Waste Management 161 (2023) 92–103

Contents lists available at ScienceDirect

Waste Management

journal homepage: www.elsevier.com/locate/wasman

Filling in the gaps in biowaste biorefineries: The use of the solid residue after enzymatic hydrolysis for the production of biopesticides through solid-state fermentation

Esther Molina-Peñate [a,b], María del Carmen Vargas-García [c], Adriana Artola [a,*], Antoni Sánchez [a]

[a] GICOM Research Group, Department of Chemical, Biological and Environmental Engineering, School of Engineering, Edifici Q, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[b] Aeris Tecnologías Ambientales S.L, Carrer Santa Rosa, 38, local, 08290 Cerdanyola del Vallès, Barcelona, Spain
[c] Microbiology Unit, Department of Biology and Geology, University of Almeria, International Excellence Campus of the Sea (CEI·MAR), Crta. Sacramento s/n, La Cañada de San Urbano, 04120 Almería, Spain

ARTICLE INFO

Keywords:
Biorefinery
Biowaste
Biopesticide
Enzymatic hydrolysis
Solid-state fermentation

ABSTRACT

Alternative production processes using waste are necessary to preserve non-renewable resources and prevent scarcity of materials for future generations. Biowaste, the organic fraction of municipal solid waste, is abundant and easily available. It can be fractionated into building blocks for which fermentative processes can be designed. By using solid-state fermentation, this paper proposes a method of valorizing biowaste's residual solid fraction after enzymatic hydrolysis. In a 22 L bioreactor, two digestates from anaerobic digestion processes were evaluated as cosubstrates to modify the acidic pH of the solid residue after enzymatic hydrolysis and promote the growth of the bacterial biopesticide producer *Bacillus thuringiensis*. Regardless of the cosubstrate used, the final microbial populations were similar indicating microbial specialization. The final product contained 4×10^8 spores per gram of dry matter and also crystal proteins of *Bacillus thuringiensis* var *israelensis*, which have insecticidal activity against pests. This method allows for the sustainable use of all materials liberated during the enzymatic hydrolysis of biowaste, including residual solids.

1. Introduction

The transition from the current linear economic system toward a more sustainable scheme that reduces the use of non-renewable resources can only be accomplished through incentives for waste valorization and technological developments. Municipal solid waste (MSW) management has emerged as one of the century's greatest challenges for municipalities worldwide and a paradigm shift seems inevitable to offset the expense of implementing selective collection systems (Sánchez et al., 2015; Tonini et al., 2013). The organic fraction of municipal solid waste (OFMSW) could be used in biorefineries through a cascade of biological processes to obtain a variety of bioproducts (Budzianowski and Postawa, 2016). OFMSW comprises food waste from households, retail and restaurants as well as green waste from parks and gardens. Its highly variable composition is rich in carbohydrates and fibers, representing up to 85%, and also includes lipids, proteins, lignin and macro/micronutrients (Campuzano and González-Martínez, 2016).

Enzymes, which have been widely used in second-generation biorefineries (based on lignocellulosic materials), can fractionate the complex polysaccharides of the OFMSW and the other macromolecules into monomeric sugars and other functional units (Pleissner and Peinemann, 2020). Recently, fermentative pathways for these enzymatic hydrolysates that result in high-value bioproducts have been investigated (Molina-Peñate et al., 2022a). These novel pathways can coexist with the current most implemented treatment technologies for OFMSW, composting and anaerobic digestion (AD) (Cerda et al., 2018) in a biorefinery-like scenario.

After the enzymatic hydrolysis, the remaining solid fraction contains partially hydrolyzed and non-hydrolyzed fibers that can also support microbial growth in solid-state fermentation (SSF) (Molina-Peñate et al., 2022b). SSF is a simple and cost-effective biotechnological process based on the culture of microorganisms on moist solid substrates under aerobic conditions (Soccol et al., 2017). The main bottleneck to its successful establishment as an industrial alternative is the difficulty of

* Corresponding author.
E-mail address: adriana.artola@uab.cat (A. Artola).

https://doi.org/10.1016/j.wasman.2023.02.029
Received 31 October 2022; Received in revised form 31 January 2023; Accepted 23 February 2023
Available online 3 March 2023

scaling up the process due to heat and mass transfer limitations. At large scales, the accumulation of heat and diffusion problems cause gradients in temperature, humidity, and concentration, as well as oxygen and nutrient deficiencies (Soccol et al., 2017). At the time of writing, a Scopus®-based bibliometric analysis on the topic "solid state fermentation" limited to articles in English of the last 20 years (2002–2022) resulted from 5,667 documents, from which <2% also included the terms "pilot" or "bench" (October 4, 2022). Therefore, more research on representative scales is necessary to make SSF a commercially competitive technology.

For the use of the solid residue after enzymatic hydrolysis of OFMSW as an SSF substrate, the selection of robust microorganisms capable of thriving in not sterile environments, such as *Bacillus thuringiensis* (Bt) (Ballardo et al., 2016; Cerda et al., 2019), can facilitate the process implementation in urban waste management plants. Bt is a microbial biopesticide producer that benefits from solid cultivation as it leads to increased spore production, viability and infectibility due to reduced osmotic stress and increased surface for gas exchange (Flores-Tufiño et al., 2021). However, the acidic pH of the solid residue after enzymatic hydrolysis may hinder Bt growth and sporulation because this microorganism thrives at pH near neutrality. Moreover, pH tends to acidify at the early stages of fermentative processes due to the release of short-chain fatty acids, which inhibits microbial activity, as reported for the OFMSW composting process (Sundberg et al., 2004). The monitoring and control of pH during SSF is another major bottleneck of this technology due to the heterogeneity of the solid matrix, a limited amount of free water and a lack of suitable online solid-state pH measurement methods (Kumar et al., 2021). Besides, the solid substrate itself can present a buffering effect due to its complex chemical composition, as for the OFMSW. Therefore, efficient operational strategies are required to prevent sharp pH changes and the overuse of chemicals during the fermentation course.

In the AD of food waste, the use of cosubstrates is a common practice to prevent an inhibitory pH from the rapid hydrolysis step and to improve process efficiency (Karki et al., 2021). On the contrary, in SSF, cosubstrates have been researched to a lesser extent, and the use of nutrient supplementation or chemicals for pH control is more common, though rarely reported at larger scales (Soccol et al., 2017). Thus, the present work aimed to evaluate the use of high-buffer capacity cosubstrates as a strategy to control pH in a 22 L SSF bioreactor. Two abundant biomaterials (digested sewage sludge and digested OFMSW) were tested as cosubstrates for the production of *Bacillus thuringiensis* var. *israelensis* on the solid residue after enzymatic hydrolysis of OFMSW. This strain is especially attractive as the solid material can be used as biopesticide and no excessive downstream is necessary. SSF performance was evaluated on the basis of oxygen consumption rate, temperature and Bt sporulation, which is closely related to toxicity (Angelo et al., 2015). Further, the final product was also evaluated in terms of microbial community for safety assessment. Finally, an OFMSW biorefinery scheme based on these technologies was proposed, including overall mass balances.

2. Materials and methods

2.1. Raw materials

The OFMSW was collected upon arrival at the MSW treatment plant of Mancomunitat La Plana (Malla, Barcelona) in February (winter season). This material is obtained by a well-established door-to-door collection system hence ensuring high quality with a low level of impurities (<1%). After collection, bags were opened manually and screened for inert materials (plastic, metal, glass or textile). Also, excess paper, hard shells, hair and bones were removed. Then, around 12 kg of OFMSW were shredded mechanically using a home composting shredder (Tecoinsaen SL, Spain), homogenized and stored at −20°C for a maximum period of three months. In previous work, it was demonstrated that freezing for even longer times does not have a significant effect on material properties according to aerobic respiration indices (Pognani et al., 2012).

As cosubstrates of the SSF process, two types of digested materials were used: (i) digestate from a source selected OFMSW (DOF) treatment plant (Granollers, Barcelona), which was obtained from a mesophilic wet AD process followed by a solid–liquid separation using a screw press, and (ii) digested sewage sludge (DSS) from a municipal wastewater treatment plant (Sabadell, Barcelona), which was obtained from a mesophilic wet AD followed by a solid–liquid separation using a centrifuge. They were stored at −20°C for a maximum period of three months. Before use, both materials were defrosted and subjected to a hygienization step to pasteurize them as specified in the European Regulation N° 142/2011., Materials were kept at 70°C for 1 h using a previously heated oven and covering them to prevent moisture losses. They were stored in the fridge (5°C) until their use for <24 h.

Raw materials were characterized upon arrival at our facilities (Table 1). Both digestates were similar in the physicochemical properties evaluated. However, DOF content in organic matter was higher than that of DSS, which is a relevant parameter when using it as a cosubstrate of SSF.

2.2. Enzymatic hydrolysis

OFMSW samples were defrosted overnight at 5°C and sterilized by autoclaving at 121°C for 30 min before use. The enzymatic hydrolysis step was conducted under sterile conditions in 2 L Erlenmeyer flasks loaded with 0.7 kg of autoclaved OFMSW. n enzymatic cocktail tailor-made for OFMSW-like materials was supplied by ASA Spezialenzyme GmbH (Wolfenbüttel, Germany). It was made up of a mixture of cellulases and pectinases, but also includes hemicellulase, ß-glucosidase and

Table 1
Characterizations of the OFMSW, the digested materials and the enzymatic hydrolysates used in this study.

	OFMSW[a]	Digested OFMSW[a]	Digested sewage sludge[a]	Hydrolysate[b]
MC (%)	76.4 ± 1.1	77.2 ± 0.4	83.5 ± 0.1	77.4 ± 2.4
DM (%)	23.6 ± 1.1	22.8 ± 0.4	16.5 ± 0.1	22.6 ± 2.4
OM (%*)	89.7 ± 0.7	72.3 ± 6.3	68.8 ± 1.1	86.9 ± 1.7
RS (%*)	16.6 ± 0.9	NM	NM	12.6 ± 1.7
pH	5.6 ± 0.1	8.5 ± 0.2	8.3 ± 0.2	5.3 ± 0.1
Conductivity (mS/cm)	2.2 ± 0.1	2.6 ± 0.1	1.1 ± 0.1	2.6 ± 0.1
DRI_{24h} (g O_2 kg^{-1} DM h^{-1})	3.5 ± 0.3	1.4 ± 0.3	1.6 ± 0.2	1.2 ± 0.1
AT_4 (g O_2 kg^{-1} DM)	179 ± 18	91 ± 2	95 ± 4	40 ± 2

*dry basis. OFMSW, organic fraction of municipal solid waste. MC, moisture content. DM, dry matter. OM, organic matter. RS, reducing sugars. 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. NM, not measured. [a]Data presented as mean values ± standard deviation of the sample analysis. [b]Data presented as mean values ± standard deviation of two independent hydrolysis samples.

α-amylase activities. Enzyme dosage was 0.05 mL of enzymatic cocktail per g of initial dry matter according to manufacturer's instructions, the solid-to-liquid ratio was set to 10% (w v^{-1}), and the initial pH to 4.5 using 0.05 M sodium citrate buffer. Flasks were incubated at 50 °C and 180 rpm for 24 h. Then, hydrolysates were centrifuged at 6000 rpm for 15 min at 4°C. Samples from both fractions were taken to measure sugar content and the solid fraction was collected and stored at 5°C until its use in the SSF for a maximum of three days. The mass balance for the enzymatic hydrolysis is presented in Section 3.4.

2.3. Microbial strain and inoculum preparation

Bacillus thuringiensis var *israelensis* (Bti) CECT 5904 was obtained from "Colección Española de Cultivos Tipo" (Valencia, Spain) and preserved at −80°C using a seed lot system in cryo-pearls (DeltaLab, Barcelona). Inoculum preparation was carried out according to the methodology presented by Mejias et al. (2020). Briefly, one cryo-pearl was inoculated in 100 mL of sterile Nutrient Broth n°2 (Oxoid CM0067B) and incubated at 130 rpm and 30°C for 20 h, until an optical density of 2.5–3.0 was reached. Then, the culture was centrifuged for 10 min at 3500 rpm. The obtained pellet was resuspended in 3 mL of the exhausted media and then, diluted 1:10 (v v^{-1}) to reach approximately a concentration of 10^8 CFU mL^{-1}. This suspension was used as inoculum for SSF experiments. No spores were detected at this point.

2.4. Solid-state fermentation process

Two SSF experiments were conducted in a 22L packed-bed bioreactor to evaluate the effect of each cosubstrate on Bti growth and sporulation.

2.4.1. Experimental set-up

The SSF bioreactor was a packed-bed bioreactor made of stainless steel with a removable inner basket of 22 L and an automatic vertical helical ribbon mixer, as showed in Fig. 1 and the supplementary material (Fig. S1) (Martínez et al., 2018). The working volume was approximately 85% of the reactor capacity, corresponding to 4.5 kg of solid materials. The substrate mixture consisted of 1.75 kg of the solid residue after enzymatic hydrolysis, 1.75 kg of cosubstrate (digestate from OFMSW or digested sewage sludge) and 1 kg of sterile wood chips, corresponding to a 39%, 39% and 22% in wet weight ratio, respectively. The addition of wood chips (1:2 volumetric ratio) as bulking agent is essential to provide porosity and ensure proper airflow and oxygen availability. The mixture was thoroughly mixed and inoculated with 25 mL of Bti inoculum suspension per kg of solid material, to reach an initial concentration of around 10^7 colony-forming units (CFU) per gram of dry matter (DM) (Mejias et al., 2020). To prevent compaction at the lower part of the reactor where the air inlet is, a 5 cm wood chips layer was added to the basket before loading the inoculated mixture. The reactor is connected to a mass airflow meter (Bronkhorst, The Netherlands) that supplies and controls the specific airflow rate of 730 mL min^{-1}. The airflow goes first through a humidifier to saturate the air with water and then enters the reactor from the bottom. Experiments were monitored for 96 h and samples were taken each 24 h from the upper part of the reactor. Intermittent mixing (10 min at 12 rpm) was applied each 24 h before sampling. Last day, when the reactor was stopped, two additional sampling points from the middle and lower parts (Fig. 1) were included to evaluate the material homogeneity.

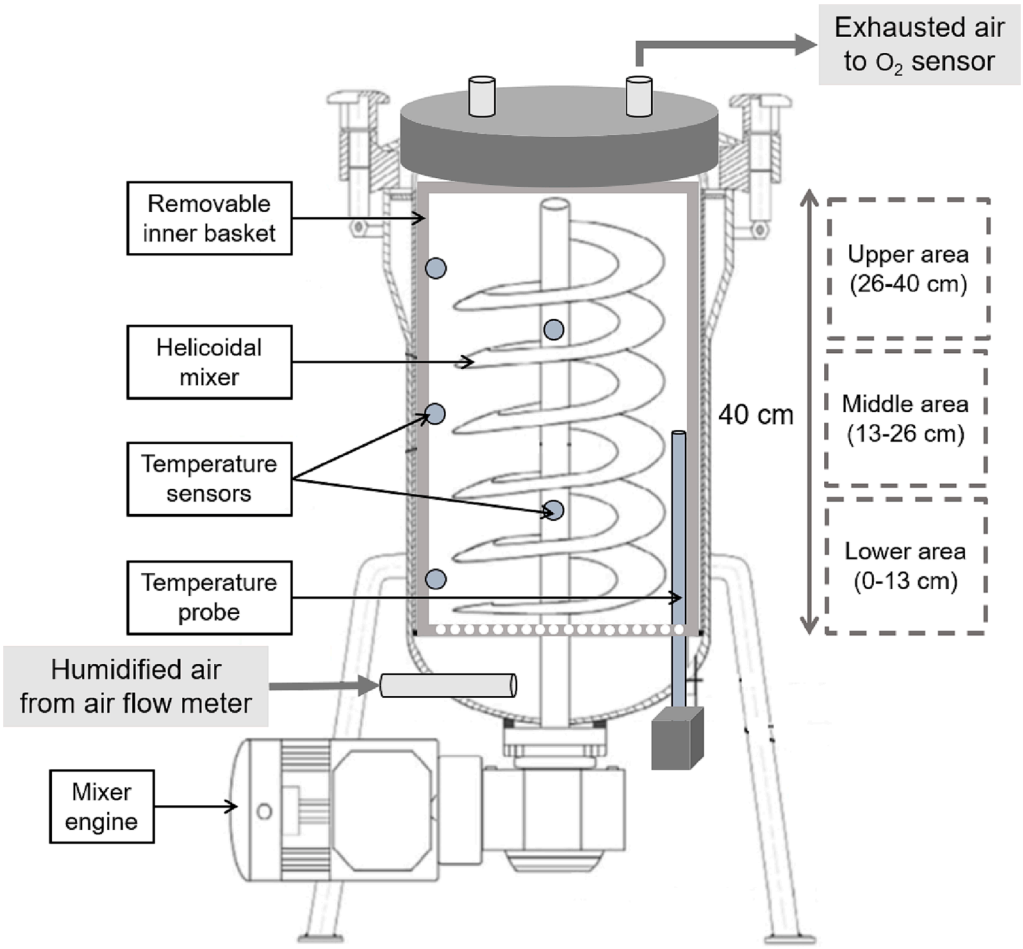

Fig. 1. Experimental set-up of the solid-state fermentation 22 L bioreactor.

2.4.2. Monitored parameters

Microbial growth was assessed using different parameters related to microbial activity. Oxygen consumption was online monitored by measuring oxygen concentration at the outlet port of the reactor by 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; Ponsá et al., 2010). The specific oxygen uptake ratio (sOUR) was calculated according to:

$$sOUR = F \times \left(0.209 - y_{O_2}\right) \times \frac{P \times 32 \times 60 \times 1000^{a}}{R \times T \times DM \times 1000^{b}}$$

where sOUR is the specific oxygen uptake rate (g O_2 kg^{-1} DM h^{-1}); F, airflow rate into the reactor (mL min^{-1}); y_{O2}, oxygen molar fraction in the exhaust air (mol O_2 mol^{-1}); P, the pressure of the system assumed constant at 101,325 (Pa); 32.6, oxygen molecular weight (g O_2 mol^{-1} O_2); 60, the conversion factor from minute to hour; 1000^{a}, conversion from ml to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, the temperature at which F is measured (K); DM, dry matter of solids in the reactor (g); 1000^{b}, conversion factor from g to mg.

The cumulative oxygen consumption represented by the area below the O_2 consumption curve was also calculated as another indicator of the biological activity in the SSF bioreactor.

Temperature is another indicator of microbial activity as a consequence of the metabolic heat produced during microbial growth (Arora et al., 2018). This parameter was online monitored in the lower half of the reactor bed employing a temperature probe (Pt-100 sensors, Sensotrans) located in the bioreactor. Also, accurate temperature profiles at different heights of the reactor bed were obtained using temperature sensors (Maxim Integrated, U.S.). Sensors were placed at both the center of the packed bed (at 18 cm and 30 cm height) and the edges close to the basket wall (at 12 cm, 24 cm and 36 cm height). Room temperature was also monitored.

Specific Bti growth was monitored by measuring viable cells and spores. First, solid samples were subjected to a solid–liquid extraction using Ringer solution in a 1:10 (w v^{-1}) ratio at 150 rpm for 20 min. Then, the extract was appropriately diluted and 50 μL plated in triplicate onto Petri dishes containing a Nutrient agar medium (Oxoid CM0003B, England). To measure spores, 20 mL of the previous extract were submitted to a thermal shock by incubating them at 80°C for 10 min and then placing them into ice before plating (Mejias et al., 2020). All plates were incubated at 30°C for 20 h and viable cells or spores were estimated in terms of CFU and related to the DM of the sample. The sporulation ratio at a certain time is calculated considering that the viable cell count includes both vegetative cells and spores according to the following equation:

$$Sporulation\ ratio(\%) = \frac{spores\ g^{-1}DM}{viable\ cells\ g^{-1}DM} \times 100$$

The sporulation yield that expresses the spores produced per initial viable cell inoculated is calculated using the viable cell count at time 0 h as follows:

$$Sporulation\ yeild = \frac{spores\ g^{-1}DM}{initial\ viable\ cells\ g^{-1}DM}$$

2.5. Analytical methods

2.5.1. Sugar content

Reducing sugar content of the enzymatic hydrolysis fractions was quantified using the 3,5-dinitrosalicylic acid colorimetric method (DNS) (Miller, 1959). The liquid fraction was centrifuged (10,000 rpm, 20 min), filtered through a 0.45 μm membrane filter and properly diluted before processing. For the solid fraction, a solid–liquid extraction with distilled water in a 1:10 (w v^{-1}) ratio was performed at 50°C for 30 min. Then, it was centrifuged and processed like the liquid fraction.

2.5.2. Routine parameters

Raw materials and fermentation samples were characterized in terms of moisture content, DM, organic matter (OM), pH and conductivity, which were measured following standard procedures (Leege, 1998).

2.5.3. Biodegradability

Biodegradability was assessed through two respiration indices and compared among the different substrates: the dynamic respiration index (DRI_{24h}), which represents the average oxygen uptake rate during the 24 h of maximum activity observed expressed in g O_2 kg^{-1} DM h^{-1}, and the cumulative oxygen consumption index (AT_4), which is the cumulative oxygen consumption of the four days after the lag phase expressed in g O_2 kg^{-1} DM, as described elsewhere (Ponsá et al., 2010).

All measurements were conducted in triplicates.

2.6. Scanning electron microscopy

Scanning Electron Microscope (SEM) (Zeiss EVO) was used to visualize Bti cells, spores and crystals produced. Samples of fermented material of each SSF were taken after the process was finished, and a solid–liquid extraction was performed with Ringer solution (1:10 w v^{-1}) for 20 min and sonicated (10 rounds of 1 min and 30 s of ice). Samples were fixed on adhesive paper and dried for further sample metallization with gold (Loutfi et al., 2021).

The dimensions of spores and crystals were determined by measuring 25 spores and 50 crystals in each sample on the screen of the SEM at a magnification of ×20,000. These dimensions were used to identify Bti spores according to the literature (Loutfi et al., 2021).

2.7. Microbial community analysis

Sequencing was performed by the Genomic Service of the Universitat Autònoma de Barcelona. Samples from the raw materials, the initial SSF mixtures and the final fermented products of both cosubstrates were processed for DNA extraction using the Soil DNA Isolation Plus Kit (Norgen Biotek, Canada). DNA extracts were tested for concentration and quality using a NanoDrop spectrophotometer and used to construct the corresponding genomic libraries by analyzing the variable regions V3-V4 of the prokaryotic 16S rRNA gene sequences, which gives 460 bp amplicons in a two-round PCR protocol. First amplification was done with the specific primers with overhang adapters attached that flanks regions of interest, forward (5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and reverse (5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Then, using a limited-cycle PCR, sequencing adapters and dual index barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA), were added to the amplicon for sequencing pooled together in the MiSeq sequencer with the MiSeq® Reagent Kit v2 (500 cycles) MS-102-2003. Sequencing analysis was carried out at the BaseSpace (Illumina, Inc, USA) with the 16S Metagenomic App that performs taxonomic classification using a taxonomic database. The algorithm used was a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang et al. (2007).

The alpha diversity indices of the different microbial communities were obtained from the EzBioCloud microbiological research platform (https://www.ezbiocloud.net), while the dendrogram and the Principal Component Analysis were performed using the PAST software for statistical analysis of biological data (PAST 4.05). The phylogenetic tree showing the evolutionary relationship of the dominant ASVs was generated from MEGA version 10.1.6 software, using the Neighbour-Joining method combined with the Maximum Composite Likelihood method to compute evolutionary distances.

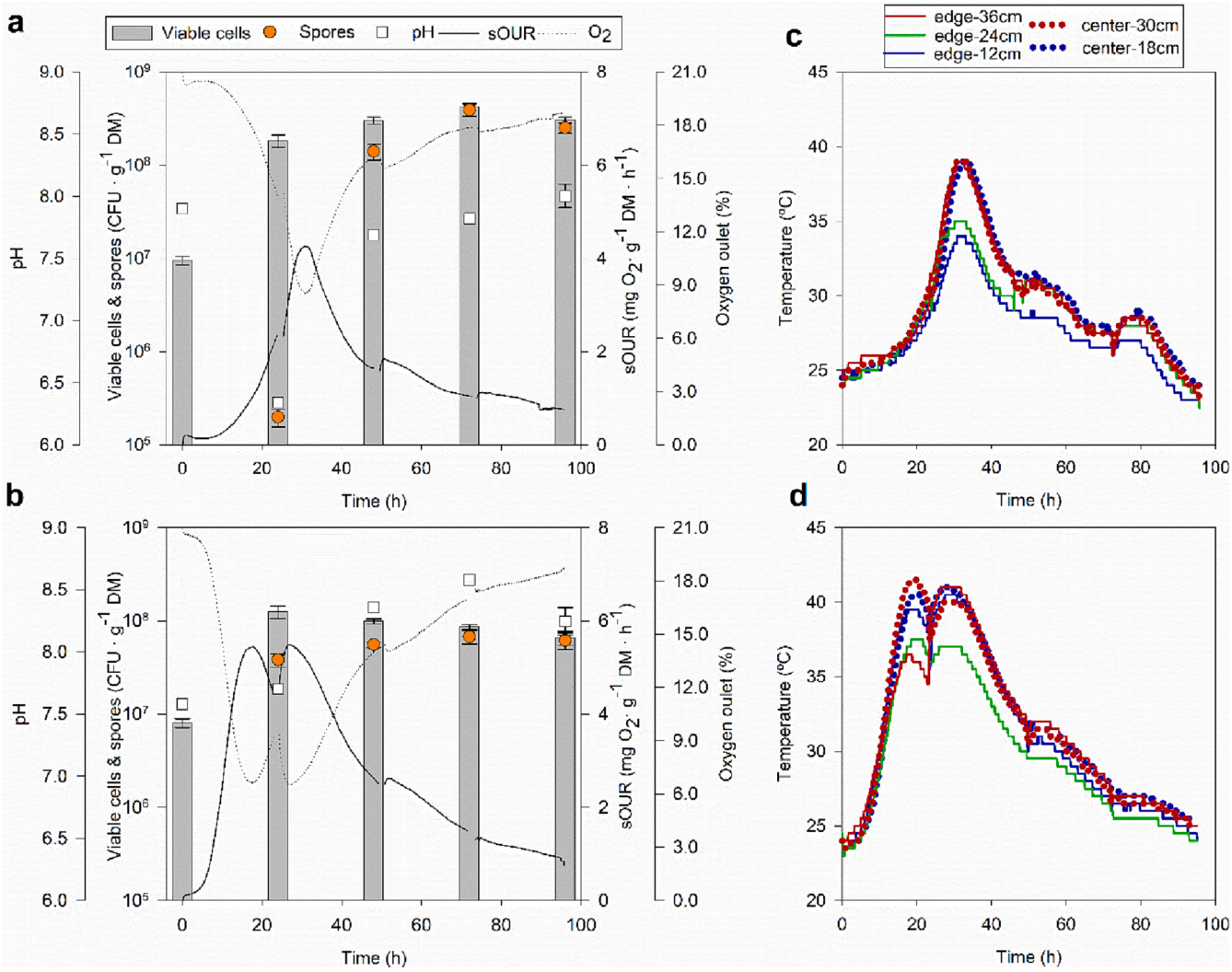

Fig. 2. Monitored parameters during 22 L SSF to produce Bti spores from OFMSW hydrolysate using cosubstrates and thermal behavior inside the respective packed-bed (a, c) digested sewage sludge and (b, d) digested OFMSW.

3. Results and discussion

3.1. Process performance at 22 L

The effect of each cosubstrate on the growth and sporulation of Bti, as well as other operational parameters, was monitored during the SSF of the solid residue after enzymatic hydrolysis of OFMSW in a packed-bed bioreactor of 22 L. As shown in Fig. 2, both cosubstrates increased Bti growth by an order of magnitude when compared to inoculated cells. Digested sewage sludge (DSS) (Fig. 2**a**) showed a slower increase in biological activity (measured as sOUR) than in digested OFMSW (DOF) (Fig. 2**b**). They reached maximum activity in 30 h and 17–26 h, respectively. This maximum activity was one sOUR unit higher for the DOF and coincided with the maximum production of viable Bti cells, whereas for the DSS, Bti continued to grow for 72 h. This indicates less microbial competition in the latter scenario, promoting the growth of Bti. Another remarkable difference between both cosubstrates is that while the sporulation ratio after 24 h of processing was practically zero for DSS, it was 30% for DOF, which can also be attributed to a more competitive environment that promotes faster sporulation.

Considering that most crystal proteins associated with toxicity are produced during sporulation and that spores also act as insecticides, achieving a high sporulation percentage was the ultimate goal (Angelo et al., 2015). The maximum spore production was achieved at 72 h of the fermentation (Fig. 2), as has been previously reported for Bt in SSF (Cerda et al., 2019; Mejias et al., 2020). This was 3.9×10^8 spores g^{-1}

Table 2
Performance parameters of the SSF of OFMSW hydrolysate and each cosubstrate to produce Bti spores at 22L-scale.

Cosubstrate	Lag phase (h)	Total oxygen consumption (mg $O_2 \cdot g^{-1}$ DM)	Sporulation (%)		Sporulation yield (spore/initial cell)	
			72 h	96 h[a]	72 h	96 h[a]
DSS	18	140.6	91.9	88.7 ± 16	43.5	26.5 ± 15.3
DOF	8	244.8	78.5	99.1 ± 17	8.4	8.5 ± 2.3

Bti, *Bacillus thuringiensis* var *israelensis*. DSS, digested sewage sludge. DOF, digested organic fraction of municipal solid waste. The lag phase is calculated as the time it takes to reach 25% of the maximum sOUR. [a]Data presented as mean values ± standard deviation of three different sampling points.

DM for DSS and 6.8×10^7 spores g^{-1} DM for DOF. This corresponds to a sporulation yield per initial viable cell of 43.5 and 8.4 respectively (Table 2). A previous study showed a spore production of 1.6×10^6 spores g^{-1} DM at a 0.5 L scale using an OFMSW solid hydrolysate that had been chemically modified with NaOH to adjust the initial pH (Molina-Peñate et al., 2022b). Therefore, the use of alkaline cosubstrates appears as a more suitable strategy at a larger scale (x44 times) to ensure a pH near neutrality for Bti growth and sporulation, while saving costs of chemical reagents. The use of the solid residue after enzymatic hydrolysis of OFMSW appears beneficial for Bt production compared to using only non-sterile digested OFMSW, which reported lower spores values (2.8×10^7 spores g^{-1} DM) when using Bt var *kurstaki* (Cerda et al., 2019). However, the mixture with non-hydrolyzed biowaste reported slightly higher spore values (4×10^8 spores g^{-1} DM) using a two-step aeration strategy (Mejias et al., 2020).

The pH of the solid residue after enzymatic hydrolysis was 5.3 (Table 1) and therefore, inhibitory for Bti (Foda et al., 1985). The use of alkaline cosubstrates increased the pH of the SSF mixtures to 7.5–8 (Fig. 2), which is in the optimal range for Bti growth and sporulation. Their high buffering capacity (Karki et al., 2021) prevented the pH from reaching acidic inhibitory values (Fig. 2), which are characteristic of OFMSW aerobic treatments (Sundberg et al., 2004). For DSS the pH dropped to 6.3 after 24 h (Fig. 2**a**) in contrast to DOF (Fig. 2**b**), which did not present a pH drop. This might be explained by the higher content of

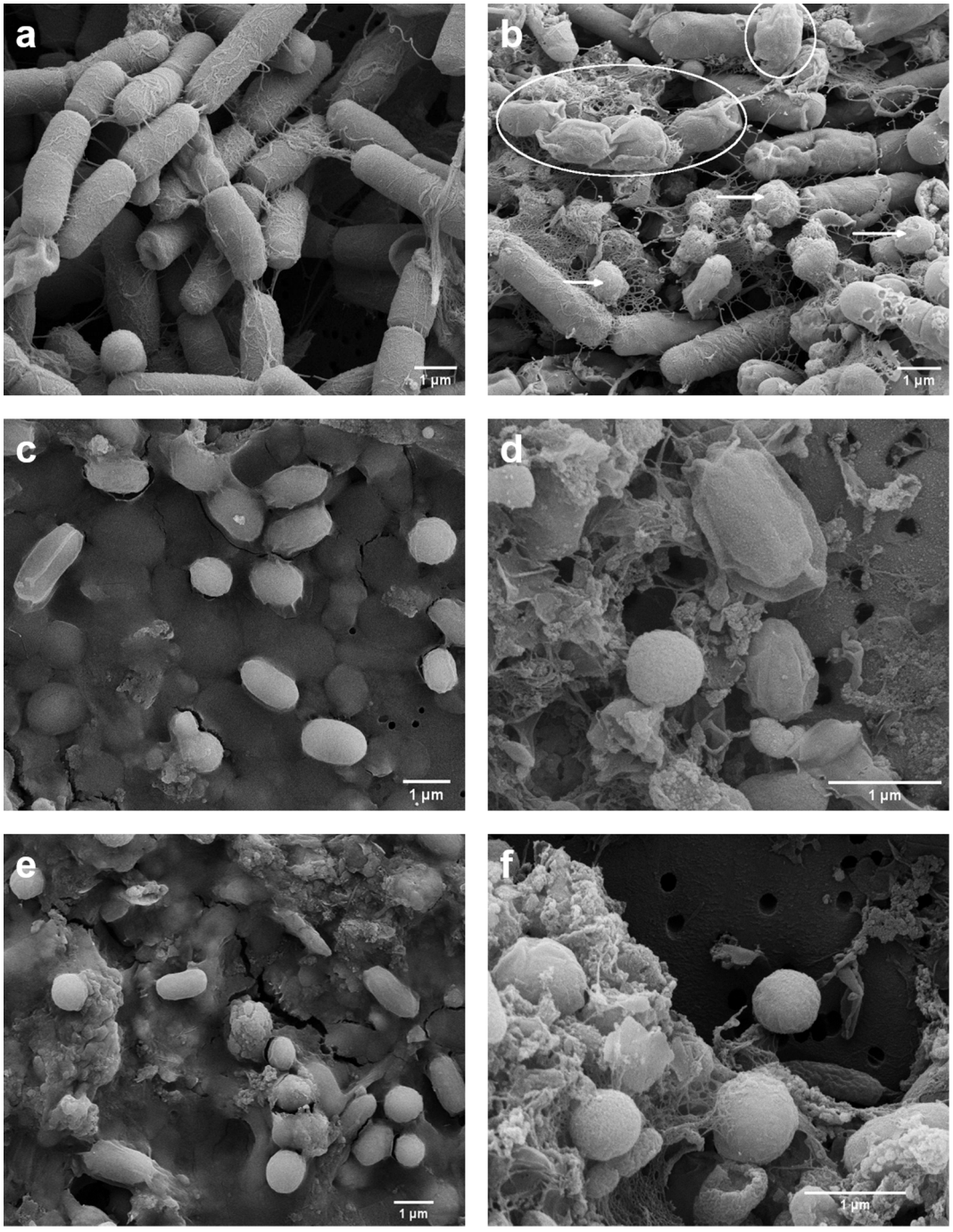

Fig. 3. SEM images of Bti pure culture (a) vegetative cells and (b) spores in circles and spherical crystals pointed with arrows; (c, d) 96 h SSF sample of digested sewage sludge as cosubstrate; (e, f) and 96 h SSF sample of digested OFMSW as a cosubstrate.

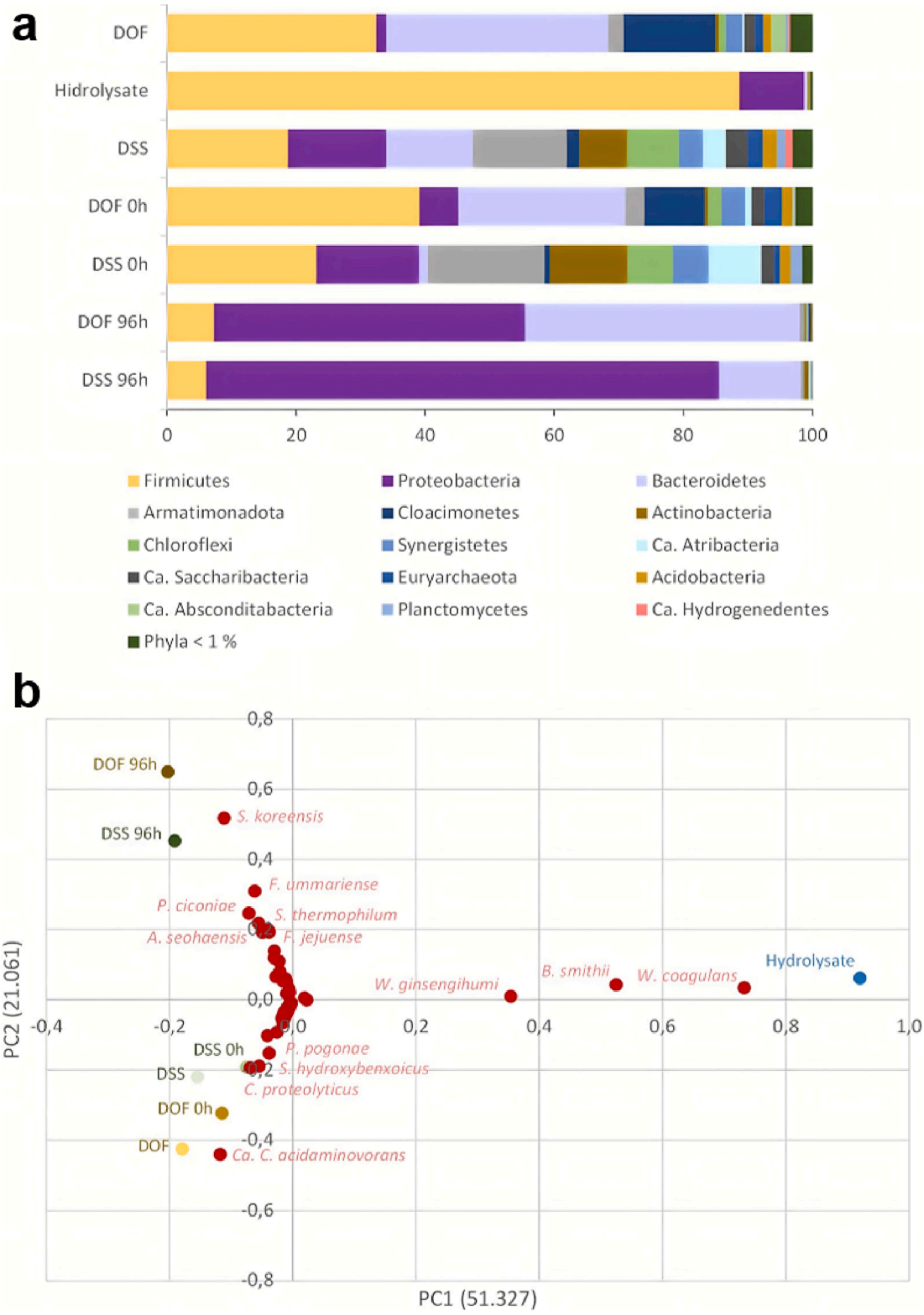

Fig. 4. Prokaryote community structure of the substrates and processes analyzed. a) Distribution by phyla expressed as a function of relative abundances. b) Principal Component Analysis. Biplot graph showing similarity between samples and relationship with dominant species (relative abundance <1 % in at least some of the samples). c) Heat map of the relative abundances for the dominant population (Firmicutes; Synergistetes; Armatimonadota; Ca. Atribacteria; Coprothermobacterota; Chloroflexi; Actinobacteria; Ca. Hydrogenedentes; Proteobacteria; Ca. Saccharibacteria; Ca. Absconditabacteria; Bacteroidetes; Ca. Cloacimonetes; Euryarchaeota). On the left, the evolutionary relationships found between these ASVs (Neighbor-Joining Method, combined with the Maximum Composite Likelihood Method to compute evolutionary distances). The upper part shows the dendrogram grouping the samples.

Fig. 4. (*continued*).

organic matter (Table 1). However, considering the faster development of the DOF fermentation process, the pH drop might have occurred during the first 24 h. The use of cosubstrates appears as an effective alternative to keep pH within safe values for Bti, contrary to the use of chemicals for pH control, which is rather difficult for SSF processes, at large scales (Kumar et al., 2021). Additionally, chemical reagents are only useful to adjust pH at the beginning of the SSF process as was observed in previous work (Molina-Peñate et al., 2022c).

3.2. Temperature considerations for further scale-up

Heat transfer is one of the major challenges for scaling up SSF processes (Kumar et al., 2021; Soccol et al., 2017). Especially for packed-bed bioreactors because axial and radial temperature and humidity gradients appear as a consequence of metabolic heat generation and heat transfer mechanisms within the bed (Casciatori et al., 2016; Rodrigues et al., 2022). Since more microbial activity and consequent temperature rise can be anticipated when working with non-sterile substrates, this overheating becomes even more important. Therefore, the temperature was monitored using button sensors dispersed in various locations throughout the bioreactor to assess the impact of each cosubstrate on the packed-bed temperature.

The temperature profile and the sOUR profile peaked at the same time for both cosubstrates (Fig. 2 **c, d**), indicating that the temperature rise was indeed caused by the metabolic heat generation of microbial growth. DOF reached slightly higher temperature values (42°C) compared to DSS (39°C) at this scale. Temperature increase for DOF was faster than for DSS, which might have also led to a lower viable cell count. In both scenarios, Bti continued to grow and sporulate (Fig. 2 **a, b**) despite the highest temperature. Even though these temperatures can be deemed as high, a previous study using soy waste as a solid substrate for Bt showed that Bt spores can tolerate temperatures as high as 60°C (Ballardo et al., 2016). Therefore, there is still some room when scaling up. However, it should also be considered that temperature can affect secondary metabolites (Odeniyi and Adeola, 2017), such as toxic proteins, and thus, the final choice might be influenced by a deep characterization of the biopesticide activity. The temperature profiles at the different locations inside the reactor's bed followed the same trend, being around 5°C lower in the middle point for both scenarios (Fig. 2**d**). This favors the exploitation of the entire packed bed and a homogeneous production. The use of digested cosubstrates with low biodegradability can explain the lower temperature variations observed according to Barrena et al. (2011).

3.3. Visualization of biopesticide crystal proteins

The presence of Bti spores and crystals in the fermented products was confirmed by scanning electron microscopy (SEM). Identification was done by visual comparison with a pure culture and by size measurements. Fig. 3 shows SEM images of the pure culture and the fermented products using DSS and DOF. Spores are clearly visible in both SSF (Fig. 3**d** and Fig. 3**e**), and presented an average size of 1.3 ± 0.2 μm × 0.8 ± 0. 1 μm (N = 25) for SSW and 1.2 ± 0.2 μm × 0.7 ± 0.1 μm (N = 25) for DOF, respectively. Both are within the expected size for Bti spores (0.71–1.93 μm long × 0.47–1.14 wide) (Loutfi et al., 2021). They also presented the characteristic exosporium of some *Bacillus* species (in detail in Fig. 3**d**) that contributes to spore survival and virulence (Peng et al., 2016). Bti toxic proteins crystallize into a spherical form that is released into the environment during sporulation as can be observed in Fig. 3**b**. In the fermented products, crystals showed a diameter of 0.7 ± 0.1 (N = 50) for SSW (Fig. 3**c**) and 0.9 ± 0.1 (N = 50) for SOF (Fig. 3**e**), with a smooth surface. During SEM analysis of the SSF products, vegetative cells (Fig. 3**a**) could not be clearly distinguished, probably due to their low abundance considering the high sporulation ratios observed (Table 2). Other microorganisms with different morphologies could also be seen, in this sense, more diversity was observed in the DOF sample, as discussed in the microbial population analysis.

3.4. Effect of cosubstrates on the microbial communities

The microbiota associated with any type of material is conditioned by the physicochemical properties of the material and by the structural and nutritional characteristics of the molecules of which it is composed. The results obtained in the present study support this, both in terms of the total population and the structure of the dominant community (Relative abundance >1%). In the case of the complete population, the analysis at the phylum level (Fig. 4**a**) showed both similarities between the starting cosubstrates used and the initial times of the fermentation process and divergences between the latter two, while at the end of fermentation, the degree of similarity between the bacterial community representative of both SSF experiments was considerably high. As fermentation proceeds, the metabolic activity derived from the microbiota promotes changes in the environment, which exert a selective pressure effect on the starting bacterial community (Shen et al., 2021). Thus, despite initial differences, the convergence towards similar conditions brought about by this activity tends to increase the similarity between the microbial populations associated with the two processes. Specifically, there is a sharp decrease in the abundance of ASVs belonging to the phylum Firmicutes, while the presence of representatives of the phyla Bacteroidetes and Proteobacteria increases considerably **(**Fig. 4**b**). The percentage increase was higher in the SSF made with DOF as cosubstrate, given that the starting levels were very low, although the relative abundance was higher in the one that used DSW. In any case, in both processes, the proteobacteria was the majority group at the end of fermentation. Members of the phyla Proteobacteria and Bacteroidetes are recognized for their ability to degrade macromolecules present in organic substrates (Ventorino et al., 2015). This capacity is also associated with bacteria belonging to the phylum Firmicutes, although the latter prevail in degradative processes where high temperatures are reached (Hosseini Koupaie et al., 2021), which is not the case. Precisely, the dominance of the phylum Firmicutes in the solid residue after enzymatic hydrolysis (relative abundance close to 90%) clearly differentiated the microbiota of this substrate from the rest. On the other hand, this clear difference shows that the microbial community of the starting materials was conditioned to a much greater extent by the cosubstrates used than by the solid residue after enzymatic hydrolysis.

The aforementioned changes in the microbial population structure, with a clear dominance of the Proteobacteria and Bacteroidetes phyla at the end of fermentation, were clearly reflected in the α diversity indices (Table 3). The microbiota associated with the final fermentation times showed lower richness and diversity, especially in the case of DOF, for which the number of ASVs and the Chao1 index value were reduced by almost 44%, while the diversity according to the Shannon index fell by 1.4 units. This is a common profile in fermentation processes (Yong et al., 2011; Yu et al., 2009), probably due to the change in conditions generated by the process itself. Such changes lead to a highly selective environment that negatively affects the diversity of the microbial community present (Yang and Wang, 2019). However, the lowest diversity was detected in the solid residue after enzymatic hydrolysis, for which the Shannon index showed a value of 1.656, typical of a poor community in terms of diversity. Similar bacterial communities have been described on substrates of this nature, obtained from the enzymatic hydrolysis of solid matter (Palomo-Briones et al., 2021) so that a loss of diversity of the solid residue after enzymatic hydrolysis in relation to its source material seems to be common.

In line with the above, the changes produced in the microbiome of the process will be fundamentally conditioned by the type of fermentation promoted, i.e. by the nature of the metabolites generated and the physicochemical conditions they favor. The results revealed by the Principal Component Analysis (PCA) seem to confirm this hypothesis (Fig. 4**b**) as it is clear that both processes, regardless of the starting

Table 3
α Diversity indices found for the substrates and processes analyzed.

	Sewage sludge	Digestate	Hydrolysate	DSS		DOF	
				0 h	96 h	0 h	96 h
Number of ASVs	1346	914	230	525	403	1043	587
Goods coverage	98.9	99.6	99.8	97.4	99.8	99.6	99.8
Chao1	1528.3	959.0	349.8	661.9	636.1	1135.6	636.1
Shannon	4.91	4.38	1.66	4.03	3.53	4.59	3.17
Simpson	0.972	0.955	0.716	0.919	0.945	0.969	0.873

DSS, digested sewage sludge. DOF, digested organic fraction. ASVs, amplicon sequence variant.

material, evolved in a similar way, as far as the taxonomic affiliation of the dominant species at the end of fermentation is concerned. This conclusion is also supported by the dendrogram generated according to the same criteria (Fig. 4**c**). The clustering of the samples points to the similarity between the initial process times and the corresponding starting materials, as well as between the final samples of both processes. The study by Shen et al. (2021), in the same direction, postulates that the initially present microbiota, as it develops its metabolic activity, generates a selective environment that gives rise to a bacterial community in which the dominant species show a high degree of phylogenetic closeness. This is the phenomenon conceptually known as homogeneous selection (Dini-Andreote et al., 2015).

In particular, in the present work, it was observed how the dominance of species belonging to the phylum Firmicutes (*Sedimentibacter*, *Weizmannia* and *Mageeibacillus*), in addition to the species *Porphyromonas pogonae* and *Candidatus Cloacimonas acidominovorans*, in the initial samples representative of DOF, and *Coprothermobacter proteolyticus* and *Levilinea saccharolytica* in those from the same DWS material, resulted in a common community at the end time of both fermentations, consisting mainly of *Stenotrophomonas*, *Acinetobacter*, and *Sphingobacterium*. Additionally, at DSW 96 h, *Psychrobacter* stood out, while *Flavobacterium* did the same at DOF 96 h. Most of the bacteria belonging to these genera are characterized by their metabolic activity associated with organic matrices, which makes them regular members of the microbial community present in fermentative processes of organic substrates from different human activities (Jung and Park, 2015; Ryan et al., 2009; Sun et al., 2013). The preferential presence of *Psychrobacter* in DSW 96 h may be due to the thermal sensitivity of the species of this genus, whose growth limit is around 37°C (Welter et al., 2021), a value close to the maximum reached in this process, but lower than that detected in DOF. *Flavobacterium*, on the other hand, groups species of cosmopolitan distribution, being among the environments in which they have been located those related to the food sector, as OFMSW, in which its powerful and diverse arsenal of extracellular enzymes is of special importance (Kolton et al., 2016).

Concerning the microbial community composition of the solid residue after enzymatic hydrolysis at the species level, as expected, clear differences were again observed with any of the other microbiomes. In this case, only three bacteria belonging to the evolutionarily close genera *Bacillus* and *Weizmannia* accounted for almost 90% of the population. The conditions under which the enzymatic hydrolysis was carried out (50°C, 24 h) probably exerted a strong selective effect on the bacterial population present, favoring the persistence and dominance of thermo-resistant species, as the three mentioned above.

3.5. Integration of the proposed strategy in an OFMSW biorefinery

Recycling OFMSW into higher-value products will directly contribute to the transition from the current fossil-based economy to a bioeconomy and more sustainable society (Budzianowski and Postawa, 2016; Sánchez et al., 2015). In this article, the use of the residual solids of enzymatic hydrolysis for biopesticide production through SSF has been tested successfully at a representative scale. An integration of this system into the current management scenario based on AD is proposed in Fig. 5. Here, each kg of dry OFMSW is converted into around 338 g of reducing sugars with potential use in liquid fermentation systems. From the residual solids, around 10^8 spores of the microbial biopesticide, Bt can be produced. Thus, by redirecting a part of the incoming high-quality OFMSW into the treatment plant, two high-value products can be obtained besides the energy produced in the AD system. This overall mass balance set the basis for future calculations of the environmental and economic impact of the process that will ultimately determine the scalability of the process. Future research should also explore the possibility of achieving a more self-sufficient SSF process, for instance by

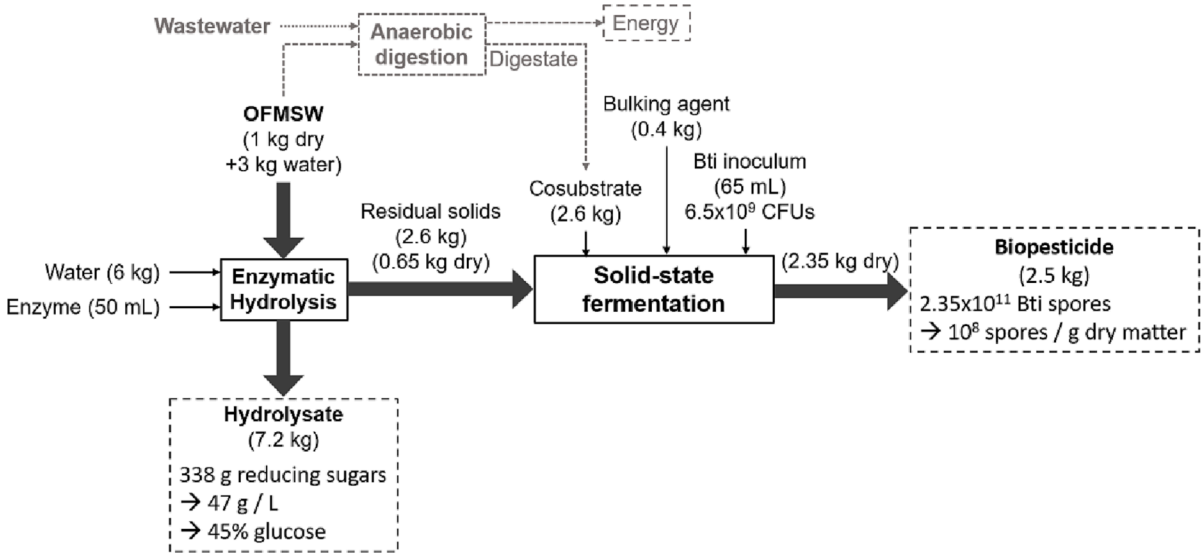

Fig. 5. Scheme and overall mass balance of the proposed OFMSW valorization route.

implementing a sequential batch operation that eliminates the inoculum preparation step and maximizes the use of the solid materials. This strategy has proved to be successful in other SSF processes such as the production of fungal biopesticides or aromas (Sala et al., 2021; Martínez et al., 2018).

4. Conclusions

Bacillus thuringiensis var *israelensis* has been successfully grown on the solid residue after enzymatic hydrolysis of OFMSW at a representative solid-state fermentation scale. A strategy based on the use of digested cosubstrates maintained pH from reaching acidic inhibitory values and prevented temperature increase to thermophilic conditions (<45 °C). Thus, two significant challenges of SSF: scale-up and pH control, were overcome. A maximum of 4×10^8 spores per g of DM was obtained when using digested sewage sludge, the presence of crystal proteins was confirmed and the microbial community was systematically analyzed.

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.

Data availability

Data will be made available on request.

Acknowledgments

The authors thank *ASA Spezialenzyme GmbH*, for providing the enzymatic cocktail; *Mancomunitat de la Plana*, for the OFMSW; *Consorci per a la Gestió dels Residus del Vallès Oriental*, for the digested biowaste; and *EDAR Riu Sec de Sabadell*; for the digested sewage sludge. Esther Molina thanks Generalitat de Catalunya for her industrial doctorate grant (DI 7, 2019). This work was supported by the Horizon 2020 Program within the SCALIBUR project [grant agreement No 817788] and the Spanish *Ministerio de Ciencia e Innovación* in the call *Proyectos de I+D+i en líneas estratégicas 2022,* Project **FertiLab,** reference PLEC2022-009252.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2023.02.029.

References

Angelo, E.A., Vilas-Bôas, G.T., Castro-Gómez, R.J.H., Lopes, J., 2015. Utilisation of response surface methodology to optimise the culture medium for *Bacillus thuringiensis* subsp. *israelensis*. Biocontrol Sci. Technol. 25, 414–428. https://doi.org/10.1080/09583157.2014.983457.

Arora, S., Singh, P., Rani, R., Ghosh, S., 2018. Oxygen uptake rate as a tool for on-line estimation of cell biomass and bed temperature in a novel solid-state fermentation bioreactor. Bioprocess Biosyst. Eng. 41, 917–929. https://doi.org/10.1007/s00449-018-1923-2.

Ballardo, C., Abraham, J., Barrena, R., Artola, A., Gea, T., Sánchez, A., 2016. Valorization of soy waste through SSF for the production of compost enriched with *Bacillus thuringiensis* with biopesticide properties. J. Environ. Manage. 169, 126–131. https://doi.org/10.1016/j.jenvman.2015.12.029.

Barrena, R., Gea, T., Ponsá, S., Ruggieri, L., Artola, A., Font, X., Sánchez, A., 2011. Categorizing raw organic material biodegradability via respiration activity measurement: a review. Compost Sci. Util. 19, 105–113. https://doi.org/10.1080/1065657X.2011.10736985.

Budzianowski, W.M., Postawa, K., 2016. Total chain integration of sustainable biorefinery systems. Appl. Energy 184, 1432–1446. https://doi.org/10.1016/j.apenergy.2016.06.050.

Campuzano, R., González-Martínez, S., 2016. Characteristics of the organic fraction of municipal solid waste and methane production: a review. Waste Manag. 54, 3–12. https://doi.org/10.1016/j.wasman.2016.05.016.

Casciatori, F.P., Bück, A., Thoméo, J.C., Tsotsas, E., 2016. Two-phase and two-dimensional model describing heat and water transfer during solid-state fermentation within a packed-bed bioreactor. Chem. Eng. J. 287, 103–116. https://doi.org/10.1016/j.cej.2015.10.108.

Cerda, A., Artola, A., Font, X., Barrena, R., Gea, T., Sánchez, A., 2018. Composting of food wastes: status and challenges. Bioresour. Technol. 248, 57–67. https://doi.org/10.1016/j.biortech.2017.06.133.

Cerda, A., Mejias, L., Rodríguez, P., Rodríguez, A., Artola, A., Font, X., Gea, T., Sánchez, A., 2019. Valorisation of digestate from biowaste through solid-state fermentation to obtain value added bioproducts: a first approach. Bioresour. Technol. 271, 409–416. https://doi.org/10.1016/j.biortech.2018.09.131.

Dini-Andreote, F., Stegen, J.C., Van Elsas, J.D., Salles, J.F., 2015. Disentangling mechanisms that mediate the balance between stochastic and deterministic processes in microbial succession. Proc. Natl. Acad. Sci. U.S.A. 112, E1326–E1332. https://doi.org/10.1073/pnas.1414261112.

Flores-Tufiño, B., Figueroa-Martínez, F., Viniegra-González, G., Loera, O., 2021. Polyurethane foam as an inert support using concentrated media improves quality and spore production from *Bacillus thuringiensis*. World J. Microbiol. Biotechnol. 37, 1–9. https://doi.org/10.1007/s11274-021-03122-2.

Foda, M.S., Salama, H.S., Selim, M., 1985. Factors affecting growth physiology of *Bacillus thuringiensis*. Appl. Microbiol. Biotechnol. 22, 50–52. https://doi.org/10.1007/BF00252156.

Hosseini Koupaie, E., Lin, L., Bazyar Lakeh, A.A., Azizi, A., Dhar, B.R., Hafez, H., Elbeshbishy, E., 2021. Performance evaluation and microbial community analysis of mesophilic and thermophilic sludge fermentation processes coupled with thermal hydrolysis. Renew. Sustain. Energy Rev. 141, 110832 https://doi.org/10.1016/j.rser.2021.110832.

Jung, J., Park, W., 2015. *Acinetobacter* species as model microorganisms in environmental microbiology: current state and perspectives. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-015-6439-y.

Karki, R., Chuenchart, W., Surendra, K.C., Shrestha, S., Raskin, L., Sung, S., Hashimoto, A., Kumar Khanal, S., 2021. Anaerobic co-digestion: current status and perspectives. Bioresour. Technol. https://doi.org/10.1016/j.biortech.2021.125001.

Kolton, M., Erlacher, A., Berg, G., Cytryn, E., 2016. The *Flavobacterium* genus in the plant holobiont: ecological, physiological, and applicative insights. In: Microbial Models: From Environmental to Industrial Sustainability. Springer, Singapore, pp. 189–207. https://doi.org/10.1007/978-981-10-2555-6_9.

Kumar, V., Ahluwalia, V., Saran, S., Kumar, J., Patel, A.K., Singhania, R.R., 2021. Recent developments on solid-state fermentation for production of microbial secondary metabolites: challenges and solutions. Bioresour. Technol. 323, 124566 https://doi.org/10.1016/j.biortech.2020.124566.

Leege, P.B., 1998. Introduction of test methods for the examination of composting and compost, in: Beneficial co-utilization of agricultural, municipal and industrial by-products. Springer, Dordrecht, pp. 269–282. <https://doi.org/10.1007/978-94-011-5068-2_22>.

Loutfi, H., Fayad, N., Pellen, F., Jeune, B.L., Chakroun, M., Benfarhat, D., Lteif, R., Kallassy, M., Brun, G.L., Abboud, M., 2021. Morphological study of *Bacillus thuringiensis* crystals and spores. Appl. Sci. 11, 1–15. https://doi.org/10.3390/app11010155.

Martínez, O., Sánchez, A., Font, X., Barrena, R., 2018. Enhancing the bioproduction of value-added aroma compounds via solid-state fermentation of sugarcane bagasse and sugar beet molasses: operational strategies and scaling-up of the process. Bioresour. Technol. 263, 136–144. https://doi.org/10.1016/j.biortech.2018.04.106.

Mejias, L., Komilis, D., Gea, T., Sánchez, A., 2017. The effect of airflow rates and aeration mode on the respiration activity of four organic wastes: implications on the composting process. Waste Manag. 65, 22–28. https://doi.org/10.1016/j.wasman.2017.04.008.

Mejias, L., Estrada, M., Barrena, R., Gea, T., 2020. A novel two-stage aeration strategy for *Bacillus thuringiensis* biopesticide production from biowaste digestate through solid-state fermentation. Biochem. Eng. J. 161, 107644 https://doi.org/10.1016/j.bej.2020.107644.

Molina-Peñate, E., Artola, A., Sánchez, A., 2022a. Organic municipal waste as feedstock for biorefineries: bioconversion technologies integration and challenges. Rev. Environ. Sci. Biotechnol. 21, 247–267. https://doi.org/10.1007/s11157-021-09605-w.

Molina-Peñate, E., Sánchez, A., Artola, A., 2022b. Enzymatic hydrolysis of the organic fraction of municipal solid waste: Optimization and valorization of the solid fraction for *Bacillus thuringiensis* biopesticide production through solid-state fermentation. Waste Manag. 137, 304–311. https://doi.org/10.1016/j.wasman.2021.11.014.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030.

Molina-Peñate, E., Arenòs, N., Sánchez, A., Artola, A., 2022c. *Bacillus thuringiensis* production through solid-state fermentation using organic fraction of municipal solid waste (OFMSW) enzymatic hydrolysate. Waste Biomass Valor. https://doi.org/10.1007/s12649-022-01978-5.

Odeniyi, O.A., Adeola, O.J., 2017. Production and characterization of polyhydroxyalkanoic acid from *Bacillus thuringiensis* using different carbon substrates. Int. J. Biol. Macromol. 104, 407–413. https://doi.org/10.1016/j.ijbiomac.2017.06.041.

Palomo-Briones, R., de Montoya-Rosales, J., Razo-Flores, E., 2021. Advances towards the understanding of microbial communities in dark fermentation of enzymatic hydrolysates: diversity, structure and hydrogen production performance. Int. J. Hydrogen Energy 46, 27459–27472. https://doi.org/10.1016/j.ijhydene.2021.06.016.

Peng, Q., Kao, G., Qu, N., Zhang, J., Li, J., Song, F., 2016. The regulation of exosporium-related genes in *Bacillus thuringiensis*. Sci. Rep. 6, 1–12. https://doi.org/10.1038/srep19005.

Pleissner, D., Peinemann, J.C., 2020. The challenges of using organic municipal solid waste as source of secondary raw materials. Waste Biomass Valoriz. 11, 435–446. https://doi.org/10.1007/s12649-018-0497-1.

Pognani, M., Barrena, R., Font, X., Sánchez, A., 2012. Effect of freezing on the conservation of the biological activity of organic solid wastes. Bioresour. Technol. 104, 832–836. https://doi.org/10.1016/j.biortech.2011.11.097.

Ponsá, S., Gea, T., Sánchez, A., 2010. Different indices to express biodegradability in organic solid wastes. J. Environ. Qual. 39, 706–712. https://doi.org/10.2134/jeq2009.0294.

Rodrigues, N.A., Katayama, E.T., Casciatori, F.P., 2022. Alternative strategies to perform solid-state cultivation in a multilayer packed-bed bioreactor: continuous and cyclic operations. Chem. Eng. J. 448, 137726 https://doi.org/10.1016/j.cej.2022.137726.

Ryan, R.P., Monchy, S., Cardinale, M., Taghavi, S., Crossman, L., Avison, M.B., Berg, G., van der Lelie, D., Dow, J.M., 2009. The versatility and adaptation of bacteria from the genus *Stenotrophomonas*. Nat. Rev. Microbiol. 2009 77 7, 514–525. <https://doi.org/10.1038/nrmicro2163>.

Sala, A., Barrena, R., Sánchez, A., Artola, A., 2021. Fungal biopesticide production: Process scale-up and sequential batch mode operation with *Trichoderma harzianum* using agro-industrial solid wastes of different biodegradability. Chem. Eng. J. 425, 131620 https://doi.org/10.1016/j.cej.2021.131620.

Sánchez, A., Artola, A., Gea, T., Barrena, R., Font, X., 2015. A new paradigm for waste management of organic materials. Waste Manag. 42, 1–2. https://doi.org/10.1016/j.wasman.2015.05.002.

Shen, D., Shen, H., Yang, Q., Chen, S., Dun, Y., Liang, Y., Zheng, J., Zhao, S., 2021. Deciphering succession and assembly patterns of microbial communities in a two-stage solid-state fermentation system. Microbiol. Spectr. 9 https://doi.org/10.1128/spectrum.00718-21.

Soccol, C.R., da Costa, E.S.F., Letti, L.A.J., Karp, S.G., Woiciechowski, A.L., de Vandenberghe, L.P., S.,, 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71. https://doi.org/10.1016/j.biori.2017.01.002.

Sun, L.N., Zhang, J., Chen, Q., He, J., Li, S.P., 2013. *Sphingobacterium caeni* sp. *nov*., isolated from activated sludge. Int. J. Syst. Evol. Microbiol. 63, 2260–2264. https://doi.org/10.1099/ijs.0.046987-0.

Sundberg, C., Smårs, S., Jönsson, H., 2004. Low pH as an inhibiting factor in the transition from mesophilic to thermophilic phase in composting. Bioresour. Technol. 95, 145–150. https://doi.org/10.1016/j.biortech.2004.01.016.

Tonini, D., Martinez-Sanchez, V., Astrup, T.F., 2013. Material resources, energy, and nutrient recovery from waste: Are waste refineries the solution for the future? Environ. Sci. Technol. 47, 8962–8969. https://doi.org/10.1021/es400998y.

Ventorino, V., Aliberti, A., Faraco, V., Robertiello, A., Giacobbe, S., Ercolini, D., Amore, A., Fagnano, M., Pepe, O., 2015. Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application. Sci. Reports 2015 51 5, 1–13. <https://doi.org/10.1038/srep08161>.

Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R., 2007. Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267. https://doi.org/10.1128/AEM.00062-07.

Welter, D.K., Ruaud, A., Henseler, Z.M., De Jong, H.N., van Coeverden de Groot, P., Michaux, J., Gormezano, L., Waters, J.L., Youngblut, N.D., Ley, R.E., 2021. Free-Living, psychrotrophic bacteria of the genus *Psychrobacter* are descendants of pathobionts. mSystems 6. <https://doi.org/10.1128/mSystems.00258-21>.

Yang, G., Wang, J., 2019. Changes in microbial community structure during dark fermentative hydrogen production. Int. J. Hydrogen Energy 44, 25542–25550. https://doi.org/10.1016/j.ijhydene.2019.08.039.

Yong, X., Cui, Y., Chen, L., Ran, W., Shen, Q., Yang, X., 2011. Dynamics of bacterial communities during solid-state fermentation using agro-industrial wastes to produce poly-γ-glutamic acid, revealed by real-time PCR and denaturing gradient gel electrophoresis (DGGE). Appl. Microbiol. Biotechnol. 92, 717–725. https://doi.org/10.1007/s00253-011-3375-3.

Yu, Z., Dong, B., Lu, W., 2009. Dynamics of bacterial community in solid-state fermented feed revealed by 16S rRNA. Lett. Appl. Microbiol. 49, 166–172. https://doi.org/10.1111/j.1472-765X.2009.02636.x.