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Research article

Bench-scale solid-state fermentation of digested sewage sludge to produce *Bacillus thuringiensis*: influence of digestate characteristics

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ABSTRACT

The solid fraction of wastewater digested sludge is a material rich in nutrients and organic matter that is typically applied to the soil, composted or disposed of. To further exploit its potential, it can be used as a secondary raw material for the production of marketable products, such as biopesticides, through solid-state fermentation (SSF). In this study, a thorough characterisation in terms of pyrolysis gas chromatography-mass spectrometry analysis, physicochemical methods and biodegradability analysis was carried out on four different digested sewage sludges to understand their inherent properties. The effect of these properties on the growth and sporulation of *Bacillus thuringiensis* during a laboratory-scale SSF process was then evaluated. Sporulation yields ranged from a maximum of 11.3 to a minimum of 0.4 (expressed as spores per initial viable cell), highlighting the influence of digestate variability on the fermentation performance. Finally, the SSF process was scaled up to bench-scale using the two solid digestates with the highest *Bacillus thuringiensis* viable cell and spore production to assess the robustness and reproducibility of the process. By scaling up, both sporulation yield and viable cell yield increased compared to the laboratory-scale trials with a maximum spore production of 2.3×10^8 spores g^{-1} DM. The results confirmed the feasibility of growing *Bacillus thuringiensis* by SSF in three of the four solid digestates studied, highlighting the importance of advanced techniques to obtain reliable characterisation of the digestate as a substrate for SSF and the production of valuable bioproducts. These results help to determine the most effective valorisation pathway for the solid digestate within the circular economy paradigm.

1. Introduction

Anaerobic digestion (AD) is widely used to treat and manage organic waste streams because it stabilises organic matter (OM) while producing biogas, a renewable energy source. AD is a process in which microorganisms degrade OM in the absence of oxygen, producing biogas and a nutrient- and lignocellulose-rich by-product known as digestate (Lema and Omil, 2001). Biogas production can make a significant contribution to achieving the EU's clean energy objectives, reducing dependence on fossil fuels and accelerating the transition to renewable energy. The rapid expansion of the biogas sector is leading to a corresponding increase in the amount of digestate to be managed. In 2022, 31 million tonnes of dry matter (DM) of digestate will be produced in Europe (European Biogas Association, 2023), which is expected to increase exponentially in the coming years.

Digestate contains nutrients and, if properly managed, can be used as an organic amendment or fertiliser to recycle these nutrients and protect the environment, reducing reliance on synthetic fertilisers. However, to obtain a safe fertilizer, it is necessary to sanitise the material to remove pathogens and hazardous biological agents that can cause nutrient loss through volatilisation or leaching (Chojnacka and Moustakas, 2024). Due to its high water content, digestate is usually mechanically separated into liquid and solid fractions to facilitate handling and transport (Carraro et al., 2024). The liquid fraction contains most of the nitrogen and potassium, while the solid fraction is rich in residual fibres and phosphorus (Monlau et al., 2015). The solid fraction of the digestate is used directly on the soil, composted or dried for further transport or management, but has the potential to be used as a secondary raw material to produce marketable products.

In this context, SSF is emerging as a promising technology to further stabilise the OM of solid digestate while producing bioproducts of interest. SSF is defined as the growth of microorganisms that occurs in a

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solid matrix in the absence or near absence of free water, where the solid substrate is considered as the carbon and nutrient source for the microorganisms (Sadh et al., 2018). In recent years, research interest in SSF has increased due to its ability to use organic residues as a substrate to produce high-value bioproducts, such as fungal and bacterial biopesticides (Ballardo et al., 2016; Mejias et al., 2020; Qiu et al., 2019; Zhang et al., 2013), flavourings (Martínez-Avila et al., 2021) and biosurfactants (Jiménez-Peñalver et al., 2016), among others (Sadh et al., 2018).

However, although many studies have focused on the agronomic value of digestate for soil application, the assessment of its composition and properties, beyond being a source of nutrients, has rarely been investigated. In this sense, pyrolysis coupled with gas chromatography-mass spectrometry (Py-GC-MS) analysis may be a promising tool to thoroughly characterise the solid digestate and accurately assess its value for SSF and other advanced uses. Py-GC-MS is usually used in environmental analysis to characterise OM, humic substances and contaminants (Picó and Barceló, 2020), but it can also be used to characterise digested sludge (Wang et al., 2020). Controlled thermal degradation (pyrolysis) breaks down macromolecules into small molecules, which can then be separated by GC and detected by MS. The major advantage of this method is that it can be applied directly to the organic material to be analysed without the need for prior extraction (Hernández et al., 2006).

Solid fraction of digestate from sewage sludge has been found to contain a significant proportion of residual biodegradable OM with high microbial activity (Maynaud et al., 2017). Therefore, the production of biopesticides via SSF requires the use of robust microorganisms that can thrive in non-sterile environments, such as *Bacillus thuringiensis* (Bt) (Ballardo et al., 2016; Zhang et al., 2013). Bt-based biopesticides, which account for nearly 90 % of the global biopesticide market, are widely used due to their selective toxicity to pest insects, especially from the orders Lepidoptera, Diptera and Coleoptera (Bravo et al., 2011; Jallouli et al., 2020). During the sporulation phase, Bt produces endotoxin proteins that form parasporal crystals that are lethal to insect larvae when ingested (Malovichko et al., 2019). Among Bt varieties, *Bacillus thuringiensis* var. *israelensis* has been identified as one of the most environmentally friendly agents for mosquito larval control, providing a sustainable alternative to chemical pesticides (Bravo et al., 2011; Duarte Neto et al., 2020).

The aim of this work is, on the one hand, to characterise the composition and properties of different solid digestates from full-scale AD of wastewater treatment sludge using Py-GC-MS. On the other hand, the behaviour of these characterised digestates as substrates of the SSF process for the production of Bt-derived biopesticides will be investigated at laboratory and bench-scale. To our knowledge, this is the first study to report this novel approach to the characterisation and use of digested sewage sludge, an organic waste whose production is expected to increase, requiring alternative and sustainable waste management strategies within the framework of a circular bioeconomy.

2. Materials and methods

2.1. Substrates and materials

The solid fraction of four digestates was obtained from the AD treatment of primary and secondary sewage sludge of four standard municipal wastewater treatment plants (WWTPs). In all cases the AD was carried out under mesophilic conditions (35–37 °C), and the solid-liquid separation was performed using centrifugation. The physico-chemical and biodegradability characterisation of the digestates was performed as collected from the WWTP. They were then stored at −20 °C for a maximum of three months. Before use, each digestate was sanitised at 70 °C for 1 h, as required by European Regulation No. 142/2011. Throughout the work, the substrates are designated as follows: SD1, SD2, SD3 and SD4.

Wood chips (Acalora, Palets Pla d'Urgell, Barcelona, Spain) were used as a bulking agent and were manually mixed with the solid digestate to ensure proper air distribution and oxygen availability, providing porosity to the solid matrix during fermentation. The wood chips were autoclaved before use.

2.2. Substrates characterisation

2.2.1. Biodegradability analysis

The biodegradability of the substrates was assessed using different respiration indices: dynamic respiration index, DRI_{24h} and DRI_{1h}, which are the average oxygen uptake rate measured during the 24 h and 1 h of maximum activity (g O_2 kg^{-1} DM h^{-1}) respectively, and AT_4, being the cumulative oxygen consumption in four days after the lag phase (g O_2 kg^{-1} DM) (Ponsá et al., 2010). DRI_{24h} is a very sensitive measure of the maximum biodegradability level while AT_4 quantifies the overall biodegradable OM content of a given sample. The respirometric tests were conducted in triplicate for each digestate.

2.2.2. Py-GC-MS

The analyses were performed using a Pyroprobe 5000 coupled to an Agilent Technologies 5977 GC-MS instrument. The pyrolysis step was performed at 650 °C for 20 s, with a heating rate of 10 °C ms^{-1}. The oven program of the GC was from 80 °C to 325 °C at a rate of 30 °C min^{-1}, and a final hold time of 3 min. The MS operated in EI mode of 70 eV, scanning in the *m/z* 50–500 range. Compounds were identified based on previous studies with the same procedure aided by a NIST14 library (Kaal et al., 2023).

The detected 148 pyrolysates are specified in the Supplementary Material (Table S1). The relative abundance of the pyrolysis products was semi-quantified by normalizing the peak areas of each individual compound relative to the total area for all the peaks of the detected products and expressed as the percentage (%) of the total quantified peak area (TQPA). These percentages indicate the contribution of each compound to the total signal, allowing evaluating differences between samples.

2.3. Solid-state fermentation

2.3.1. Microorganism and inoculum preparation

Fermentations were carried out using *Bacillus thuringiensis* var *israelensis* strain CECT 5904, obtained from *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain). The strain was stored at −80 °C in cryovials containing cryo-pearls impregnated with Bt (DeltaLab, Barcelona, Spain) using a seed lot system. For the inoculum preparation, 100 mL of sterile Nutrient Broth N° 2 (Oxoid CM0067B, England) were added in a 500 mL Erlenmeyer and then inoculated with one Bt cryo-pearl. The culture media was incubated for 20 h, at 30 °C and 130 rpm, reaching a final optical density of 2.5–3.0. After the incubation, the inoculum was centrifuged at 4 °C and 3500 rpm for 10 min. The supernatant was separated by decantation and the pellet was resuspended with 3 mL of the supernatant (exhausted medium) and finally diluted 1:10 (v v^{-1}) also with the supernatant to reach approximately a concentration of 10^8 CFU mL^{-1} (Colony Forming Units) (Molina-Peñate et al., 2023). No spores were detected at this point.

2.3.2. Experimental set-up

SSF was carried out in laboratory and bench-scale packed bed reactors (Fig. 1). Both were completely sealed, but with an air inlet at the bottom and an air outlet at the top. A constant humidified air flow was provided and controlled by a mass airflow meter (Bronkhorst, The Netherlands) set at 0.2 mL min^{-1} g^{-1} total wet weight to ensure aerobic conditions (Mejias et al., 2017). Fermentations were carried out for 72 h, which has been established as the time for maximum sporulation of Bt (Cerda et al., 2019). The proportion of wood chips used was 30 % of the total weight (Mejias et al., 2020). For the laboratory-scale reactors, the

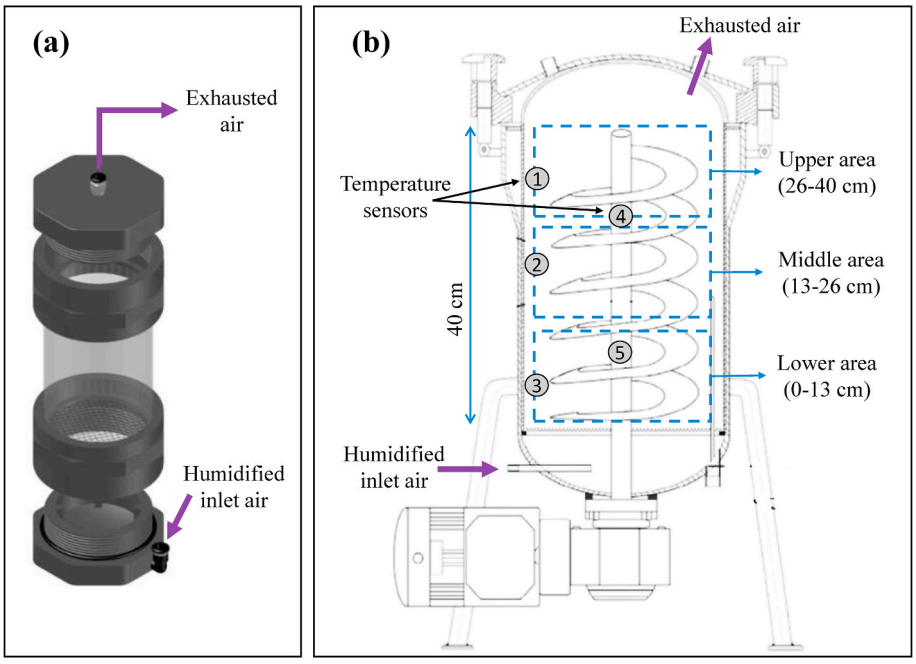

Fig. 1. Experimental set-up of the 0.5-L (a) and 22-L (b) packed-bed reactors.

size of the wood chips used ranged from 710 μm to 3.1 mm, whereas for the bench-scale reactors, the size of the wood chips used ranged from 710 μm to 7.1 mm.

Laboratory-scale SSF was carried out in 0.5 L cylindrical polyvinyl chloride reactors (Fig. 1a) with 100 g of solid substrate initially inoculated with 5 mL of Bt inoculum to reach an initial concentration of about 10^7 CFU per gram of DM (Mejias et al., 2020). At this scale, in order to perform an initial screening, the reactors were placed in a thermal bath to ensure a constant temperature (30 °C) throughout the fermentation. SSF was performed in triplicate with each solid digestate to evaluate the effect of each substrate on Bt growth and sporulation. Initial and final samples were analysed.

Two bench-scale SSF experiments were carried out using two of the substrates that showed the highest viable cell and spore production in 0.5L reactors: SD1 and SD2. Bench-scale SSF was performed in a 22-L stainless steel packed bed reactor with a 22-L removable inner basket and a fixed vertical helical ribbon mixer (Fig. 1b). The working volume was approximately 85 % of the reactor capacity, equivalent to 4.4 kg of solid substrate. The mixture was inoculated with 132 mL of Bt inoculum suspension to achieve an initial concentration of approximately 10^7 CFU g^{-1} DM. The experiments were monitored throughout the process and samples were taken from the top of the reactor every 24 h to follow the growth of Bt, while minimising disturbance to the solid bed. Finally, at the end of the fermentation, two additional points were sampled from the middle and lower zones of the reactor (Fig. 1b). A statistical analysis of variance (ANOVA) was performed to assess the final homogeneity throughout the packed bed, based on Tukey test statistical analysis. All experiments were carried out at room temperature and the temperature in the reactor was monitored using button sensors (Maxim Integrated, USA) placed at different points in the reactor to obtain an accurate time and position profile of the temperature (Fig. 1b).

Images of the experimental set-up, for both 0.5 and 22-L scales, are presented in Supplementary Material (Figure S1).

2.3.3. Process monitoring

The oxygen consumption was monitored by measuring the oxygen concentration of the exhaust air with an O_2-A_2 oxygen sensor (Alphasense, UK) connected to a custom-built data acquisition system (Arduino® based) as detailed elsewhere (Mejias et al., 2017). The specific oxygen uptake rate (sOUR) was calculated as an indicator of the biological activity according to eq. (1) (Ponsá et al., 2010).

$$sOUR = F \cdot \left(0.209\text{-}y_{O_2}\right) \cdot \frac{31.98 \cdot 60 \cdot 1000^{a}}{22.4 \cdot DM \cdot 1000^{b}} \quad \text{eq. 1}$$

where, sOUR is the specific oxygen uptake rate for a given time (g O_2 kg^{-1} DM h^{-1}); F, airflow rate into the reactor (mL min^{-1}); y_{O_2}, oxygen molar fraction in the exhaust air (mol O_2 mol^{-1}); 31.98, oxygen molecular weight (g mol^{-1}); 60, conversion factor (minutes h^{-1}); 1000^a, conversion factor (mg g^{-1}); 22.4, volume occupied by one mol of ideal gas under normal conditions (1 atm and 273 K) (L); DM, dry mass of sample loaded in the reactor (g); 1000^b, conversion factor (mL L^{-1}). The results are expressed as $sOUR_{1h}$, which is the average sOUR per hour and the COC, the total Cumulative Oxygen Consumption (g O_2 kg^{-1} DM) during the entire fermentation.

Specific Bt growth, monitored by determining the number of viable cells and spores, was performed as an indirect measure of biopesticide activity (De Lourdes Tirado Montiel et al., 2001). For viable cells, an extraction was performed with 10 g of solid sample mixed with 90 mL of Ringer's solution at 180 rpm for 20 min. For spores, 20 mL of the previous extraction was subjected to a thermal shock at 80 °C for 10 min and immediately immersed in ice (Mejias et al., 2020). The extracts were diluted as required, plated in triplicate and incubated at 30 °C for 20 h. The results for viable cells and spore concentration are expressed per gram of DM.

The sporulation ratio at a specific time is calculated considering that the viable cells count includes both spores and vegetative cells, as eq. (2) shows:

$$\text{sporulation ratio } (\%) = \frac{\text{spores } g^{-1}DM}{\text{viable cells } g^{-1}DM} \cdot 100 \quad \text{eq. 2}$$

The sporulation yield expresses the number of spores produced per initial viable cell inoculated, and the viable cells yield represents the increment of the viable cells relative to the initial viable cell count, as described in eq. (3):

$$\text{sporulation} \Big/ \text{viable cells yield} = \frac{\text{final spores}/\text{final viable cells}}{\text{initial viable cells}} \quad \text{eq. 3}$$

During sporulation, Bt produces spores containing parasporal crystal

inclusions composed of toxic proteins, which are responsible for the insecticidal effect (Bravo et al., 2011). While spore count and toxicity are not always strictly proportional, each spore is typically associated with a corresponding toxic crystal, making it a reasonable indicator for estimating biopesticidal potential (De Lourdes Tirado Montiel et al., 2001; Malovichko et al., 2019). Moreover, in the commercial production of Bt-based biopesticides, spores are the principal active ingredient and are used as the formulation basis.

Additionally, substrates and fermentation samples were physicochemically characterised in terms of DM, OM and pH following standard methods (Leege, 1998).

3. Results and discussion

3.1. Solid digestate general properties

The solid fraction of digested sewage sludge is a highly variable material whose specific characteristics depend directly on the AD feedstock, operating conditions and solid-liquid separation process. Therefore, a physicochemical and biodegradability characterisation was carried out to better understand how the inherent variability between digestates affects the SSF.

The results of this characterisation are presented in Table 1, together with other values reported in the literature. The DM content ranged from 19.6 to 26.2 % and the OM was around 60–69 % for the solid digestate samples, values in line with the literature. The pH was above 8 for all the digestates analysed.

The determination of the stability of organic wastes is a crucial aspect to fulfil the legal requirements necessary for their final use (European Commission, 2019). In this sense, respirometric tests are recognised as accurate methods for determining biological stability, as they provide a direct measure of microbial activity during the biodegradation of OM (Baffi et al., 2007; Teglia et al., 2010) and can be successfully applied to heterogeneous solid wastes (Barrena et al., 2009a). According to Ponsá et al. (2010), who presented a qualitative classification of stability levels, all four solid digestates can be defined as moderately biodegradable waste (2–5 g O_2 kg^{-1} DM h^{-1}). It should be noted that SD1 and SD4 have higher values of DRI_{24h} and AT_4, indicating a higher biodegradability and therefore a less stable material leading to higher microbial activity. Despite the anaerobic digestion process, the solid fraction of digested sludge still contains a significant amount of residual slowly biodegradable OM, as previously reported (Bayard et al., 2015; Teglia et al., 2011). Although the use of dynamic respirometric tests to assess the biodegradability of organic waste is gradually increasing, especially in composting processes (Barrena et al., 2009b; Cossu and Raga, 2008), there are few studies on the solid fraction of digestate from sewage sludge. For example, Maynaud et al. (2017) evaluated the biodegradability of seven solid fractions of digestate from different feedstocks and different AD operating conditions. They reported DRI_{24h} values ranging from 1 to 3 g O_2 per hour per kg volatile solids and AT_4 values from 100 to 250 g O_2 per kg volatile solids, demonstrating the variability of digestate biodegradability and highlighting the lack of agreement in the use of terms to describe waste biodegradability.

These results are valuable for understanding the nature of the digestates and show a slight but not remarkable difference between the substrates in terms of respirometric indices. Therefore, a more detailed characterisation of the solid digestates was carried out using Py-GC-MS.

Table 1
Physicochemical and biodegradability characterisation of the solid digestates used in the SSF and comparison with some literature values.

Substrate	DM (%)	OM (%)[a]	pH	DRI_{24h} (g O_2 kg^{-1} DM h^{-1})	AT_4 (g O_2 kg^{-1} DM)	reference
SD1	26.2 ± 0.1	60.3 ± 0.1	8.3	3.9 ± 0.3	206 ± 22	this study
SD2	21.0 ± 0.1	63.0 ± 0.1	8.1	3.5 ± 0.2	143 ± 21	
SD3	19.6 ± 0.1	64.8 ± 0.2	8.0	2.5 ± 0.2	136 ± 13	
SD4	25.3 ± 0.2	69.2 ± 0.1	8.2	4.9 ± 0.3	219 ± 12	
solid fraction of digested sewage sludge	20.9	56.7	n. p.	1.1	35	Teglia et al. (2011)
	18.9	59.9	n. p.	1.9	85	
	25.8	63.9	7.3	n.p.	n.p.	Cristina et al. (2019)

[a] dry basis. SD, solid digestate. DM, dry matter. OM, organic matter. DRI_{24h}, dynamic respiration index average in the 24 h of maximum activity. AT_4, cumulative oxygen consumption during the 4 days after the lag phase. n.p., not provided. Data presented as mean values ± standard deviation of the sample analysis.

3.2. Py-GC-MS analysis

In order to understand the composition of the studied solid digestate matrices and to evaluate the differences between them, a more detailed characterisation was carried out using Py-GC-MS. Table 2 shows the proportions (%) of the main compounds relevant for interpretation, categorised in different groups and subgroups.

Methylene chain compounds (MCC; alkanes, alkenes, fatty acids, etc.) were the most abundant group (24.9 % mean ± 8.9 % standard deviation) (Table 2). MCC were most abundant in sample SD4, mainly due to the elevated proportions of fatty acids, which are an indicator of the presence of oils and fats. Conversely, pyrolysis products derived from carbohydrates (10.9 ± 2.3 %) and nitrogen compounds (22.9 ± 3.9 %) are relatively abundant in SD1, SD2 and SD3. The N-compounds reflect microbial material, mainly protein, but also chitin, which is present in fungal cell walls (Stankiewicz et al., 1996). Given the high proportion of N-containing material, the carbohydrate products are also likely to be predominantly microbial in origin, in addition to an

Table 2
Characterisation of the solid digestates by Py-GC-MS. Relative proportions are expressed as percentage of total quantified peak area.

Group	Subgroup	SD1	SD2	SD3	SD4
Carbohydrate products	sum	11.56	13.15	11.26	7.63
Cholesterol products	sum	4.79	7.96	7.09	4.07
Nitrogen compounds	sum	25.51	22.14	26.28	17.66
	protein	20.73	17.91	21.38	14.16
	chitin	2.55	1.80	3.01	1.77
	unid. Aromatic N	2.23	2.44	1.89	1.73
Lignin products	sum	2.45	1.59	3.44	3.09
	p-hydroxyphenyl (H)	1.13	0.67	0.90	0.88
	guaiacyl (G)	0.98	0.68	1.69	1.53
	syringyl (S)	0.34	0.24	0.85	0.68
MAHs	sum	21.77	23.33	15.06	14.92
	polystyrene markers	5.54	6.53	3.70	3.62
MCC	sum	18.42	20.67	22.64	37.97
	n-alkanes	1.94	2.25	2.61	1.83
	n-alkenes	1.60	2.30	1.76	1.15
	fatty acids	6.56	12.44	12.52	29.10
	isoprenoids	0.47	0.21	1.69	0.12
	amides/nitriles	0.77	1.57	1.13	3.28
	propenyl esters	2.29	1.73	2.80	1.98
	wax esters	4.79	0.16	0.13	0.51
PAHs	sum	2.01	1.37	1.00	1.41
	polystyrene markers	0.82	0.54	0.57	0.67
Phenols	sum	9.11	6.47	6.87	7.18
Other compounds	sum	4.39	3.33	6.35	6.06

MAH, monocyclic aromatic hydrocarbons. MCC, (poly) methylene chains. PAH, polycyclic aromatic hydrocarbons.

unknown contribution from structural plant polysaccharides such as cellulose (Pouwels et al., 1989). Therefore, SD4 is N-deficient and has the highest levels of fatty acids (oils and fats), which may indicate lower microbial conversion. Less degradation of OM during the AD process may have resulted in a less stable digestate and therefore higher AT_4, as previously observed in the biodegradability analysis (Table 1).

Minor product groups highlight some further differences between samples (Table 2). Lignin products from vascular plant material (4-vinylphenol, guaiacols and syringols) (van der Hage et al., 1993) account for 2.6 ± 0.8 % and decrease in the order SD3 > SD4 > SD1 > SD2. Some mono- and polyaromatic markers of polystyrene (together 5.5 ± 1.4 %) suggest that samples SD1 and SD2 in particular contain some plastics. Sample SD1 is the only sample with significant contributions of wax esters (part of MCC). These waxes can be produced by bacteria in response to stressful conditions and their presence has been previously reported in activated sludge systems (Modin et al., 2016; Revellame et al., 2012). Finally, samples SD2 and SD3 have higher proportions of cholesterol-based compounds (cholestenes and cholesterol), probably indicating faecal material (6.0 ± 1.8 %).

The use of Py-GC-MS for more detailed characterisation of solid digestates was found to be a useful tool to reveal more significant differences in their composition. To the best of the authors' knowledge, this is the first work to present a detailed characterisation of different digested sludges, as this information is important for further uses of this material, which is increasingly being produced due to the implementation of AD as a renewable energy source in the world. In this study, the next step was to assess whether the observed differences in the composition of SD have an impact on its use as a substrate in an SSF process for Bt cultivation, a novel strategy in the use of digestate.

3.3. Evaluation of solid digestates as substrates for SSF used for Bt production

The influence of the nature of each digestate on Bt growth and sporulation was evaluated in 0.5-L reactors. The results of these experiments are summarised in Fig. 2, which shows the sOUR profile (Fig. 2a), the viable cells on the first and last day and the spores on the last day (Fig. 2b). The results are presented as the average of three SSF independent experiments performed for each substrate. In addition, the viable cell yield, sporulation yield, and percentage of sporulation are shown in Table 3.

The sOUR profiles in Fig. 2a show the oxygen consumption profile for each substrate. SD1, SD2 and SD3 show similar profiles with maximum oxygen consumption around 1.5 g O_2 kg^{-1} DM h^{-1} at 24 h. In contrast, the sOUR profile for SD4 clearly shows a longer lag phase, peaking 24 h later. Despite the high oxygen consumption of SD4, no Bt growth can be seen after 72 h (Fig. 2b), only sporulation. However, an increase in viable cells of about one order of magnitude was obtained with SD1, SD2 and SD3. Other important data related to Bt sporulation are presented in Table 3 for the four digestates. For further comparison, the values obtained with bench-scale SSF bioreactors are also shown in Table 3, as discussed later.

Table 3
Evolution of pH and Bt viable cells and spore production during the solid-state fermentation of digested sewage sludge in 0.5-L and 22-L reactors.

Substrate	scale	pH (initial-final)	Viable cells yield	Sporulation yield	Sporulation (%)
SD1	0.5 L	7.7–8.5 ± 0.1	9.0 ± 2.1	5.0 ± 1.5	55 ± 5
SD2		7.5–8.4 ± 0.1	13.8 ± 3.1	11.3 ± 3.3	84 ± 15
SD3		7.9–8.6 ± 0.1	4.3 ± 0.3	3.7 ± 0.9	74 ± 16
SD4		8.2–7.8 ± 0.1	0.8 ± 0.6	0.4 ± 0.1	65 ± 32
SD1	22 L	7.8–8.3	20.2 ± 1.3	13.0 ± 2.7	64 ± 14
SD2		7.9–8.4	45.8 ± 7.6	35.8 ± 8.7	78 ± 12

Data presented as mean values ± standard deviation (n = 3).

As explained in the previous section, the Py-GC-MS analysis (Table 2) revealed clear compositional differences among the digestates, particularly in terms of nutrient content. These differences are reflected in the growth of Bt across the four digestates. Carbohydrate-derived products and nitrogen compounds were more abundant in SD1, SD2, and SD3, which appears to have favoured Bt growth and sporulation. In contrast, SD4 exhibited lower levels of these compounds but a higher concentration of fatty acids, suggesting a limited availability of readily assimilable substrates. This may have hindered Bt growth and sporulation while promoting the proliferation of digestate native microbial populations that may have survived the sanitation process and competed with Bt during fermentation. Differences in the availability of carbon, nitrogen, and other key nutrients across digestates likely influenced specific growth rates and spore production. In any case, Py-GC-MS appears to be a useful tool for predicting microbial growth, and could be applied to other organic materials intended for solid-state fermentation.

Nevertheless, Bt was successfully cultured in SD1, SD2 and SD3 with viable cell yields of 9, 14 and 4, respectively, and sporulation yields relative to initial viable cells of 3–11 (Table 3). A direct comparison with

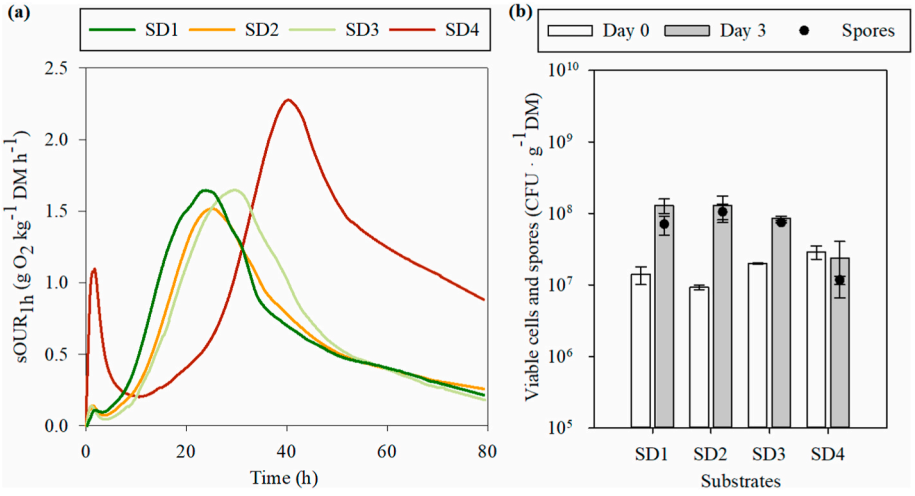

Fig. 2. Monitored parameters during the solid-state fermentation in 0.5-L reactors for the production of Bt with each solid digestate: (a) $sOUR_{1h}$ profiles, (b) initial and average final viable cells and final spores (n = 3). At the beginning of the experiments, spores were not detected in any of the digestate.

literature values is not possible due to the lack of studies using digested sewage sludge as a substrate for SSF in Bt production. However, the final values of viable cells (Fig. 2b) obtained with SD1, SD2 and SD3 are in close agreement with those previously obtained by Cerda et al. (2019), where 1.1×10^8 CFU g^{-1} DM of *B. thuringiensis* var. *kurstaky* (Btk) viable cells were obtained using digested biowaste as substrate, representing a 1.34-fold increase of the initial cell number. Similar results were observed when organic fraction of municipal solid waste (OFMSW) mixed with digested sewage sludge was used as a cosubstrate for Bt cultivation, with 5×10^8–10^9 CFU g^{-1} DM of final viable cells obtained (Molina-Peñate et al., 2023).

Although the optimal pH reported for Bt sporulation is close to neutral (Özkan et al., 2003), pH was not controlled before or during SSF. Adjustment of pH is complicated in solid matrices due to the absence of free water, the complexity and heterogeneity of the solid matrix, and the lack of online pH meters for solids (Kumar et al., 2021). El-Bendary et al. (2016) tested a range of initial pH (from 6.5 to 8.5) for the SSF process using sugar beet pulp and sesame meal and observed higher Bt growth and spore production when the initial pH was set at 6.5. However, in this study, the high initial pH (Table 3) did not inhibit Bt growth in most of the solid digestates evaluated.

Overall, these results demonstrate the feasibility of growing Bt on a complex solid matrix such as solid digestate from sewage sludge while achieving good sporulation rates. They also show that the inherent characteristics of the digestate influence the SSF process and highlight the need to use appropriate waste characterisation techniques for its correct valorisation. Furthermore, it is important to assess the scalability and robustness of the process at larger volumes, as further explained.

3.4. Evaluation of the SSF at bench-scale

The aim of the bench-scale work is to evaluate the performance of Bt at a more representative scale and to assess the reproducibility of the results with increasing scale. To this end, substrates SD1 and SD2 were selected for scale-up as they gave the highest viable cell and sporulation yields, in 0.5-L reactors. One of the main obstacles in scaling up SSF processes lies in the limitations of heat transfer of OM in a solid state, which can lead to temperature increases in the reactor that can be harmful to the microorganisms of interest (Casciatori et al., 2016; Rodrigues et al., 2022). This heating may become more important when working with non-sterile substrates such as digestate, as higher microbial activity and consequent temperature increases would be expected during the SSF process. Therefore, temperature was monitored using button sensors distributed throughout the reactor (Figs. 1b and 3) to assess the effect of the substrates on the process temperature.

The results of monitoring temperature (as mean temperature inside the reactor), sOUR, viable cells and spores during the bench-scale SSF using a 22-L packed bed reactor for the production of Bt-derived biopesticides are shown in Fig. 3 and the temperature profiles inside the reactor are shown in Fig. 4. The values of Bt viable cells and spore yields were presented in Table 3.

Temperature and sOUR profiles peaked almost simultaneously in both fermentations (Fig. 3), as the temperature increase is directly related to the metabolic heat produced during microbial growth. As shown in Fig. 3a, the maximum oxygen consumption with SD1 was reached after 24 h, while an average of 2.3×10^8 spores g^{-1} DM and a 64 % of sporulation (Table 3) were reached after 72 h, whereas the maximum oxygen consumption with SD2 was reached after 54 h and the maximum sporulation after 96 h (Fig. 3b), two days later than the previous fermentation with SD1. As the oxygen consumption profile clearly showed a higher lag phase during SSF with SD2, it was decided to continue the fermentation until the oxygen consumption dropped to 0.3 g O_2 kg^{-1} DM h^{-1}, which was the value obtained at the end of the fermentation with SD1. A stationary phase was reached after 96 h with SD2 (Fig. 3b), in which the percentage of sporulation practically did not increase, with an average of 8.8×10^7 spores g^{-1} DM and a 78 % sporulation at the end of the fermentation.

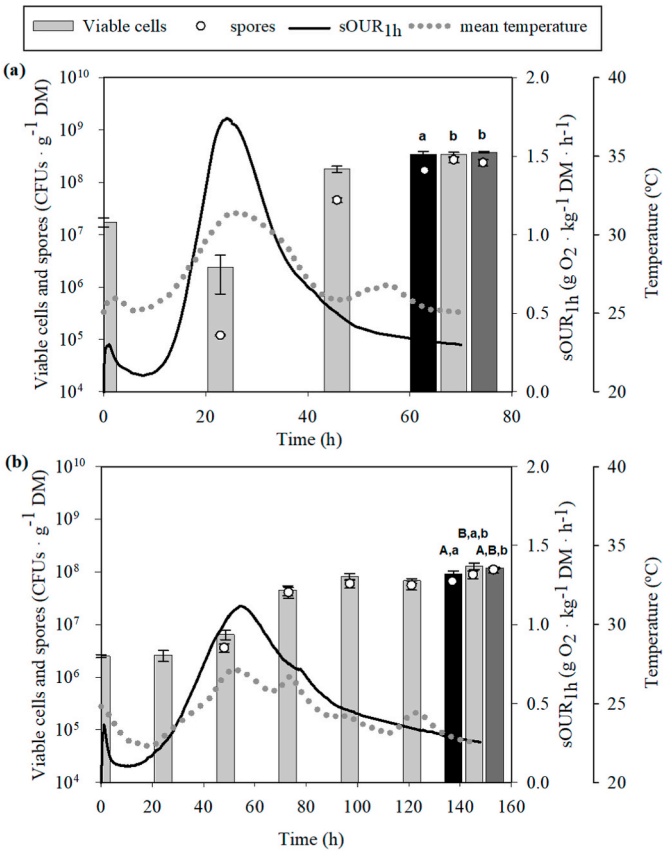

Fig. 3. Evolution of the main parameters during SSF at bench-scale: time course plot with a triplicate at the final day of fermentation corresponding, in order, to the samples of the upper, middle, and lower part of the reactor for SD1 (a) and SD2 (b). Significant difference ($p < 0.05$) between final samples is shown in uppercase (viable cells) and lowercase (spores), based on Tukey test statistical analysis.

At laboratory scale, both SD1 and SD2 exhibited a short lag phase, with the peak oxygen consumption occurring before 30 h. However, by scaling up the process, only SD1 maintained a similar oxygen consumption profile. In contrast, SD2 showed a markedly longer lag phase, with the maximum oxygen consumption peak delayed until nearly 60 h, and a lower maximum rate of less than 1.5 g $O_2 \cdot kg^{-1}$ DM$\cdot h^{-1}$. These observations highlight the influence of digestate characteristics on the scalability of the SSF process, specifically in process time.

The percentage of sporulation achieved at bench-scale was in line with the results obtained with the 0.5-L reactors, while the yield of viable cells and spores was increased, especially with SD2 (Table 3). These results are interesting as it is expected that the growth and sporulation of Bt will be limited when the process is scaled up due to air transfer limitations for compaction or overheating. For example, Zhang et al. (2013) achieved a successful fermentation process using 35 kg of kitchen waste as substrate, but Btk spore production decreased rapidly when 40 kg of substrate was used.

Temperature variations during fermentation are shown in Fig. 4. The maximum temperature reached in the centre of the reactor with SD1 was 35 °C (Fig. 4a), whereas with SD2 it did not exceed 30 °C (Fig. 4b). For SD1, a difference of about 5 °C can be observed between the centre (s4 and s5) and the sides of the reactor (s1, s2 and s3) during the period of higher microbial activity, highlighting the presence of radial temperature gradients. Along the lateral side, at axial level, there is no difference higher than 2 °C with respect to the centre. In contrast, SD2 (Fig. 4b) does not show large axial or radial temperature gradients. It is consistent that the SSF with the substrate with the highest oxygen consumption and

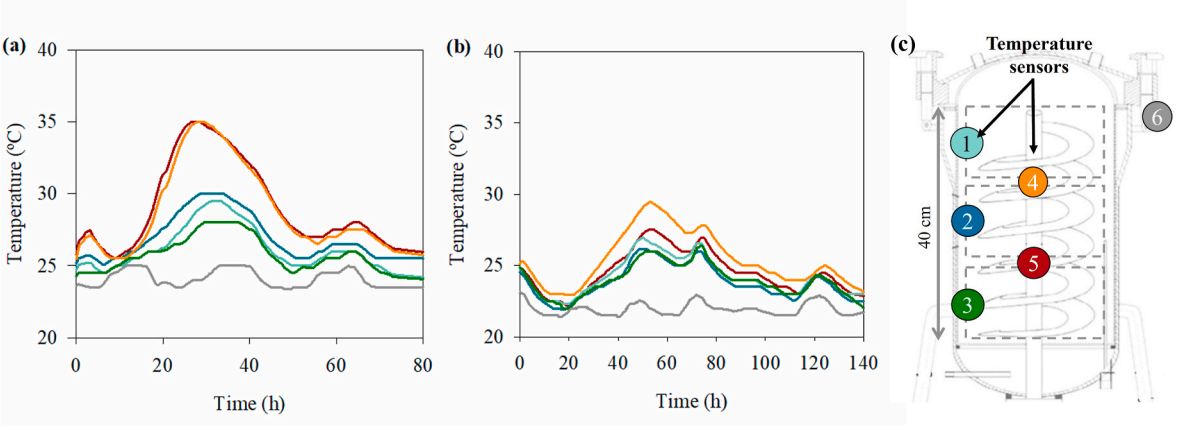

Fig. 4. Temperature profiles inside the reactor during the SSF fermentation at bench-scale with SD1 (a) and SD2 (b). Distribution of temperature sensors inside (1–5) and outside (6) the reactor.

respiration activity (SD1) is the one that has generated more heat inside the reactor (Fig. 3). In addition, the effect of room temperature and its daily variability on the SSF process must be considered. As can be seen in Fig. 4, the room temperature slightly affects the temperature profiles inside the reactor. The SSF with SD1 was carried out with an average room temperature of 24 °C, with highs of 25 °C and lows of 23 °C, whereas lower temperatures were reached during the SSF with SD2, with highs of 24 °C and lows of 21 °C. Fig. 4b shows how the low room temperatures during the SSF, especially in the early h of the process, may have affected Bt growth and would explain the delay in the lag phase (Fig. 3b).

One of the limiting factors in scaling is the high temperatures that can be reached when working with a highly biodegradable waste. However, the moderately low biodegradability of digested sludge can explain the small temperature variations observed in this study (Barrena et al., 2011). Therefore, the solid fraction of digested sludge allows to work at SSF temperatures that are not too high to be detrimental for Bt growth and sporulation, thus facilitating the process of further scaling. According to Ballardo et al. (2016), Bt spores can withstand temperatures up to 60 °C.

At the end of fermentation, the reactor was divided into three sections and the number of viable cells and spores in each section was measured. Statistical analysis was then performed to test for significant differences between the sections (Fig. 3). For the fermentation using SD1, there was no significant difference in viable cell production between the three sections. However, spore production differed significantly ($p < 0.05$), with slightly higher sporulation observed in the middle and lower sections of the packed-bed reactor (Fig. 3a). In contrast, SD2 showed significant differences ($p < 0.05$) for both viable cell and spore production. The top section of the reactor had slightly lower production compared to the other sections, as shown in Fig. 3b. Scaling up SSF using packed bed reactors presents inherent challenges of heterogeneity, particularly in terms of temperature, moisture and oxygen distribution, which can affect the uniform growth of Bt. Choosing the right substrate, using an appropriate amount of bulking agent and adjusting the air flow can help to create a more uniform process by preventing compaction, improving oxygen flow and reducing heat accumulation. In this study, only radial temperature variation (5 °C) was observed with one of the substrates, while Bt production remained relatively homogeneous throughout the height of the reactor. Similar results have been reported by other researchers growing fungi on different substrates such as rice husk and beer draff (Sala et al., 2024).

The cost of raw materials represents one of the major components of overall Bt production at commercial scale (Jallouli et al., 2020). In this context, SSF has been successfully applied to valorise a wide range of agricultural and organic residues, including spent sugar beet pulp-sesame meal and wheat germ meal-linen meal (El-Bendary et al., 2016), as well as maize stover and wheat bran (Gálvez et al., 2025). Additional examples include non-sterile biowaste (Ballardo et al., 2016), biowaste mixed with digestate from the OFMSW (Mejias et al., 2020), and OFMSW mixed with digested sewage sludge (Molina-Peñate et al., 2023). These studies demonstrate the potential of using nutrient-rich organic wastes to support Bt growth and sporulation. Substrates with a well-balanced nutrient profile, particularly those rich in readily available carbon and nitrogen sources, such as wheat bran or hydrolysed OFMSW, tend to a higher spore production. However, to fully exploit these substrates, SSF process parameters such as aeration, porosity or inoculation should be adapted accordingly. Unfortunately, there are no references regarding the growth of Bt on digestate from wastewater treatment. Therefore, a direct comparison is not possible, but some studies have used digestate from the organic fraction of municipal solid waste.

Overall, Bt has been successfully grown on the solid fraction of digested sewage sludge in a bench-scale SSF. Future research should focus on performing further tests using the 22-L packed bed configuration to evaluate both the consistency and repeatability of the SSF process. This is particularly important when using naturally variable substrates such as digestate. In addition, further characterisation of different solid digestates would help to build a database to better understand their properties. This may help to determine the most effective methods for their valorisation. To fully validate the potential of the final product as a biopesticide, future work should also include bioassays to assess insecticidal efficacy against relevant target pests. This would allow for a direct correlation between spore production and biopesticide activity, confirming the practical value of the SSF derived material and further support the development of digestate based biopesticide formulations.

4. Conclusions

This study shows that the solid fraction of digested sewage sludge is a promising substrate for the production of *Bacillus thuringiensis* var. *israelensis* spores by SSF. These results provide a new route for the valorisation of digestate, a material generated in large quantities and often requiring treatment before safe disposal.

A key takeaway from this work is that prior characterisation of digestate helps to optimise its valorisation process. Given the inherent variability between sludge digestates, policies and valorisation strategies should focus on the quality of the final product rather than its source to ensure effective management. In this regard, Py-GC-MS proves to be a valuable technique for analysing digestate properties and determining the most appropriate treatment approach.

Overall, this study provides an alternative strategy for the valorisation of the solid fraction of digested sewage sludge, producing a nutrient-rich material containing *Bacillus thuringiensis* spores that may have valuable applications in sustainable agriculture and pest control.

CRediT authorship contribution statement

J. Font-Pomarol: Writing – original draft, Methodology, Investigation, Data curation. **J. Kaal:** Writing – original draft, Investigation, Data curation. **E. Molina-Peñate:** Writing – original draft, Supervision, Investigation, Data curation, Conceptualization. **A. Sánchez:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **A. Artola:** Writing – original draft, Supervision, Data curation, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2025.127478.

Data availability

Data will be made available on request.

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