process (67.5ºC). Moreover, it was shown that the temperature probe installed at the bottom of the vessel was not representative, as an important gradient of temperatures was observed. Heat removal dynamics are associated with convective cooling, which lead to an increase in temperature in the upper parts of the reactor if the bed height is higher than the critical height. Nonetheless, the critical height is directly proportional to the applied superficial air velocity (Mitchell et al., 1999).

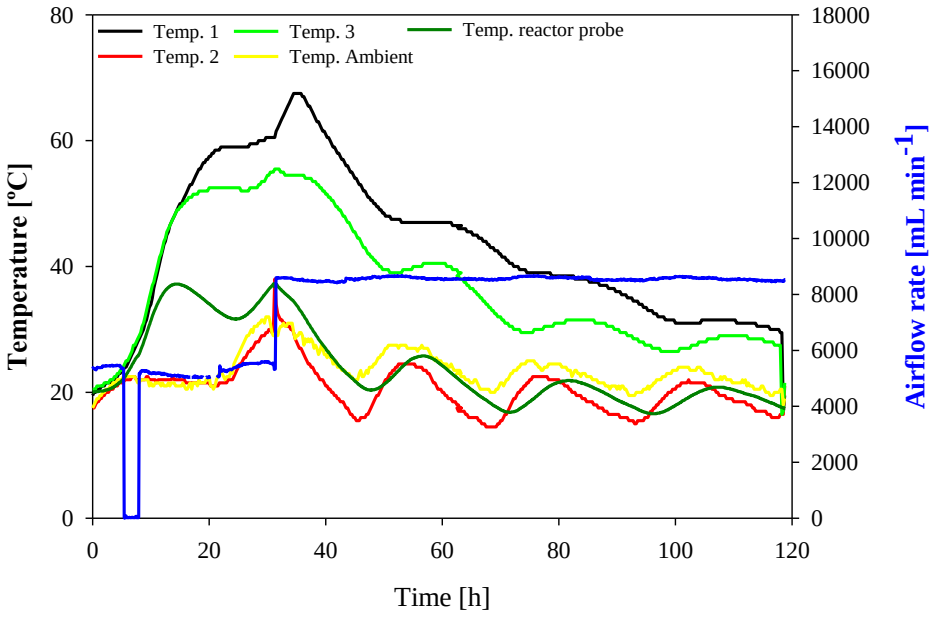


Figure 56: Temperature evolution at different points of the packed bed reactor over the composting process.

In our case, as a microaeration stage is required, low superficial air velocity is applied, affecting the convective cooling and the critical height. The ambient temperature condition was also strongly modified when working on the biowaste treatment facilities. Maximum temperatures of 32ºC and minimum of 17ºC were recorded, obtaining a temperature gradient of 15ºC, while room temperature fluctuated 5ºC in the laboratories. That is to say, ambient temperature fluctuations would be strongly dependent on season. Higher ambient temperatures hinder the cooling effect of outer surfaces of the reactor. Thus, the metabolic heat removal is negatively affected, and thermophilic temperatures are more likely to be achieved. However, when working in winter, low ambient temperatures could decrease the microbial activity, also having negative consequences on the process.

Lastly, during the material discharge, we realized that the materials mixture was not as good as expected in the lower parts of the reactor. An important amount of solids was compacted and unmixed in the lower part of the reactor, which can be defined as a mix dead volume. Additionally, the typical structure of packed bed was inexistent, as the digestate was stuck over the helical stirrer, and paper and plastics coming from biowaste were coiled around the shaft (Figure 57).

Figure 57: Compacted material at the bottom part and over the stirrer after the composting process.

These results meant important decisions had to made regards the operation of the plant. It was clear that the mixing device should be improved, and a different impeller should be tested. However, the project timing was tight, and this study has been postponed. So, on the one hand, it was decided to perform the mixing of substrates and the bulking agent manually, outside the reactor. In this way, the correct integration of those three materials could be ensured, as well as the correct formation of the packed bed. On the other hand, the problem of temperature gradients was revealed. Lastly, we decided

to avoid using the input biowaste of the treatment plant, due to the important amount of impurities and its effect on the stirrer system. To try to achieve a higher quality final product, fruit and vegetable biowaste from a local marked was selected for further test performances, since it was considered the best quality biowaste option.

8.3. References

Mitchell, D. A., Pandey, A., Sangsurasak, P., Krieger, N., 1999. Scale-up strategies for packed-bed bioreactors for solid-state fermentation. Process Biochemistry. 35, 167-178.

CHAPTER 9: OPERATION OF THE DECISIVE SSF PILOT PLANT

9.1. Overview

The DECISIVE project aims to scale-up the valorization processes developed and the use of the obtained products at pilot scale for their validation. The technologies targeted in the project, i.e. anaerobic digestion, biogas valorization with Stirling engine, and SSF, were proposed to be developed at a compatible scale with the decentralized network system. Furthermore, products from these processes were expected and adapted to urban farming needs. Two demonstration cases were raised by two partners of the consortium: Lyon (reFARMERS) and Catalunya (ARC). These real demonstration locations would enable to couple this new biowaste management system with the valorization technologies and urban farming.

This chapter targets the operation of the SSF process on the DECISIVE SSF pilot plant, and study the technology scalability in terms of spore production. Furthermore, a considered aspect concerning the quality of the final product was the analysis of microplastics (MPs) after the repeated visual observation of this pollutant in the digestate samples. Thus, an identification and quantification study of MPs present in different organic matrices over the biowaste treatment process, as well as in the SSF input and output mixtures, was performed (Figure 58).

Figure 58: MPs in compost samples.

MPs are defined as microparticles in the range of few μm to 5 mm (Andrady, 2011). Over the last years, the presence and effect of MPs in marine ecosystems have been intensively studied (Andrady et al., 2011; Yu et al., 2019; Singh et al., 2020). However, its presence on soils is still a knowledge gap, probably due to the lack of standardized analytical methods for MPs quantification for soil samples (He et al., 2018).

Besides littering, one of the possible input paths of MPs to terrestrial ecosystems is the use of sewage sludge and municipal solid waste compost as a fertilizer in agriculture (Müller et al., 2020). Concerning the case of compost, MPs are present mainly because of a non-well source selected biowaste and/or the use of non-compostable bags. One of the main issues of MP soil contamination is its capacity of changing soil properties and function, as well as microbial diversity (Rillig, 2012). The problematic is still worse when considering that MPs can interact with other pollutants, like adsorption of heavy metals and hydrophobic organic compounds on their surface.

Most common analytical methods for MP determination are Fourier transform infrared spectroscopy (FTIR), TED-GC-MS, Raman spectroscopy, and HPLC-UV (Wu et al., 2020). However, since different analytical methods provide different data and information, most of the studies carried out are not comparable.

9.2. Experimental methodology

Six batches were performed (named SSF 1-6), trying to reproduce the conditions for the correct operational strategy performance. In some cases, variations were made in co-substrate amount, initial airflow rate, and reactor insulation, compared to the protocol developed in chapter 5. The strategy was reproduced using both available Bt strains (Btk and Bti). Biowaste used was collected from a weekly local fruit and vegetable market of Granollers (Barcelona, Spain). Substrates were hygienized at the UAB laboratories before

fermentation. Batch conditions are shown in Table 21. A rotameter was used when an airflow rate below 5170 mL min^{-1} was required (Mod 2150, Tecfluid, Spain).

Table 21: Conditions tested in each SSF performed at the demonstrative scale.

Run	Biowaste (%)	Aeration condition in 1st stage	Airflow provider	Insulation in 1st stage	Month	Bt strain
SSF 1	37.5	Micro-aeration	Airflow meter	No	November	Btk
SSF 2	10	No aeration	Airflow meter	No	December	Btk
SSF 3	37.5	No aeration	Airflow meter	No	January	Btk
SSF 4	37.5	Micro-aeration	Rotameter	Yes	February	Btk
SSF 5	37.5	No aeration	Airflow meter	No	July	Bti
SSF 6	37.5	Micro-aeration (First 5 hours)	Rotameter and Airflow meter	No	September	Bti

To ensure an adequate mixing of the materials and a reproducible packed bed structure, the substrates were mixed and inoculated manually on the trolley and fed into the reactor afterward.

For MPs identification and quantification, 8 samples from the biowaste treatment plant and the SSF process were analysed following the density separation and TED-GC-MS method at IUTA laboratories (Duisburg, Germany). Samples from the biowaste treatment plant were chosen: biowaste input (coming from market waste), AD feeding (the organic matter slurry fed into the AD), fresh solid digestate, and compost (Figure

59). In addition, a sample of hygienized digestate, hygienized biowaste, SSF initial mixture, and SSF final solid were also analysed.

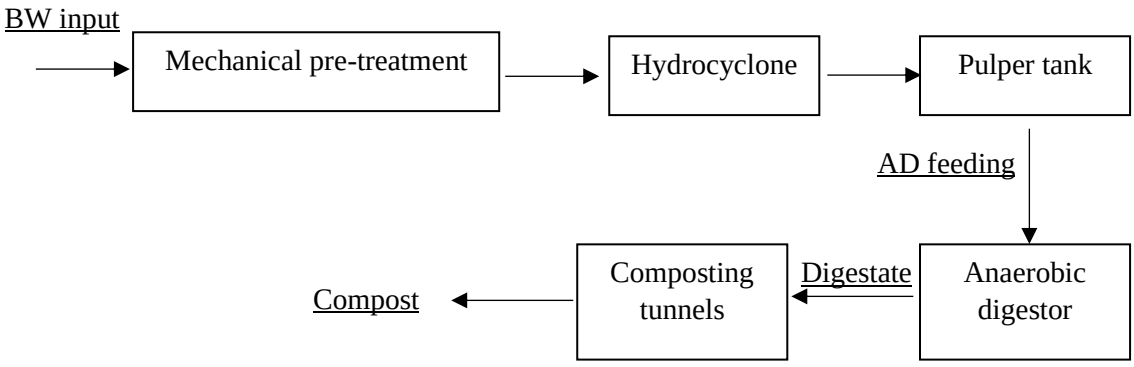


Figure 59: Biowaste treatment facility scheme.

9.3. Results

9.3.1. Operational protocol implementation at DECISIVE demonstration reactor

Figure 60 shows the process evolution of SSF 1. Initial airflow rate was set at 5400 mL min^{-1} (0.1 mL min^{-1} g^{-1}), to maintain the same specific airflow rate as the 22-L reactor performance. However, using this airflow rate, the limiting oxygen condition was not fully achieved in the first stage, and the minimum outlet oxygen measured was 10.4%. In the matter of Bt growth and sporulation, an important decrease on viable cells was monitored after 24 hours. Specifically, a reduction of one order of magnitude was observed, from $1.3x10^7$ to $1.1x10^6$ CFU g^{-1} DM. Bt spores appeared at 48 hours, as in fermentations carried out at laboratory and bench scale. However, very low spore yield was obtained, in the range of 0.1 produced spores per initial CFU.

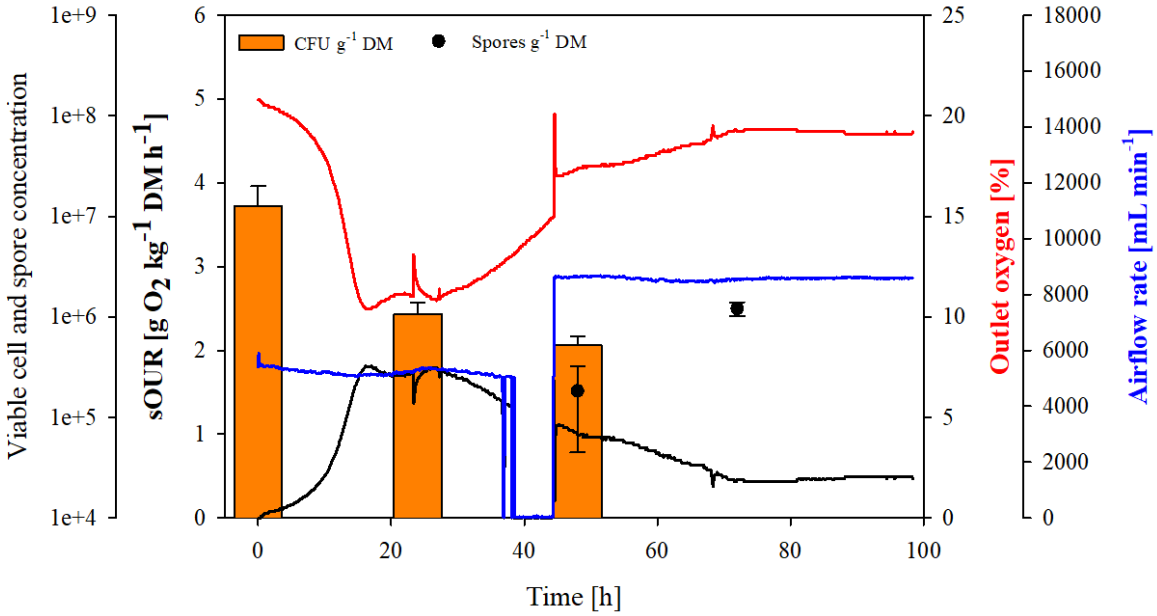


Figure 60: SSF 1 performance at the DECISIVE pilot reactor.

The main hypothesis about the low spore production was related with the inadequate Bt acclimation in the solid substrate at this scale, probably because of the fact of not reaching the oxygen-limiting condition and the temperatures reached. Higher oxygen concentration enhances aerobic biological activity, leading to an increase in autochthonous microorganism competence for Bt growth, but also an increase in temperature. Figure 61 presents the temperature evolution at different points of the packed bed. A maximum temperature gradient of 39.5ºC was registered at 37 hours. Besides, thermophilic temperatures (temperatures higher than 45ºC) were monitored in 5 of the 15 monitored positions. Therefore, under these conditions, Bt was not able to colonize and grow properly, as observed previously at laboratory and bench scale.

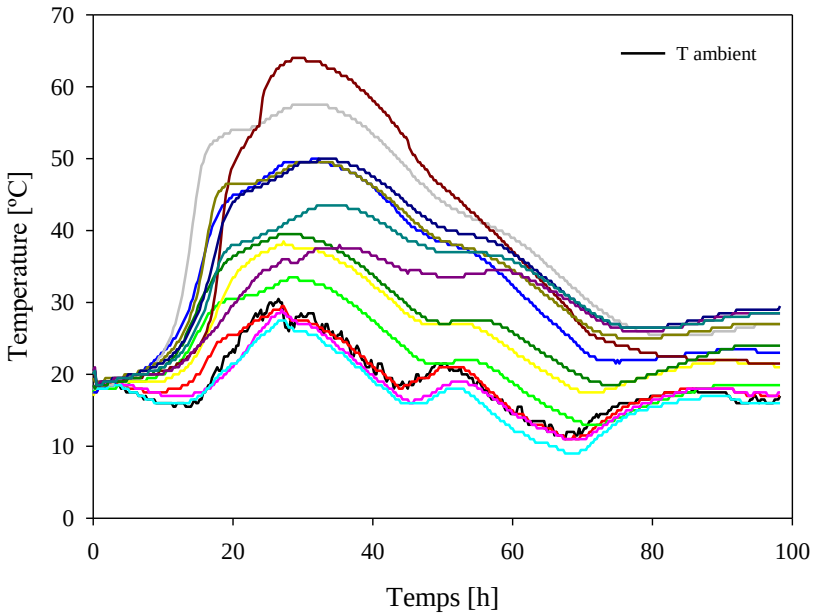


Figure 61: Temperature profiles obtained at different positions of the packed bed.

In the way of trying to mitigate the rise of temperature during the process, some operational parameters must be reconsidered. Thus, as biowaste is a rich source of highly biodegradable organic matter, and its relationship with the increase of temperature is well-known, a new batch reducing the amount of co-substrate was carried out.

Figure 62 shows the evolution of process parameters in SSF 2, where a 10% of biowaste was used, and no forced aeration was provided to force anoxic conditions in the first 30 hours.

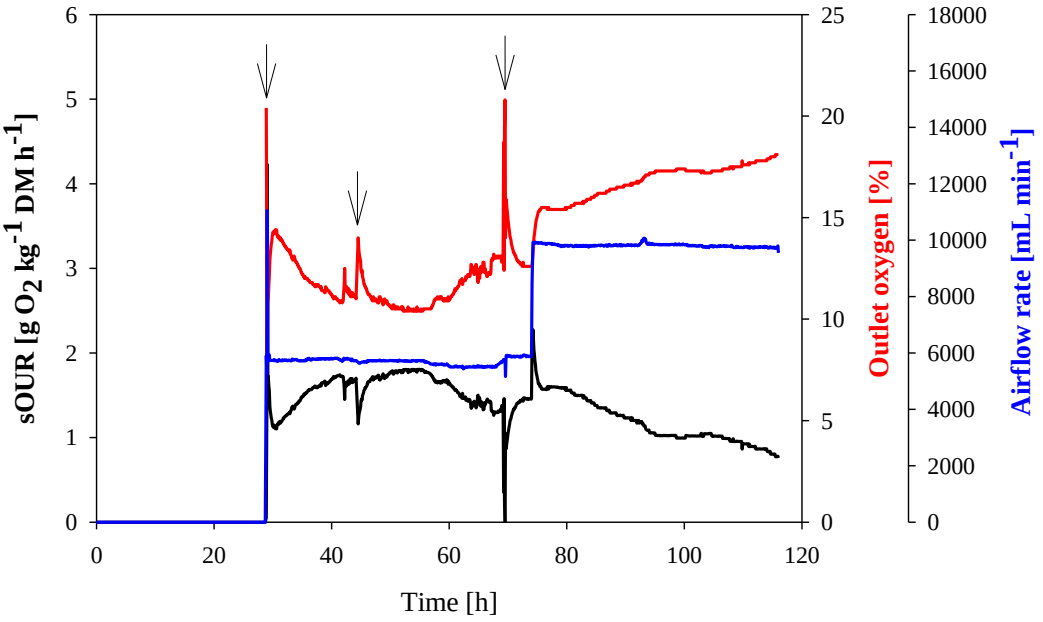


Figure 62: sOUR, outlet oxygen, and airflow rate evolution on SSF 2. Arrows indicate the samplings in the course of the process.

The oxygen consumption from the biological process could not be monitored when no forced aeration was applied, as there was not enough air pressure to read a real measure of the oxygen in the packed bed. An approximate oxygen determination started once the aeration was turned on, since the oxygen of the packed bed may be diluted with the oxygen of the airflow. However, we considered that the pilot reactor behavor similar to the reactor studied in Poyuelo et al. (2010). Therefore, the flow pattern was considered to behave as an ideal plug-flow. The expected situation was to observe very low oxygen concentrations at that point, as a result of the biological oxidation and the fact that no extra oxygen was supplied. However, a punctual oxygen concentration of 8.2% was reported and it increased rapidly to values between 10 and 15%. This range of oxygen values was maintained for 44 hours, and consequently, the airflow rate was increased again, at 74 hours, to achieve oxygen values higher than 15%.

In terms of temperature, the absence of forced aeration in the first staged caused an important delay on the temperature increase, staying mainly in the mesophilic range (Figure 63). In this sense, a better environment for Bt growth was achieved compared to SSF 1. In the second aeration stage, it was interesting to observe how higher temperatures were mainly reached in the middle part of the packed bed, with maximum temperatures of 56.5ºC.

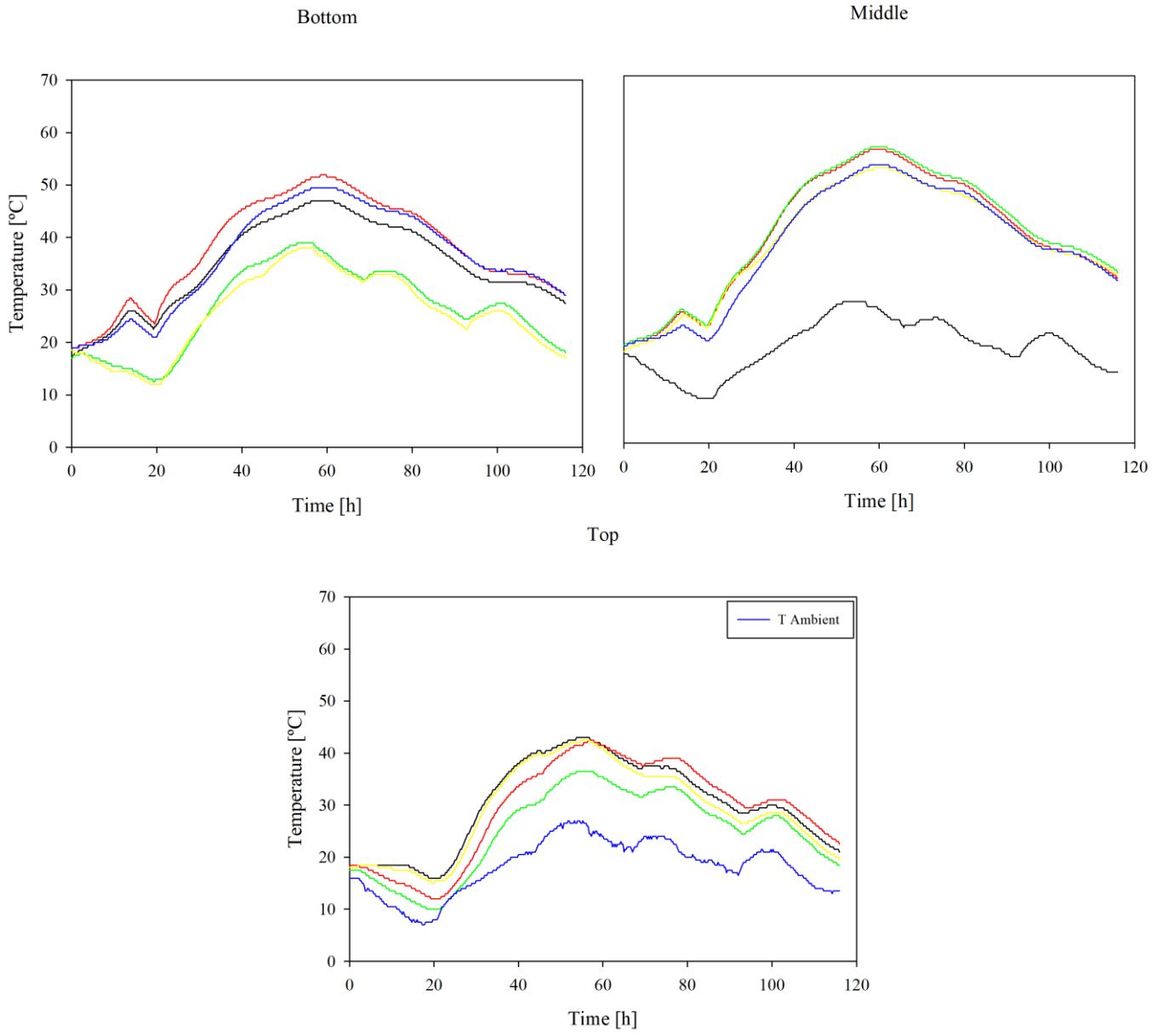
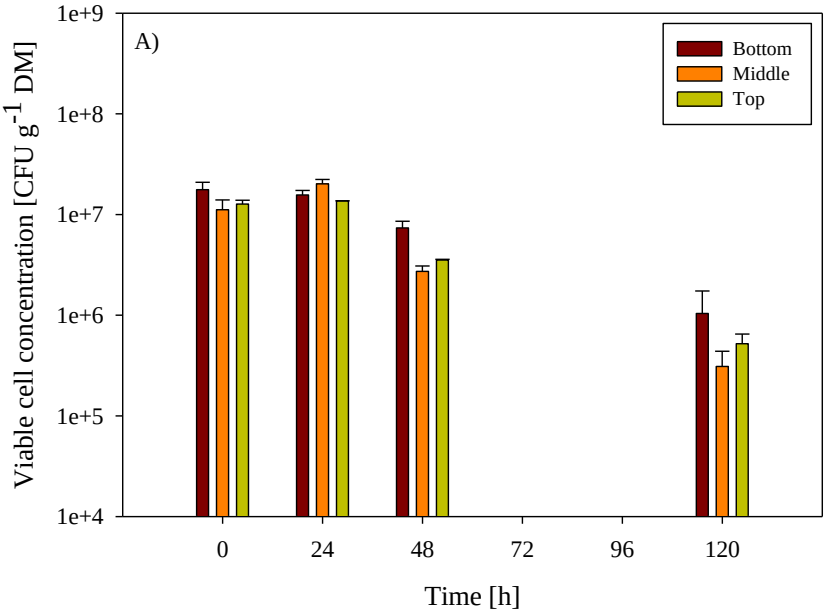


Figure 63: Temperature profiles at the bottom, middle, and top parts of the packed bed reactor in SSF 2.

This strategy improved the results, compared to SSF 1, in terms of viable cell concentration at 24 hours. The concentration was mostly maintained at the three positions sampled (Figure 64A). Nonetheless, this value decreased progressively, from $1.6x10^7$ to $6.2x10^5$ CFU g^{-1} DM, as an average value of the three positions. Regarding the spore production, spores were detected from 48 hours, coinciding with the concentration decrease of viable cells. A final concentration of $8.5x10^5$ spores g^{-1} DM was achieved, corresponding to a yield of 0.06 spores produced per initial CFU (Figure 64B).



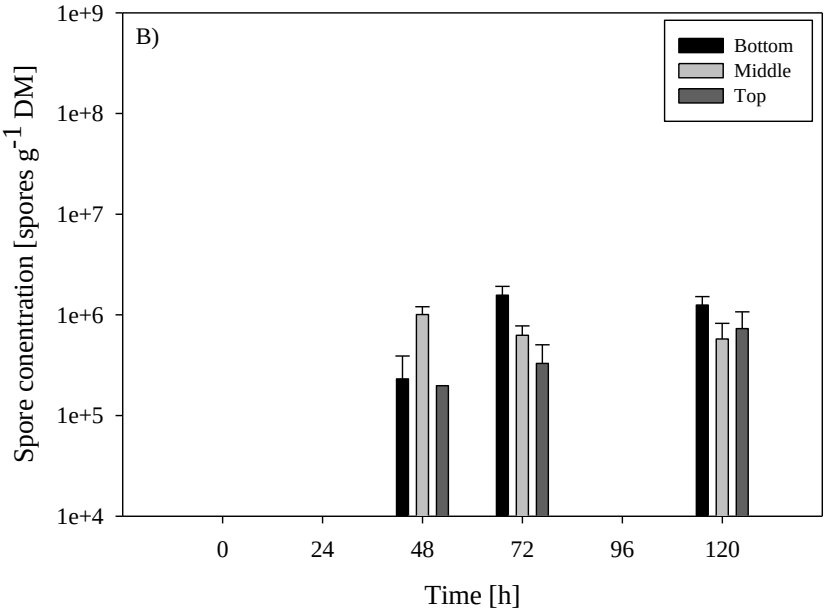


Figure 64: Viable cell (A) and spore (B) concentration at the bottom, middle, and top parts of the reactor in SSF 2.

Therefore, even though better conditions were obtained for Bt growth, the lack of a considerable Bt viable cells increase, together with a poor sporulation rate, caused a low spore yield. To boost the Bt growth, a third strategy was proposed, increasing again the biowaste amount to 37.5% (to follow the instructions of the operational protocol), but maintaining the absence of forced aeration.

The result of running a batch with these conditions (SSF 3) is presented in Figures 65, 66, and 67. The batch did not run properly in terms of oxygen profile evolution. Even though co-substrate was added, the lowest oxygen concentration (11.7%) was registered at 55 hours.

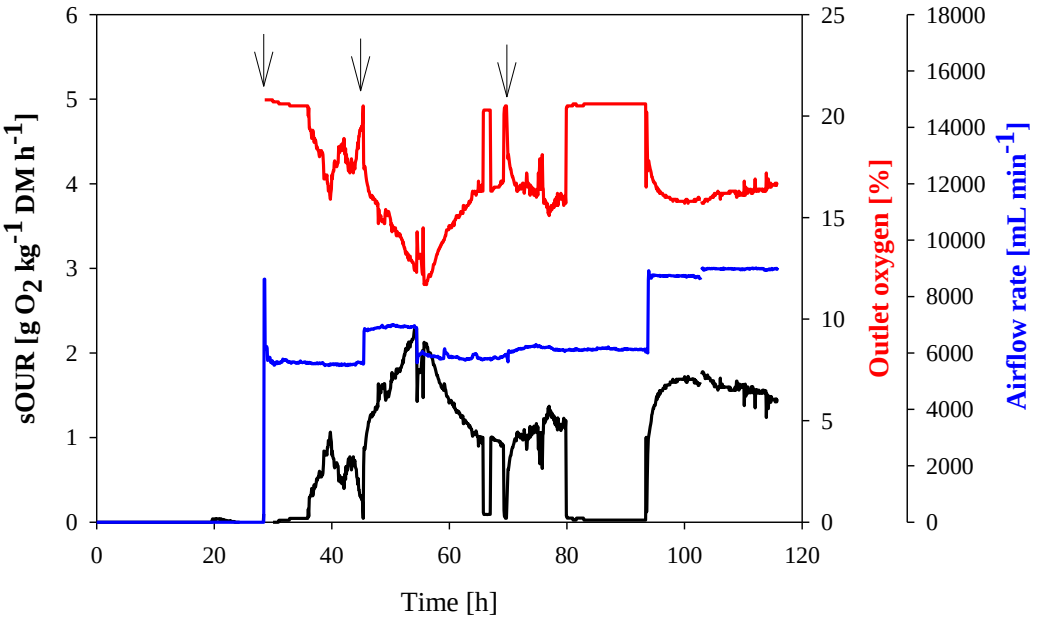


Figure 65: Monitored parameters evolution throughout SSF 3. Arrows indicate the samplings in the course of the process.

This batch was carried out during a very cold week, with temperatures between 5 and 20ºC in the pilot plant location, and it had an important impact on process temperatures (Figure 66). In the first stage, temperatures did not overcome 20ºC, affecting negatively the starting of the biological process, and impacting on the rate of oxygen consumption. Higher temperatures were recorded in the second stage in some of the monitored positions of the bottom and middle parts of the packed bed, reaching punctual maximum values of 40.5ºC.

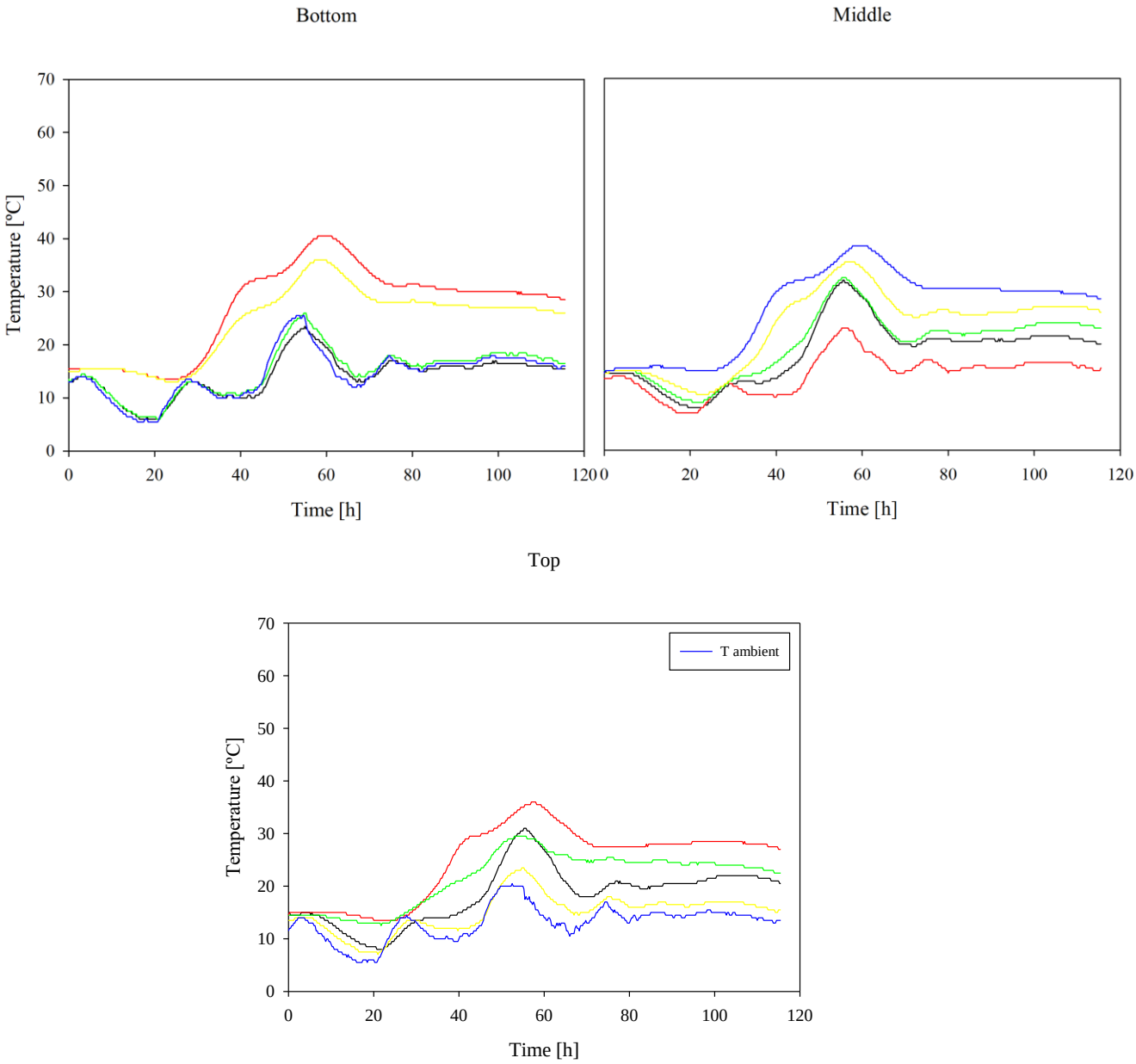


Figure 66: Temperature evolution at different positions of the bottom, middle, and top part of the packed bed in SSF 3.

Bt growth and sporulation were not improved with these conditions. A 4.2-fold increase in viable cell concentration was monitored on the top part of the packed bed at 24 hours (Figure 67A). The viable cell concentration started to decrease until being undetectable due to the abundance of other microorganisms. The maximum spore concentration ($2.7x10^6$ spores g^{-1} DM) was achieved in this batch at the upper part of the

reactor (Figure 67B). However, an important spore concentration gradient along the axial axis was also noticed at that time, from $1.5x10^5$ spores g^{-1} DM at the bottom to $2.7x10^6$ spores g^{-1} DM at the upper part. The final average spore yield was 0.1 spores per inoculated CFU, which was not higher than the productivity obtained in SSF 1.

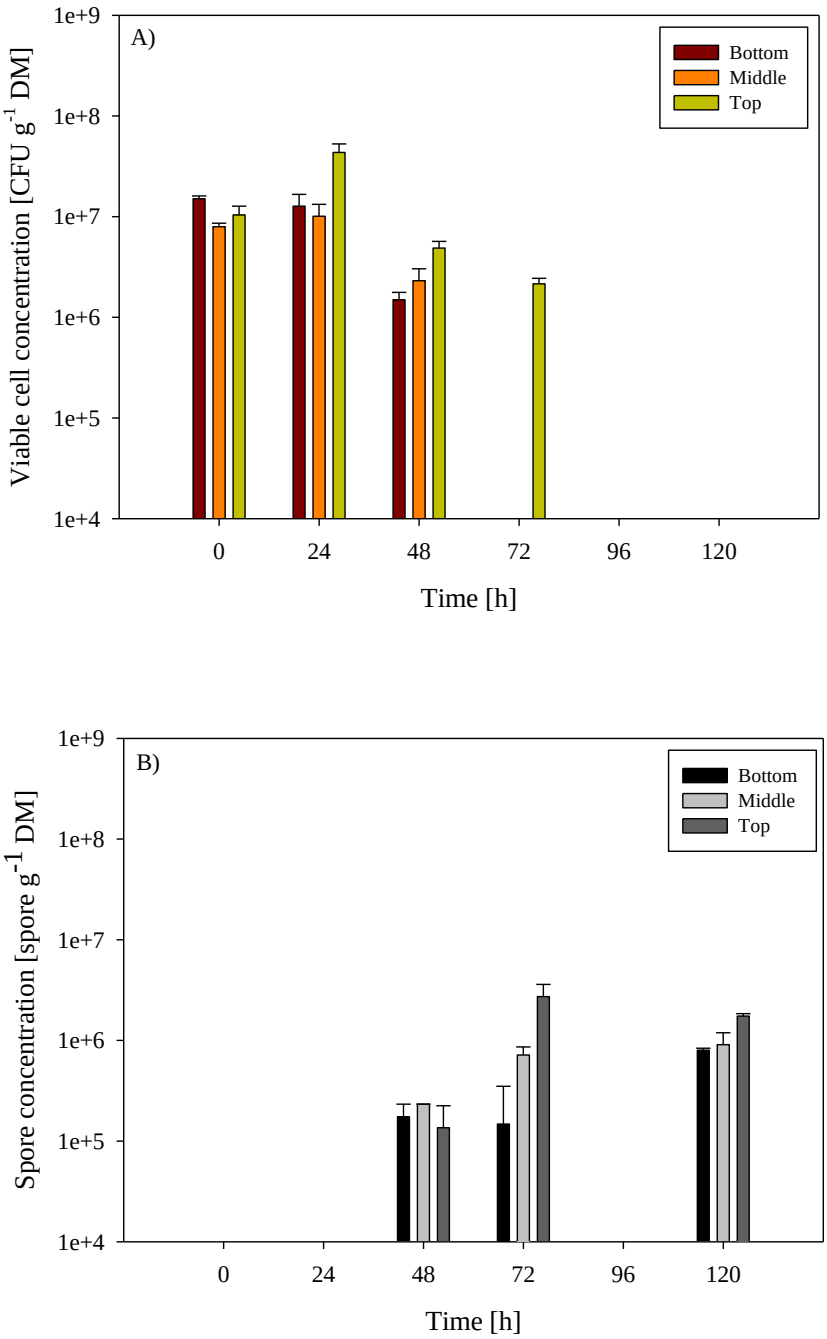


Figure 67: Viable cell (A) and spore (B) concentration in the three positions sampled in SSF 3.

The main hypothesis to explain the low spore production was related to low ambient temperatures. A 35 hours delay on the start up of the process was observed because the solid did not reach temperatures over 20ºC. The temperature raised once the aeration started. Thus, a new strategy addressed to increase the packed bed temperature but applying a very limited airflow rate to boost biological reactions was proposed. Therefore, the reactor was covered with foam during the first stage (Figure 68), and a rotameter (1-10 L min^{-1}) was installed to achieve the desired airflow rate conditions.

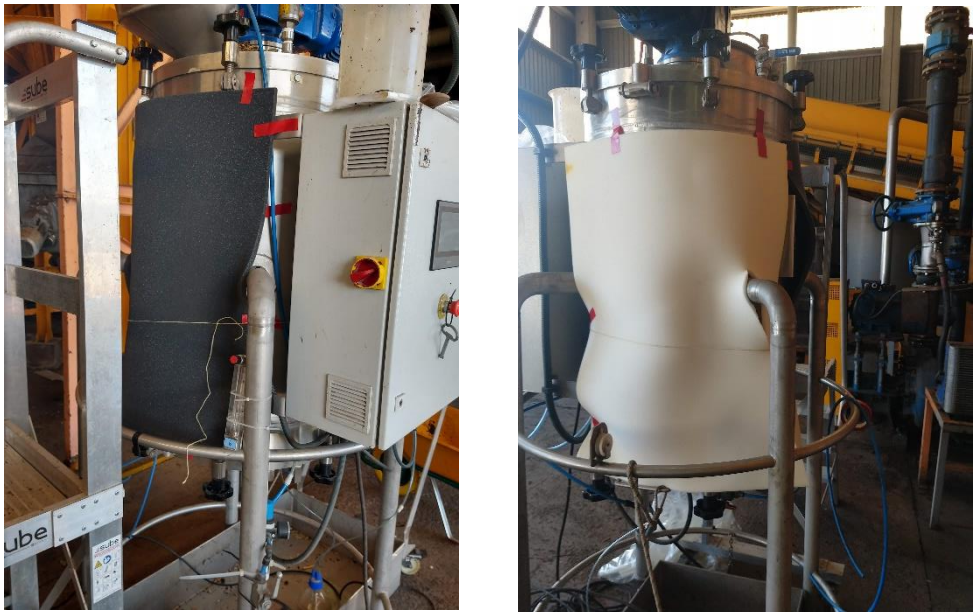


Figure 68: Reactor insulated with foam.

The results of the SSF4 performance are shown in Figures 69, 70, and 71. The initial airflow rate was set at 2000 mL min^{-1} (0.04 mL min^{-1} g^{-1}), just to provide the minimum oxygen to stimulate biological reactions, and consequently, the increase of solid' temperature. Once the aeration was increased, a minimum oxygen concentration of 5.2% was monitored. Hence, this new operational condition worked to achieve the desired oxygen concentration stablished in the operational protocol developed in this work. After

the airflow rate increase, the oxygen levels slightly recovered to full aerobic conditions, as expected.

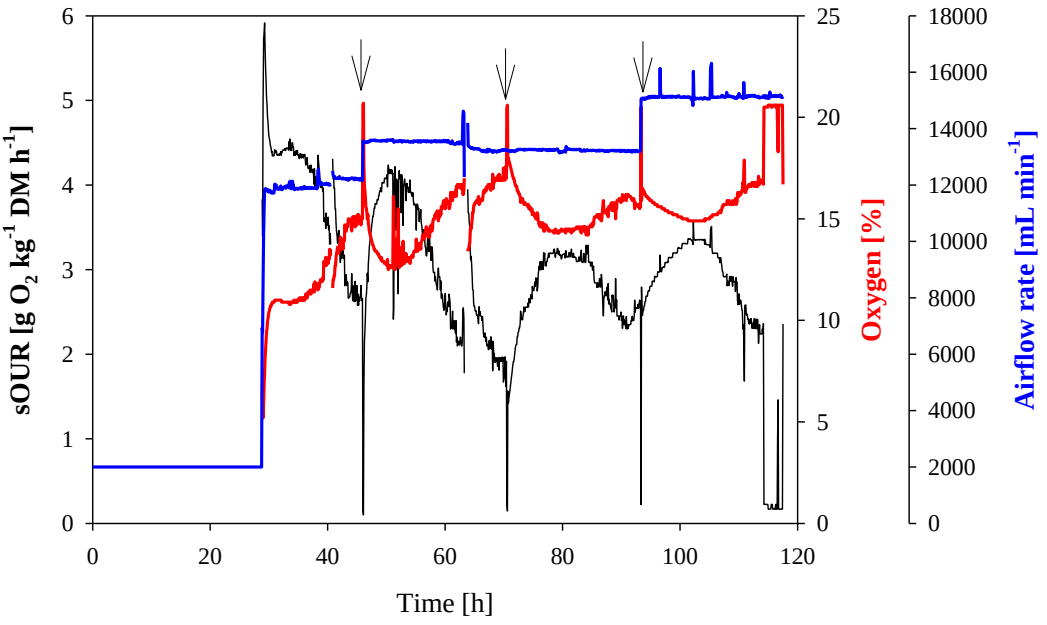


Figure 69: Monitored parameters evolution throughout SSF 4. Arrows indicate the samplings in the course of the process.

The use of foam to insulate the reactor together with the airflow rate applied shortened the delay on temperature increased observed in SSF 3. In SSF 4, the period to reach temperatures higher than 20ºC took 7 hours (Figure 70). Temperatures ranged from 9 to 48ºC, but were mostly maintained at the mesophilic range.

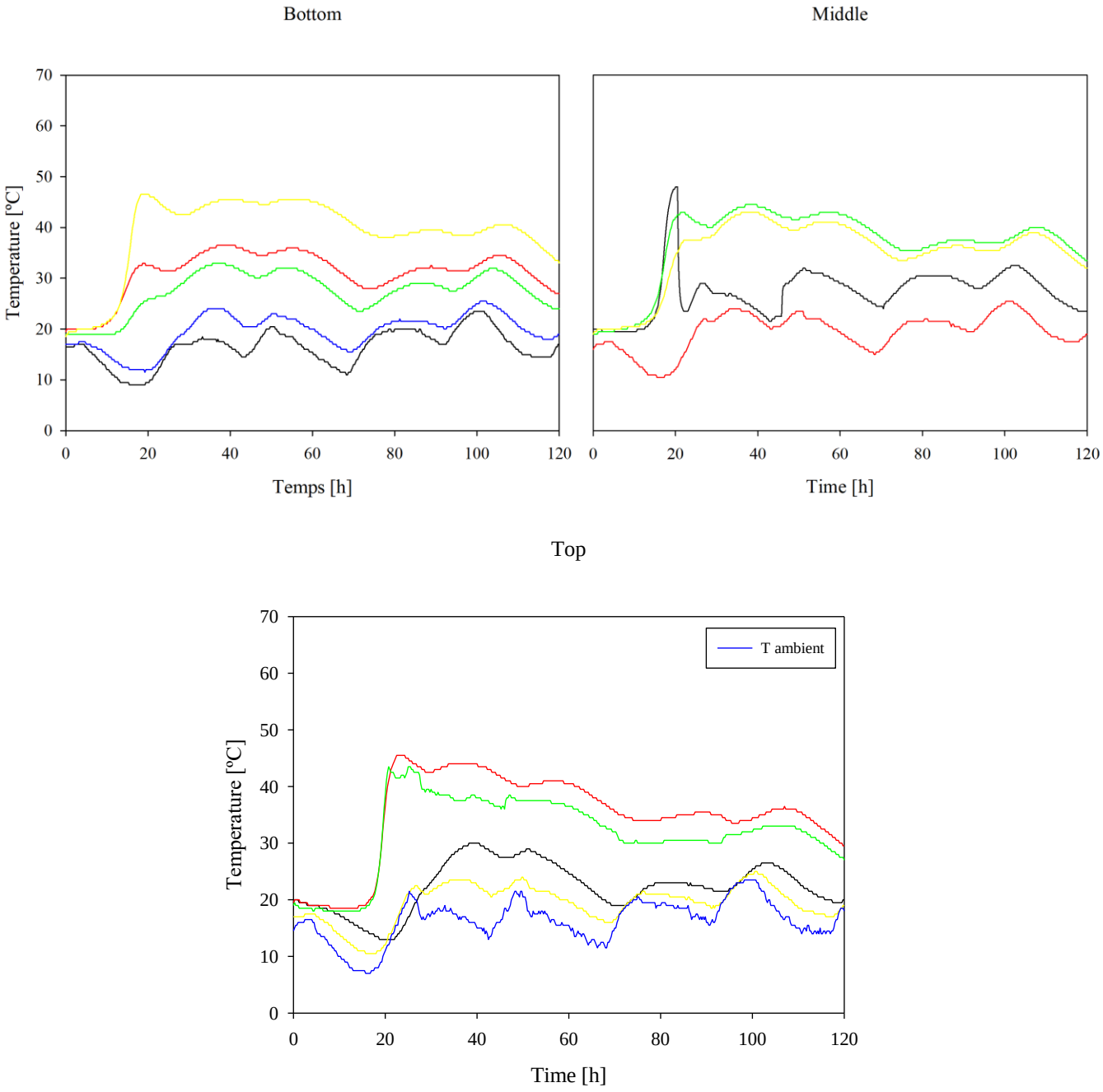
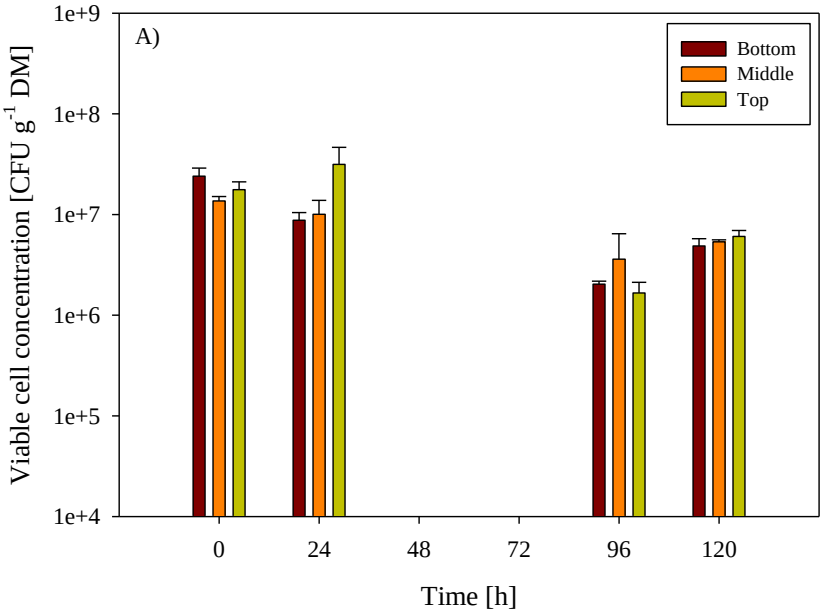


Figure 70: Temperature evolution at the top, middle, and bottom of the packed bed in SSF 4.

Although temperatures achieved were adequate for Bt growth, a slightly increase of viable cell concentration was only noticed at the upper parts of the reactor, from $1.8x10^7$ to $3.2x10^7$ CFU g^{-1} DM. Nonetheless, it decreased to an average value of $5.4x10^6$

CFU g^{-1} DM, which was the higher viable cell concentration at 120 hours of all performed batches. However, a difference in the sporulation rate was observed. The total sporulation of the remaining viable cells was monitored, with a final average spore concentration of $6.5x10^6$ spores g^{-1} DM at 120 hours, a production yield of 0.35 produced spores per inoculated CFU.

Thus, even though the total sporulation was achieved, the lack of a significant Bt growth in the first 24 hours caused a low spore concentration.



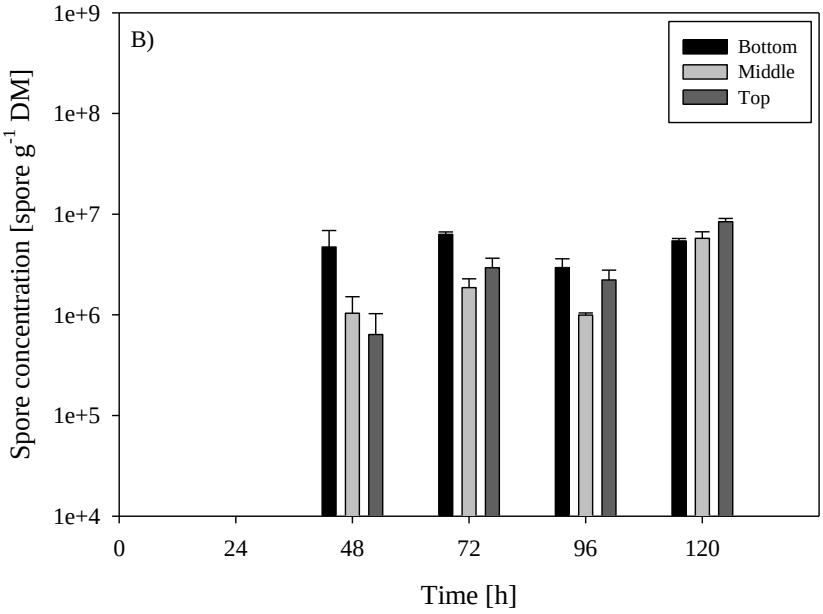


Figure 71: Viable cell (A) and spore (B) concentration at the bottom, middle, and top parts of the packed bed in SSF 4.

Due to the good performance of Bti when working at a 22-L reactor, a new batch was proposed and developed at the pilot plant using this strain, expecting higher spore yields (SSF 5, Figures 72 and 73). In this fermentation, the aeration was not provided during the first stage, and the fermentation performed as expected in terms of oxygen profile, reaching a minimum oxygen concentration of 7.1% at 25 hours, once the airflow rate was turned on.

The temperature of the anoxic phase was maintained between 20 and 30ºC (Figure 72), which was a positive point since the batch was performed in July when higher ambient temperatures can lead to higher packed bed temperatures. However, the increase in temperature occurred during the aerated phase. The temperature ranged from 27 to 55ºC, with a maximum temperature gradient of 22.5ºC at 40 hours of the process.

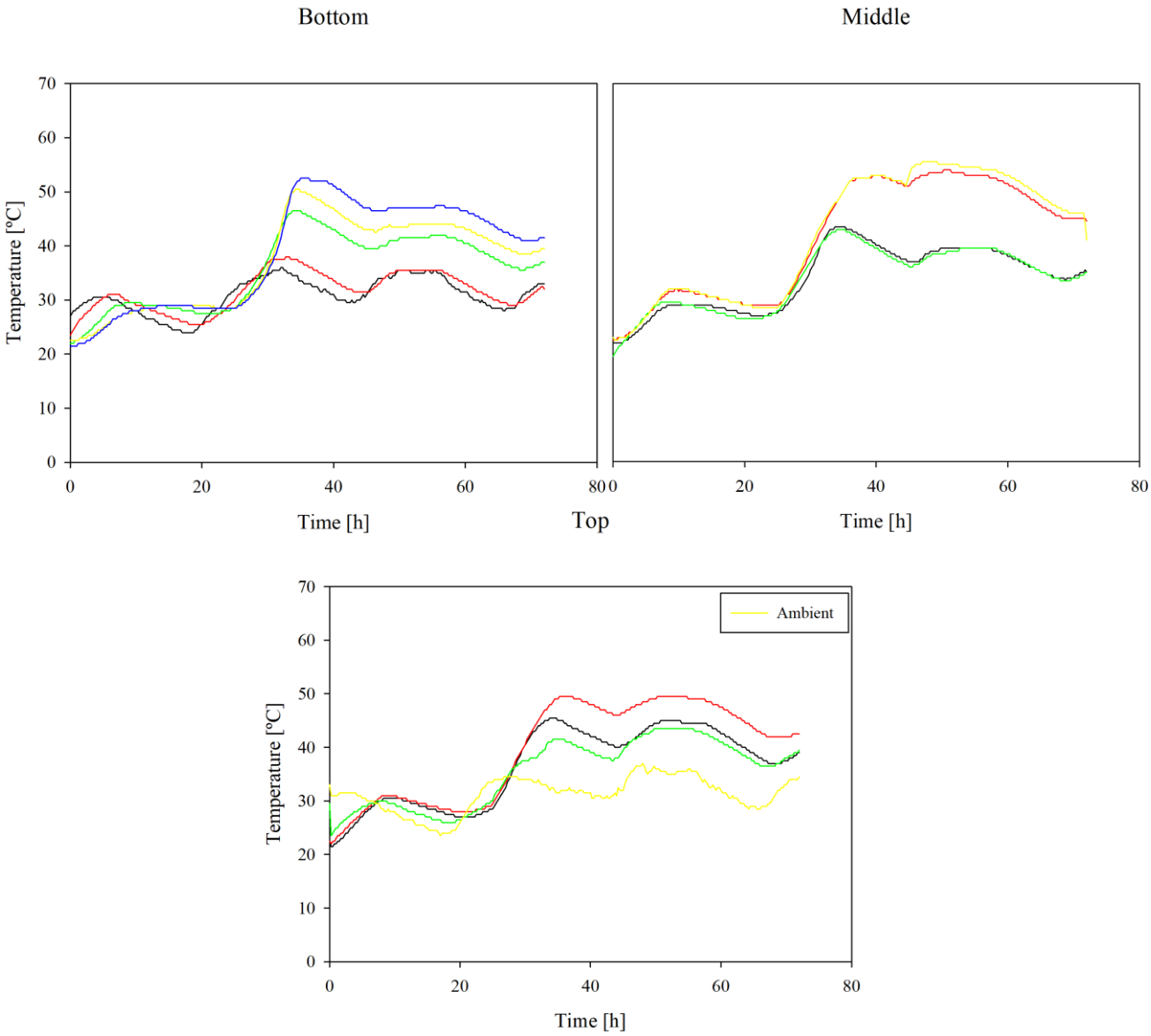


Figure 72: Temperature profile in different points of the packed bed reactor in SSF 5.

Even though mesophilic temperatures were maintained during the Bt growth phase, the viable cell and spore count did not achieve the expected values (Figure 73). The viable cell concentration was slightly higher at 24 hours (from 2.1 to 2.7x10^7 CFU g^{-1} DM, as an average value). However, the abundance of other microorganisms in the analysed samples hindered its quantification.

Concerning the spore concentration, maximum values of 2.9 and 2.4x10^5 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 between the three positions tested. This low final spore concentration affected the final spore yield, achieving an average yield of 0.05 produced spores per inoculated CFU.

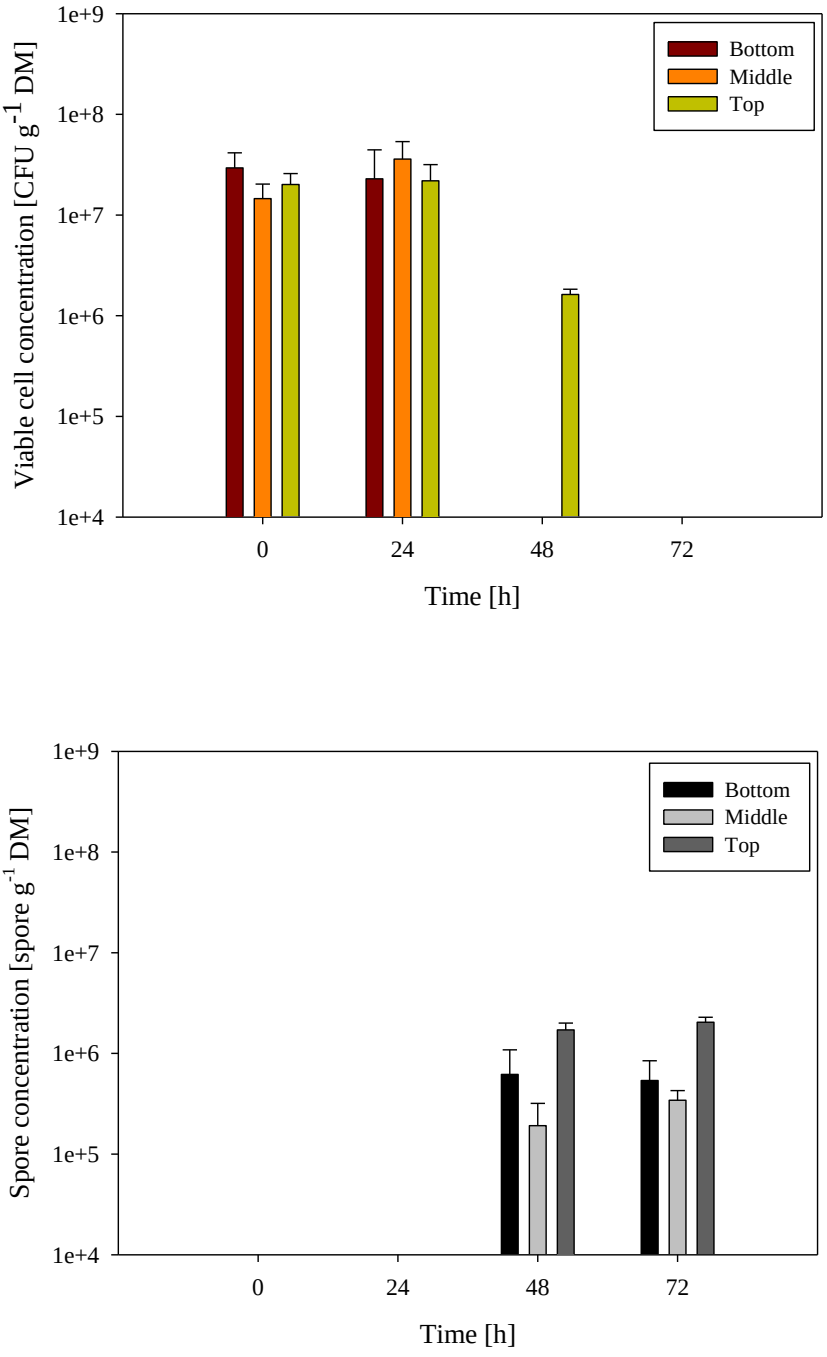


Figure 73: Viable cell and spore concentration monitored through SSF 5 at the bottom, middle and top parts of the packed bed.

This batch (SSF 5) was reproduced at a smaller scale (1.6-L) as a fermentation control. Part of the inoculated solid (500 g) was placed in the Setup 2 and the SSF was performed at laboratory scale. The main difference, apart from the reactor scale, was the ambient and packed bed temperature (Figure 74). While the ambient temperature at the pilot plant ranged from 22 to 37ºC, and thermophilic temperatures were reached during the sporulation phase, the packed bed temperature of the control experiment was maintained at mesophilic values all over the process, ranging from 24 to 32ºC. Over this condition, a spore concentration of $4.6x10^7$ spores g^{-1} DM was obtained, representing a sporulation ratio of 84% and a spore yield of 2.1 produced spores per inoculated CFU. Thus, a spore concentration 47-fold higher was obtained at 1.6-L than in the pilot reactor. Therefore, these results rule out the presence of any inhibitor that limits Bt growth.

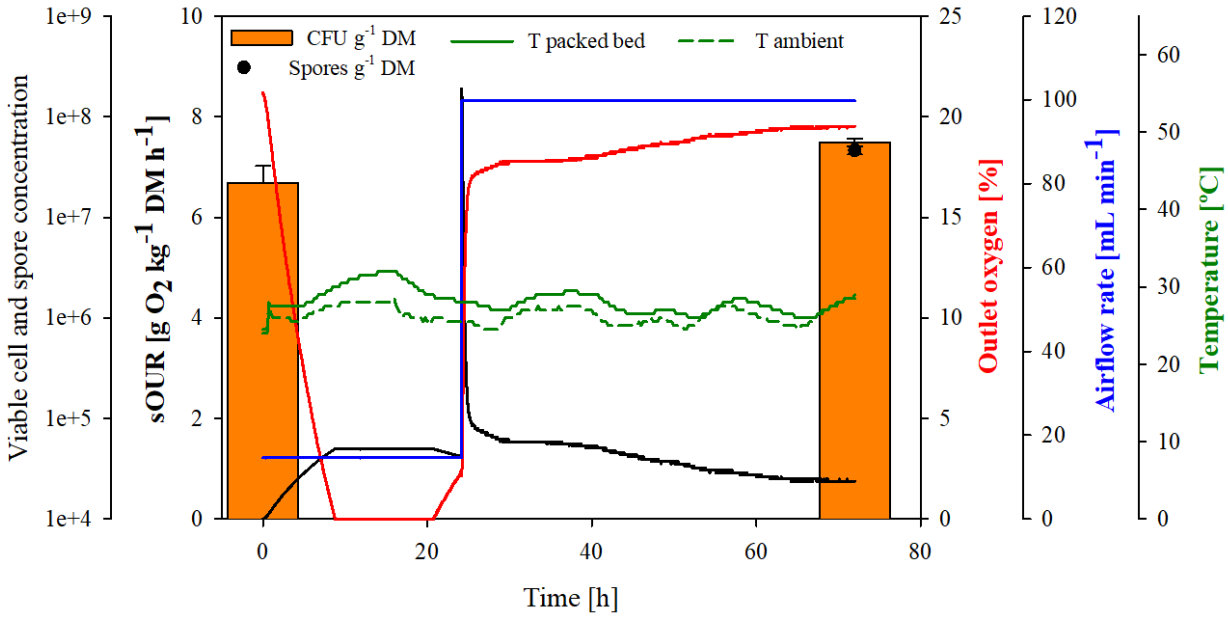


Figure 74: SSF 5 reproduction at 1.6-L. Evolution of sOUR. outlet oxygen, airflow rate, temperature and viable cell and spore concentration.

A last batch was performed (SSF 6), at the pilot plant, using again Bti as inoculum, but trying to promote and extend the anoxic phase. A low airflow rate (0.03 mL min^{-1} g^{-1}) was applied over the first 5 hours in order to activate the biological activity of the substrate (Figure 75). The air supply was cut afterward, and the anoxic phase was extended up to 44 hours of the process start. Once the aeration was turned on, an oxygen value of 2.5% was monitored, showing the success of the proposed improvement.

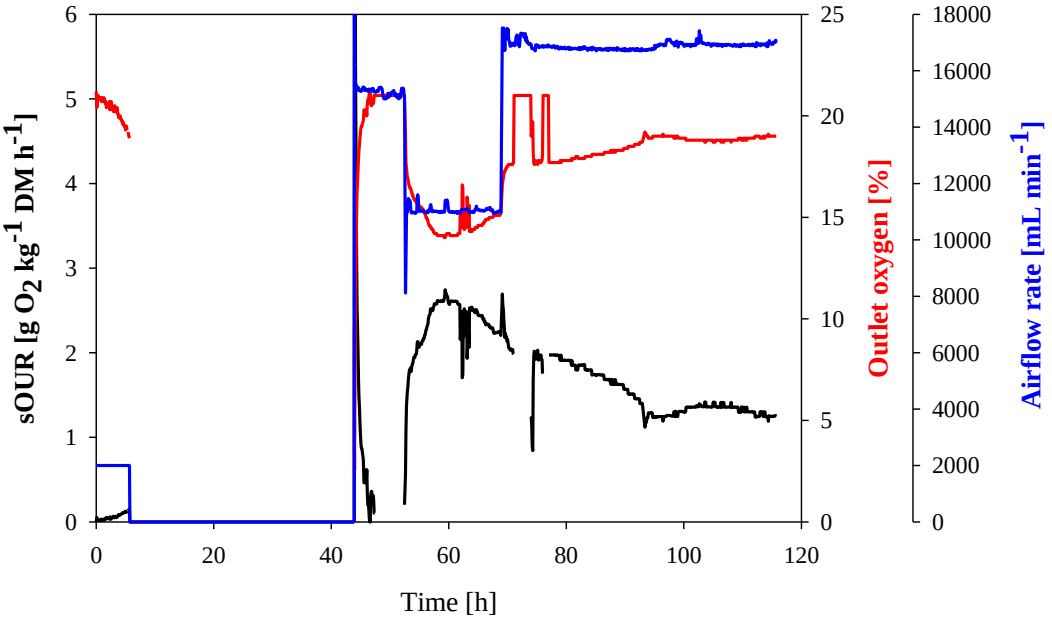


Figure 75: sOUR, outlet oxygen and airflow rate profile obtained from SSF 6.

In terms of temperature evolution, the extension of the non-aerated stage also caused a delay on the temperature increase, which was observed when a total aerobic environment was achieved (Figure 76). Temperature was kept between 20 and 30ºC over the first 44 hours, which was a suitable temperature condition for Bt growth. Maximum punctual temperatures of 46ºC were monitored at the bottom part of the packed bed once the second stage started. Even though ambient temperatures ranged from 20 to 30ºC, no thermophilic conditions were achieved over the second stage. Thus, a better reproduction of laboratory conditions in terms of temperature were achieved in SSF 6.

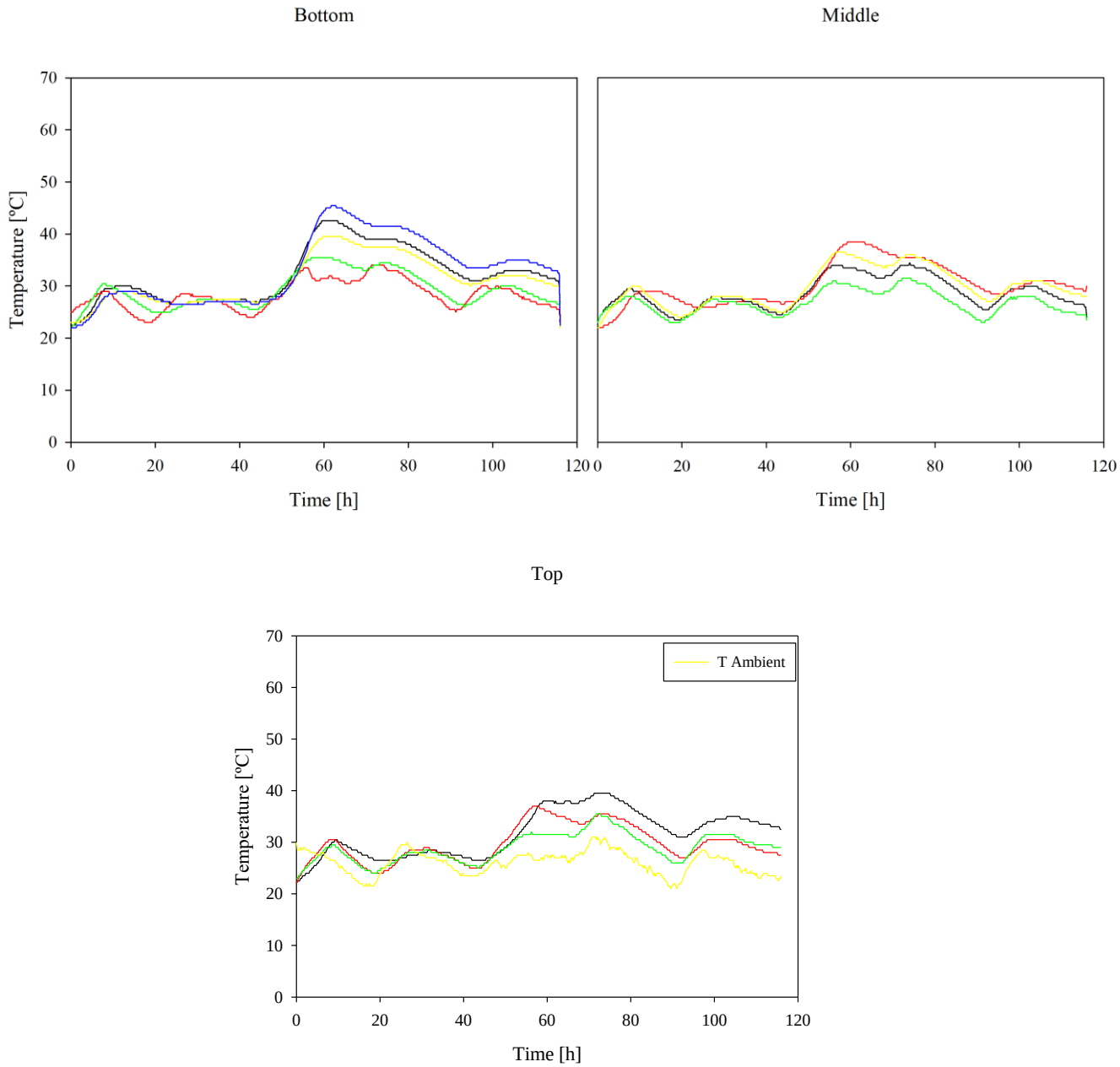


Figure 76: Temperature monitored at different positions of the bottom, middle, and top part of the packed bed in SSF 6.

The oxygen and temperature conditions achieved in the first stage promoted the Bti growth, similar to laboratory results (6.9, 8.6, and 2.4-fold increase at 22 hours on the bottom, middle, and top parts, respectively) (Figure 77A). However, this value was slightly reduced at 28 hours (from $1.2x10^8$ to $5.2x10^7$ CFU g^{-1} DM), and significantly reduced at 43h (5.9 and $2.2x10^6$ CFU g^{-1} DM). Besides, Bti was not able to be identified

on the following samplings because of the presence of others microorganism. One hypothesis for explaining this reduction in CFU concentration during the anoxic phase was the entrance of atmospheric air when samplings were performed at 22 and 28 hours. Even though Bt is a anaerobe facultative bacteria, meaning that it is an aerobe bacteria with surviving capacity in anaerobe conditions for a certain period of time, this work has demonstrated that in the DECISIVE working conditions (digestate and biowaste not sterilized but hygienized) the anoxic environment for boosting Bt growth is the fundamental advantage versus other autochthonous microorganisms. An alteration of this conditions may have undesired effects on the targeted bacteria growth. This alteration of the anoxic environment could also happen in other experiments, but its effect was not noticed at a smaller scale.

The loose of Bt vegetative cells before the sporulation phase caused again a reduction of the spore potential production (Figure 77B). Moreover, the sporulation was delayed since spores were first monitored at the 70 hours sampling. This phenomenon was in accordance with Karim et al. (1993), who observed a slowdown of spores' formation rate when working at a dissolved oxygen setpoint of 5%. However, the spore production yield was 0.05 produced spores per initial CFU at 116 hours.

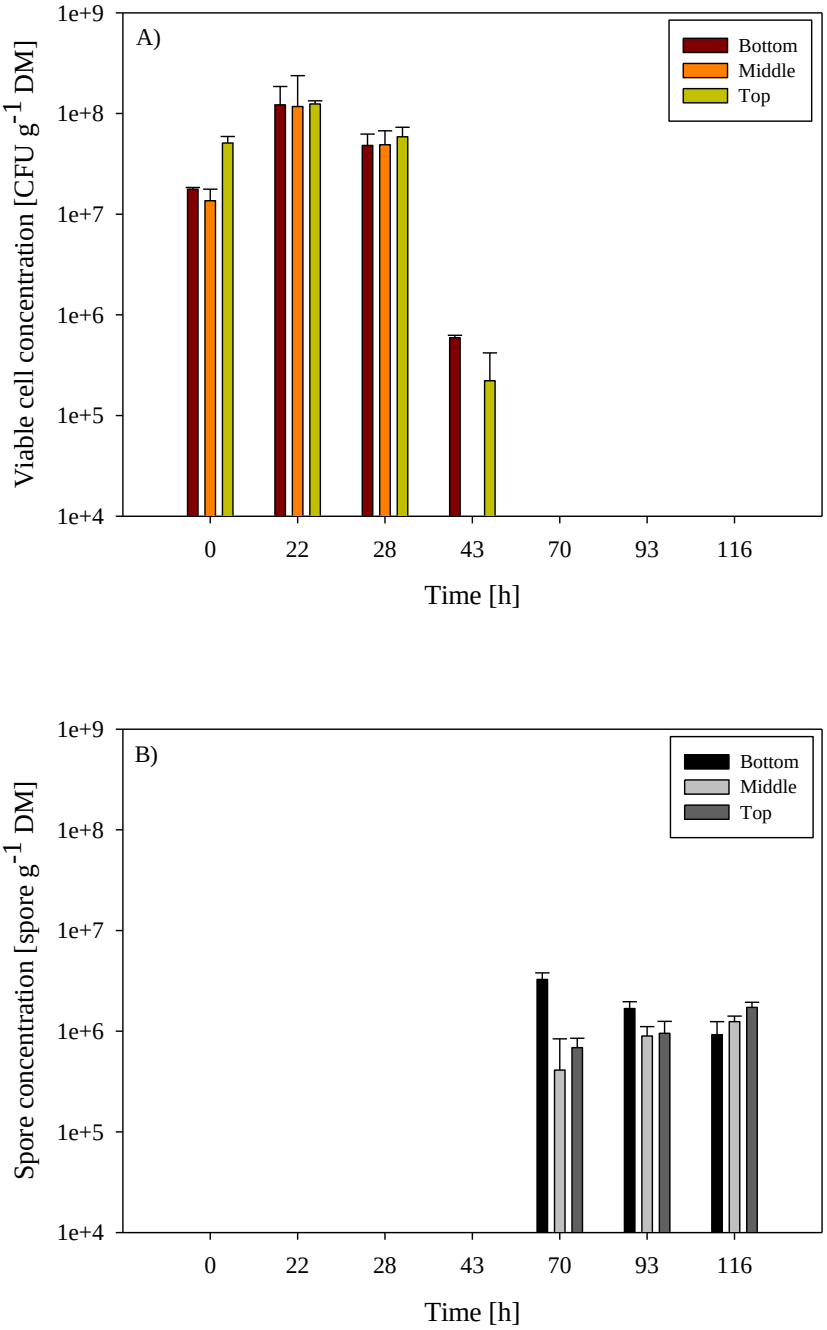


Figure 77: Viable cell and spore concentration monitored at the samplings performed in SSF 6.

A control fermentation was carried out at the laboratory in the Setup 2 (Figure 78). The same trend was observed compared to SSF 6. An increase of one order of magnitude of viable cell concentration (from $3x10^7$ to $4x10^8$ CFU g^{-1} DM) was achieved

at 22 hours, but this value decreased until values between $6x10^6$ and $1x10^7$ CFU g^{-1} DM, confirming the previous hypothesis.

A 71% of sporulation ratio was obtained at the end of the process, with a spore concentration of $4.7x10^6$ spores g^{-1} DM. Final spore concentration was slightly higher compared to the pilot plant batch, but the spore yield was the lowest obtained at this scale (0.16 produced spores per inoculated CFU). Thus, the extension of the non-aerated stage, the entrance of the atmospheric air, or a combination of those two factors could alter the spore production.

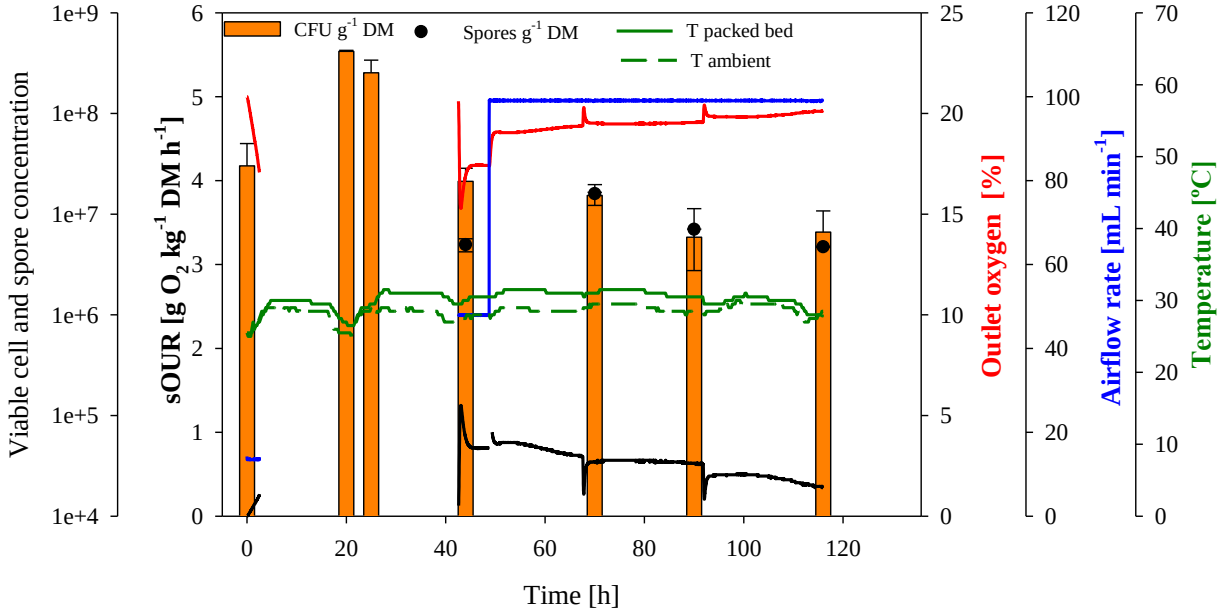


Figure 78: SSF6 reproduction at 1.6-L. Evolution of sOUR. outlet oxygen, airflow rate, temperature and viable cell and spore concentration.

A summary of the production results obtained at the pilot plant is shown in Table 22.

Table 22: Summary of the CFU and spore production in the pilot plant tests.

Run	Biowaste [%]	Aeration condition in 1st stage	Insulation in 1st stage	Bt strain	Initial CFU concentration [CFU g^{-1} DM]	Final CFU concentration [CFU g^{-1} DM]	Final spore concentration [spore g^{-1} DM]	Final spore yield [Final spore concentration per inoculated CFU]
SSF 1	37.5	Micro-aeration	No	Btk	$1.3x10^7 \pm 7.3x10^6$	n.a.	$1.2x10^6 \pm 1.9x10^5$	0.1
SSF 2	10	No aeration	No	Btk	$1.4x10^7 \pm 3.4x10^6$	$6.2x10^5 \pm 3.8x10^5$	$8.5x10^5 \pm 3.5x10^5$	0.06
SSF 3	37.5	No aeration	No	Btk	$1.1x10^7 \pm 3.6x10^6$	n.a.	$1.1x10^6 \pm 5.2x10^5$	0.1
SSF 4	37.5	Micro-aeration	Yes	Btk	$1.8x10^7 \pm 5.2x10^6$	$5.4x10^6 \pm 5.9x10^5$	$6.5x10^6 \pm 1.6x10^6$	0.35
SSF 5	37.5	No aeration	No	Bti	$2.1x10^7 \pm 7.5x10^6$	n.a.	$9.7x10^5 \pm 9.3x10^5$	0.05
SSF 6	37.5	Micro-aeration (first 5 hours)	No	Bti	$2.7x10^7 \pm 2x10^7$	n.a.	$1.3x10^6 \pm 4x10^5$	0.05

At this scale, the relevance of a strong Bt growth over the first 24 hours of the process was remarked, to increase the spore formation potential. Despite the process performed as expected in terms of aeration steps and oxygen levels, a poor spore production was obtained in all batches. As observed, a poor or null growth of vegetative cells leaded to a spore concentration two orders of magnitude lower than the results at lab and bench scale.

Limited bibliography reports the production of Bt derived biopesticides at a representative industrial scale. In Ballardo et al. (2020) similar spore concentration was reported, working with home composters of 400L of capacity. A solid Bt-enriched inoculum was used to inoculate a mixture of fruit and vegetables leftovers and pruning waste. The concentration of the spore concentration was progressively decreased until the end of the process, from $5.9x10^7$ (t = 0 days) to $7.8x10^5$ spores g^{-1} DM (t = 40 days).

The reduction of the final spore concentration was also observed by Zhang et al. (2013). A loss of 4 orders of magnitude (from 10^{10} to 10^6 spores g^{-1} DM) was monitored when increasing the fermentation media from 4 to 40 kg. The main attributed reason to explain this low production was the metabolic heat and CO_2 retention on the packed bed with increasing the medium mass, inhibiting bacterial growth.

9.3.2. Quality of the fermented material

The stability and maturity of the fermented material was analysed using respirometric indices (DRI and AT_4) and germination indices. The germination test was used to analyse the phytotoxicity of the solid. A DRI and AT_4 values of 0.4 ± 0.1 and 30 ± 7 were obtained, respectively. Furthermore, spore concentration was maintained constant along the respirometry test ($1.4x10^6$ ± $3.6x10^5$ 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 as mature material.

Eight samples from different points of the biowaste treatment plant and the SSF process were analysed to identify and quantify MPs. Polyethylene, polystyrene, polyethyleneterephthalate (PET), and polypropylene were identified (Figure 79 and Table 22). Polyethylene was the most abundant polymer in all samples (Figure 79). The principal hypothesis of polyethylene input in these samples is the presence of plastic bags in the centralized biowaste collection scheme. Those plastics 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 analysed were taken from a biowaste pile without plastic bags, and with no presence of plastics, visually. This suggest that this MP 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 MP 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 usually used in agriculture. However, MP 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).

Table 23 shows the polymers mass results. It is important to remark that, even though a representative amount of dry substrate was taken, only a few mg were analysed through TED-MS-GC, 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.

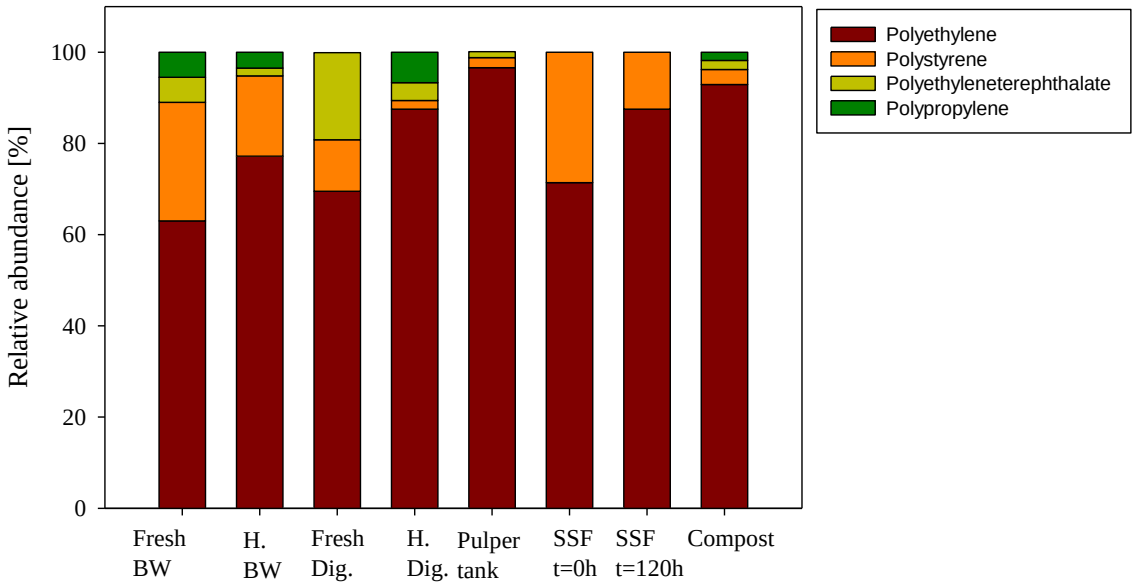


Figure 79: Relative abundance of MPs detected. BW stands for biowaste, H for hygienized, and Dig. for digestate.

Moreover, MPs concentrated in the anaerobic digestion reactor, and as consequence, in the solid part of the digestate, since 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, 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 (Urra et al., 2019).

Table 23: TED-GC-MS analysis results for polyethylen, polystyrene, polyethyleneterephthalate, and polypropylene. N.a. stands for not available.

Polymere	Fresh biowaste	Fresh digestate	Hygienized biowaste	Hygienized digestate	Pulper tank	Initial SSF sample	Final SSF sample	Compost
Dry sample amount (g)	27	80	23	90	30	45	125	150
Polyethylene (mg)	8	3428.3	50.9	392.0	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	30.0	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

9.4. Conclusions

The application of the operational protocol to run the SSF at the DECISIVE pilot plant was done. Different modifications were tested to replicate the conditions of the protocol, using the two strains of Bt (Btk and Bti). The two aeration steps allowed to mostly maintain the temperature at mesophilic ranges during the growth phase. Nonetheless, a poor Bt growth was monitored in most of the scenarios, drifting to poor spore production. An increase of the viable cell count was observed when the biological activity of the solid matrix was activated by applying a very low airflow rate for a short time followed by a cut in the air supply. However, the extension of the anoxic phase or the entrance of atmospheric air caused a significant decrease in viable cell count, losing the spore production potential. This fact highlights the importance of achieving a competitive conditions for boosting Bt growth and maximize the spore potential production. Future work should be related to improve the last performance of the pilot (SSF6) and achieve the maximum sporulation possible.

9.5. References

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CHAPTER 10: GENERAL

CONCLUSIONS AND FUTURE WORK

General conclusions

In this work, the production of Bt-derived biopesticides from digestate by using SSF technology was studied, within the framework of the DECISIVE project.

- The SSF process was optimized at a laboratory scale, by identifying the parameters with more relevance on the Bt spore production. Among all the parameters chose to study, the influence of temperature and the use of biowaste as co-substrate were remarked.
- The effect of co-substrate and temperature was studied at a higher scale (1.6-L), achieving maximum spore productions when a mixture of 62.5% of digestate and 37.5% of biowaste (wet weight basis) was used as substrate. The influence and effects of the oxygen concentration over the initial hours of the process were assessed, observing an improvement on Bt growth and spore production when a limited-oxygen environment was achieved in the packed bed. These results were used to elaborate an operational protocol with two differentiated stages in terms of air supply.
- The validation of the operational protocol developed was performed at a 22-L reactor. Successful results were obtained using both Bt available strains (Btk and Bti), identifying the presence of Bt crystals through SEM images. The operation in sequential batch and fed-batch mode was also assessed, but a significant reduction in final spore concentration was observed, compared to batch.
- A 290-L pilot reactor was built, according to DECISIVE demonstration site requirements. The developed protocol was tested, and some modifications were done to achieve an adequate competitive environment for boosting Bt growth. The fermented material was analyzed in terms of Bt spore concentration, solid

maturity (germination index and respirometry indices), and MPs identification and quantification.

Future work

As the first attempt to produce Bt-derived biopesticides in a demonstration scale in our group, much work is still to be performed.

- The duration of the two aeration stages should be optimized at the demonstration scale, without environments interruption for sampling. This could promote the improvement in Bt growth, and in consequence, increase the spore potential production.
- A significant effort is needed in terms of achieving a semi-continuous operation. It should be important to avoid the need of producing liquid inoculum before each batch, leading to a more self-sufficient process. If strategies such as sequential batch operation or fed-batch are not feasible, the option of a spore germination step with nutrient broth could be assessed.
- Lastly, it would be important to study the application of the produced biopesticide in fields. Toxicity and crystals duration tests would be useful to certificate the produced biopesticide as suitable for its use in fields.