Universitat Autònoma de Barcelona

Enzymatic hydrolysis of the Organic Fraction of Municipal Solid Waste: Valorization of the exhausted solid for biopesticide production by solid-state fermentation

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PhD Thesis

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Certifiquem:

Que la biotecnòloga **Esther Molina Peñate** ha realitzat sota la nostra direcció el treball amb títol *"Enzymatic hydrolysis of the Organic Fraction of Municipal Solid Waste: Valorization of the exhausted solid for biopesticide production by solid-state fermentation"*, que es presenta en aquesta memòria i que constitueix la seva Tesi per optar al Grau de Doctor en Ciència i Tecnologia Ambiental per la Universitat Autònoma de Barcelona.

I, per a que se'n tingui coneixement i consti als efectes oportuns, presentem a l'Escola d'Enginyeria de la Universitat Autònoma de Barcelona l'esmentada Tesi, signant el present certificat.

Dr. Antoni Sánchez Ferrer Dra. Adriana Artola Casacuberta Dr. Óscar Prado Rubianes

Bellaterra, gener de 2023

A mis abuelas,

"As a scientist I am only an ant insufficient and anonymous. But I am stronger than I look and part of something that is much bigger than I am." – Hope Jahren

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Overview

Recent European legislation requires the separate collection and the proper management of the organic fraction of municipal solid waste (OFMSW). In the framework of the EU project SCALIBUR (Horizon 2020, grant agreement No 817788), this thesis evaluates the valorization of the OFMSW in a biorefinery-like scenario based on the use of enzymatic hydrolysis to obtain building chemicals that can be further used in fermentative processes. In particular, this work focuses on the use of solid-state fermentation (SSF) as a technology for the production of biopesticides from the solid fraction obtained after an enzymatic hydrolysis of the OFMSW to obtain low molecular weight reducing sugars.

First, the enzymatic hydrolysis was studied using a high-quality OFMSW (low content of impurities). The process was optimized for key operational parameters using a commercial enzymatic cocktail and compared with a tailor-made cocktail from the SCALIBUR project.

Then, the SSF process for the production of *Bacillus thuringiensis* (Bt) biopesticides from the obtained solid enzymatic hydrolysate was developed at a laboratory scale (0.5 L). Different approaches to control pH, which was identified as a key parameter for the growth of Bt, were tested. The use of urban waste materials as cosubstrates appeared as an efficient and simple strategy to maintain pH near neutrality. 1.6 L scale bioreactors were used to evaluate the effects of non-sterile cosubstrates on process temperature. Despite the higher temperatures reached, the cosubstrate mixtures were more robust in terms of pH stability and steady growth of Bt than the solid hydrolysate. A prototype reactor (22 L) was used to validate the strategy for two cosubstrates: anaerobically digested sewage sludge and anaerobically digested OFMSW. At this scale, the final production of Bt spores, which are associated with biopesticide activity, was slightly reduced but the process remained competitive in comparison to previously published results. Moreover, the strategy was evaluated for the enzymatic hydrolysate of the SCALIBUR project at a 100 L scale although difficulties related to heat accumulation and microbial competition appeared.

Finally, preliminary mass balance and economic evaluation were performed to evaluate the viability of the proposed biorefinery scheme. Despite the high-value bioproducts produced, enzyme cost was a limiting factor. Therefore, alternative biorefinery configurations to reduce this cost are discussed, such as the production of enzymatic cocktails from organic waste.

This thesis presents a novel approach for the valorization of the OFMSW beyond composting and anaerobic digestion, to have a wider number of technological alternatives to reach the sustainability principle of securing resources for future generations.

Resumen

La reciente legislación europea requiere la recogida selectiva y gestión adecuada de la fracción orgánica del residuo sólido urbano (FORSU). En el marco del proyecto europeo SCALIBUR (Horizon 2020, grant agreement No 817788), esta tesis evalúa la valorización de la FORSU en un escenario tipo biorrefinería basado en el uso de la hidrólisis enzimática para la obtención de componentes químicos esenciales que puedan ser posteriormente usados en procesos fermentativos. En concreto, el trabajo se centra en el uso de la fermentación en estado sólido (FES) como una tecnología para producir biopesticidas a partir de la fracción sólida obtenida después de una hidrólisis enzimática para producir azúcares reductores de bajo peso molecular de la FORSU.

Primero, se estudió la hidrólisis enzimática usando una FORSU de gran calidad (bajo contenido de impropios). Fue optimizada para parámetros clave usando un cóctel enzimático comercial, que se comparó con uno desarrollado para la FORSU en el proyecto SCALIBUR.

A continuación, se desarrolló el proceso de FES para producir *Bacillus thuringiensis* (Bt) a partir del hidrolizado sólido en escala laboratorio (0.5 L). Se evaluaron métodos para controlar el pH, identificado como un parámetro clave para el desarrollo del Bt. El uso de otros residuos urbanos como cosustratos emergió como una estrategia eficiente para mantener los valores de pH cercanos a la neutralidad. El efecto del uso de cosustratos no estériles en la temperatura del proceso fue evaluado en reactores de 1.6 L. A pesar de la mayor temperatura, las mezclas con más cosustratos fueron mejores para mantener estable el pH y promover el crecimiento del Bt. Después, se utilizó un reactor prototipo (22 L) para validar la estrategia usando digestato de lodos de depuradora y de FORSU como cosustratos. La producción de esporas, asociadas con la actividad biopesticida, se vio ligeramente reducida, pero se mantuvo competitiva en comparación con resultados publicados anteriormente. Por otra parte, se evaluó la estrategia para el hidrolizado sólido proveniente del proyecto SCALIBUR en un reactor de 100 L donde aparecieron dificultades por la acumulación de calor y contaminación microbiana.

Por último, se llevó a cabo una evaluación preliminar de la propuesta de biorrefinería en términos económicos para evaluar su viabilidad. A pesar de producir bioproductos de alto valor añadido, el coste de las enzimas fue un factor limitante. Se proponen configuraciones alternativas para reducir su impacto, como la producción de las mismas a partir de residuos.

Esta tesis presenta un enfoque novedoso para la valorización de la FORSU más allá del compostaje y la digestión anaerobia, para tener más alternativas tecnológicas para alcanzar el principio de sostenibilidad de asegurar los recursos para las generaciones futuras.

Resum

La legislació europea recent requereix la recollida selectiva i la gestió adequada de la fracció orgànica del residu sòlid municipal (FORM) En el marc del projecte europeu SCALIBUR (Horizon 2020, grant agreement No 817788), aquesta tesi avalua la valorització de la FORM en un escenari tipus biorefineria basat en l'ús de la hidròlisi enzimàtica per obtenir components químics essencials que puguin ser utilitzats en processos fermentatius. En concret, l'estudi es centra en l'ús de la fermentació en estat sòlid (FES) com una tecnologia per produir biopesticides a partir de la fracció sòlida resultant d'una hidròlisi enzimàtica de la FORM per produir sucres reductors de baix pes molecular.

En primer lloc, es va estudiar la hidròlisi enzimàtica utilitzant FORM de gran qualitat (baix contingut d'impropis). Es van optimitzar els paràmetres clau del procés utilitzant un còctel enzimàtic comercial y es va comparar amb un còctel enzimàtic produit dins el projecte SCALIBUR específic per a la FORM.

A continuació, es va desenvolupar el procés de FES a escala laboratori (0,5 L) per produir *Bacillus thuringiensis* (Bt) a partir de l'hidrolitzat sòlid. Es van avaluar mètodes per controlar el pH, identificat com un paràmetre clau per al el desenvolupament del Bt. L'ús d'altres residus urbans com a cosubstrats va resultar una estratègia eficient per mantenir els valors de pH. L'efecte de l'ús de cosubstrats no estèrils en la temperatura del procés es va avaluar en bioreactors de 1.6 L. Les barreges que utilitzaven major quantitat de cosubstrats van mantenir més estable el pH i promoure el creixement del Bt, tot i que van assolir temperatures majors. Després, es va utilitzar un prototip (22 L) per validar l'estratègia utilitzant digestat de llots de depuradora i de FORM com a cosubstrats. A aquesta escala, la producció d'espores, associades a la activitat biopesticida, es va veure reduïda, però mantenint-se competitiva si es compara amb resultats publicats anteriorment. Per altra part, es va avaluar l'estratègia desenvolupada per al cas de l'hidrolitzat sòlid del projecte SCALIBUR en un reactor de 100 L i es van trobar dificultats causades per l'acumulació de calor i la contaminació microbiana.

Per últim, es va dur a terme una avaluació preliminar de la proposta de biorefineria en termes econòmics per avaluar la seva viabilitat. Tot i produir bioproductes d'alt valor afegit, el cost dels enzims és un factor limitant. Es proposen configuracions alternatives per reduir el seu impacte, com la producció dels enzims a partir de residus.

Aquesta tesi presenta un enfocament nou per a la valorització de la FORM, més enllà del compostatge i digestió anaeròbia, per tenir més alternatives tecnològiques per assolir el principi de sostenibilitat d'assegurat els recursos per a futures generacions.

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AD	-	Anaerobic Digestion	
AFP	-	Air Filled Porosity	%
AT4	-	Cumulative oxygen uptake in four days	g O_2 kg^{-1} DM
BD	-	Bulk Density	g L^{-1}
Bt	-	*Bacillus thuringiensis*	
Bti	-	*Bacillus thuringiensis* var *israelensis*	
Btk	-	*Bacillus thuringiensis* var *kurstaki*	
CFU	-	Colony Forming Units	
DM	-	Dry Matter content	%
DRI	-	Dynamic Respiration Index	g O_2 kg^{-1} DM h^{-1}
FBG	-	Fungal Beta Glucanase units	
HORECA	-	Hotel, Restaurants and Catering	G
MC	-	Moisture Content	%
MSW	-	Municipal Solid Waste	
OFMSW	-	Organic Fraction Municipal Solid Waste	
OM	-	Organic Matter content	%
PHA	-	Polyhydroxyalkanoates	
PVC	-	Polyvinyl chloride	
RS	-	Reducing Sugars	
SEM	-	Scanning Electron Microscope	
SmF	-	Submerged Fermentation	
sOUR	-	Specific Oxygen Uptake Rate	g O_2 kg^{-1} DM h^{-1}
SSF	-	Solid-State Fermentation	
WWTP	-	Waste Water Treatment Plant	

Thesis overview

The work carried out during this thesis has been conducted in the Department of Chemical, Biological and Environmental Engineering of the *Universitat Autònoma de Barcelona* (UAB), at the Composting Research Group (GICOM, https://webs.uab.cat/gicom/) and in collaboration with AERIS *Tecnologías Ambientales S.L* under the Industrial Doctorates plan of *Generalitat de Catalunya* (DI-7, 2019). This research has been developed in the framework of the H2020 project SCALIBUR (Scalable Technologies for Bio-urban Waste Recovery), which has received funding from the European Union's Horizon 2020 research and innovation Programme under grant agreement No 817788.

The main motivation for developing this thesis was to provide a valorization method for the residual solid fraction obtained after an enzymatic hydrolysis of the organic fraction of municipal solid waste (OFMSW). Enzymatic hydrolysis was one of the proposed scenarios in the SCALIBUR project for the conversion of the OFMSW into building chemicals that can be transformed into a variety of high-value bioproducts through fermentative processes. Recent regulatory requirements advocate for the selective collection of municipal solid waste, which will lead to an increase in the quality and quantity of the organic fraction. The production of high-value bioproducts could mitigate the extensive MSW management costs and avoid the use of non-renewable resources. For this reason, extended knowledge of alternative uses for OFMSW beyond the well-established composting and anaerobic digestion processes can be helpful in order to assess their viability for industrial applications and their complementarity with the consolidated technologies.

The consolidated background of GICOM on the production of *Bacillus thuringiensis* from different organic waste types was used as the starting point of this study. A protocol for obtaining sugars and a solid enzymatic hydrolysate from OFMSW was developed and implemented. The thesis, which is presented as a compilation of publications, is structured into seven chapters. The first chapter provides a brief and comprehensive introduction to the topic, including current perspectives on OFMSW management and the technologies employed. Chapters 2 and 3 describe the main objectives of this work and the materials and methods to fulfill them. The final two chapters correspond to the general conclusions, accompanied by a proposal for further research, and the references cited alongside this document. Concerning the results obtained, presented in Chapter 4, they are divided into three publications that have been published in international indexed journals and one additional manuscript. These publications

include their individual introduction, materials and methods, results, conclusions and references sections.

The first publication is a review article (*Organic municipal waste as feedstock for biorefineries: bioconversion technologies integration and challenges, Reviews in Environmental Science and Bio/Technology*, 2021) that evaluates the potential uses for the solid and liquid streams after an enzymatic hydrolysis of the OFMSW. The second scientific article (*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 Management*, 2022), presents the optimization of the OFMSW enzymatic hydrolysis for a commercial enzyme cocktail and a preliminary assessment of the use of the solid fraction as a substrate for solid-state fermentation (SSF). The third publication (*Bacillus thuringiensis production through solid-state fermentation using organic fraction of municipal solid waste (OFMSW) enzymatic hydrolysate, Waste and Biomass Valorization*, 2022), comprises the development of an operational strategy to overcome the acidic pH of solid hydrolysates as substrates for *Bacillus thuringiensis* SSF. The fourth and last scientific article, presented as a manuscript, (*Filling in the gaps in biowaste biorefineries: the use of solid hydrolysates for the production of biopesticides trough solid state fermentation*, submitted to *Waste Management*, in October 2022), shows the scale-up process and an assessment of the final product.

Finally, Chapter 5 provides a critical discussion of the results obtained from an overall perspective.

Chapter 1 **Introduction**

First, the issue of organic municipal solid waste generation and management is presented. Then, a brief overview of the SCALIBUR project, in which this thesis is embedded, is provided. Consecutively, the main actor of this thesis, *Bacillus thuringiensis*, is introduced and insights into the technologies of enzymatic hydrolysis and, especially solid-state fermentation, are given.

1.1 The organic fraction of municipal solid waste: problem or opportunity?

1.1.1 Current management practices and legislation of municipal solid waste

Waste generation rates are rising globally due to rapid urbanization, population growth and overflowed waste streams in high-income countries. By 2050, global municipal solid waste (MSW) generation is expected to reach 3.4 billion tonnes (Kaza et al., 2018). Currently, solid waste management has become one of society's greatest challenges because of its associated high cost and environmental impact.

In Europe, different legislative actions have been taken to prevent the environmental issues of MSW disposal. The Landfill Directive 1999/31/EC (Directive, 1999), defines MSW as "waste from households, as well as other waste which, because of its nature or composition, is similar to waste from households, such as waste from commerce, offices and public institutions", and limits the share of municipal waste landfilled. In this way, the Waste Framework Directive 2008/98/EC (Directive, 2008) establishes a 5-step hierarchy for managing and disposing of waste where prevention is the best option, followed by re-use, recycling, other forms of recovery, and finally disposal. Therefore, the target of these Directives is to conduct MSW away of landfills into more environmentally favorable treatment options that allow for resource recirculation. According to recent estimates, the European Union (EU) generates over 230 million tonnes of MSW per year, corresponding to 517 kg per capita (**Figure 1.1a**) (Eurostat, 2020). As a result of the legislative efforts, the MSW disposed in landfills has decreased by half in 20 years, from 54% in 2000 to 24% in 2020, despite a 5% increase in waste generation. This has been possible by shifting MSW management towards incineration, composting and, in particular, material recycling (**Figure 1.1b**).

It is estimated that nearly 40% of MSW in Europe corresponds to green and food waste (Kaza et al., 2018). This fraction is known as the organic fraction of municipal solid waste (OFMSW), also referred to as biowaste, and it does not include forestry or agricultural residues, manure, sewage sludge, paper or other biodegradable waste. It is characterized by high moisture and organic matter content, a rather acidic pH and the presence of metals and other macro or micronutrients (Campuzano and González-Martínez, 2016). Due to its high biodegradability, OFMSW is the primary source of methane and other greenhouse gas emissions from landfilled MSW. Furthermore, its natural biodegradation also involves the generation of leachates that contaminate soil and groundwater if not properly handled (Wilson, 2007). Therefore, the

previous Directives aimed to promote the application of biological treatments, such as composting or anaerobic digestion, that allow for organic matter and nutrient recirculation, and/or energy recovery from OFMSW.

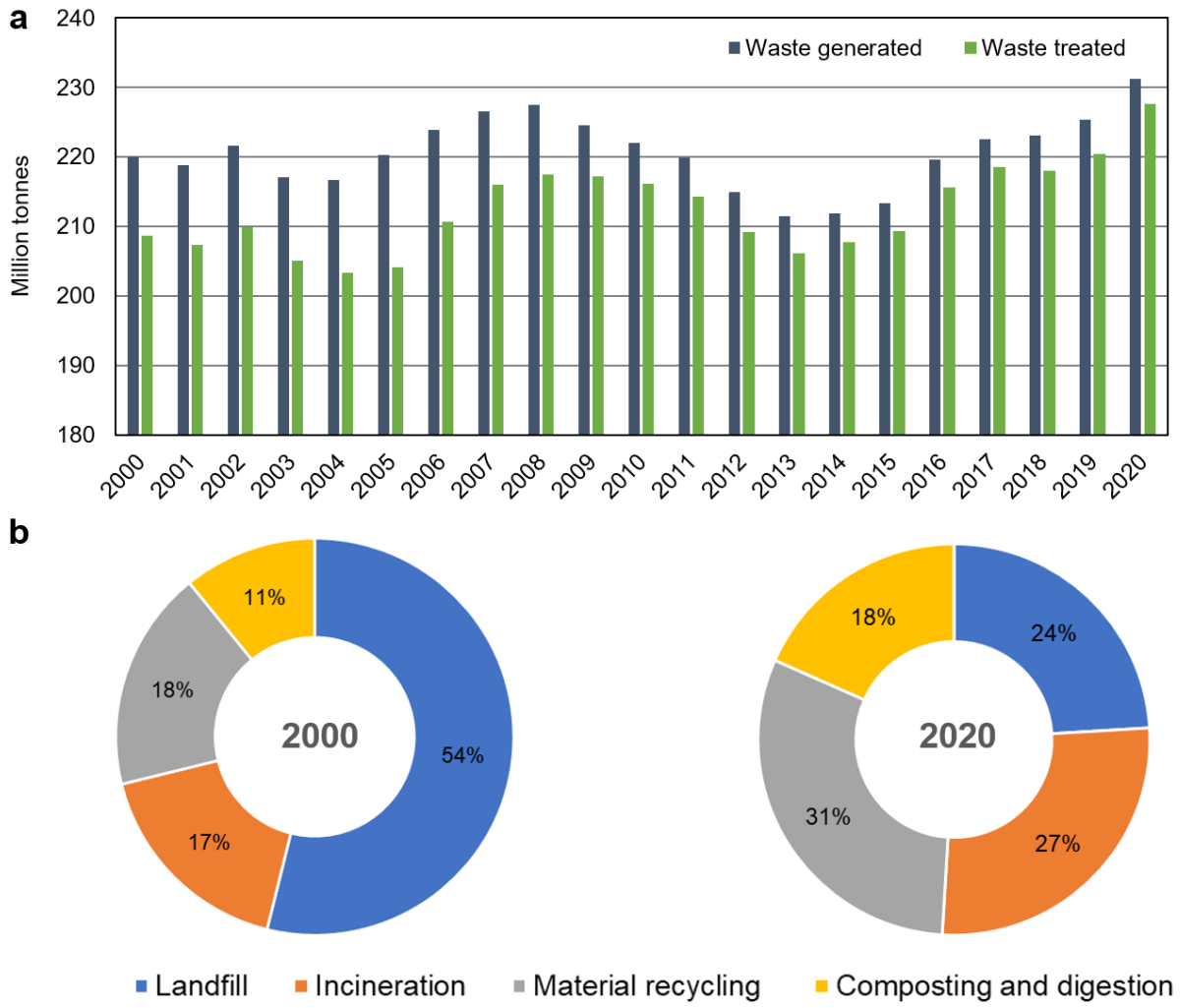

Figure 1.1 (a) Total MSW generated and treated in the EU countries from 2000 to 2020, and (b) distribution of the MSW treatment alternatives in 2000 and 2020, (Eurostat, 2020).

In contrast to material recycling performance, the increase in OFMSW recycling (composting and digestion) was more limited over the same period (**Figure 1.1b**). A reason for this trend can be that Directive 2008/98/EC (Directive, 2008) included the obligation to set up separate collection systems for paper, metal, plastic and glass by 2015 but not for biowaste, which increased materials recycling levels (European Environment Agency, 2013). In 2018, the revised Waste Framework and Landfill Directives (Directive 2018/851/EU & Directive 2018/850/EU) also required separate collection or recycling at the source for biowaste by 31 December 2023. Moreover, the incineration of separately collected biowaste is prohibited and

the amount of landfilled MSW is limited to 10% by 2030. Additionally, from 1 January 2027, only separately collected biowaste entering composting or anaerobic treatment may count as recycled to ensure good quality of compost and digestate. Consequently, the selective collection of OFMSW is going to increase in Europe in the coming years which, in turn, will lead to a greater quantity and quality of this MSW fraction.

1.1.1 New perspectives for the organic fraction of municipal solid waste: SCALIBUR H2020 project

Efficient MSW management is part of a bigger framework: the Roadmap to a Resource Efficient Europe (EC, 2011) and the Action Plan towards Circular Economy (EC, 2015), which aim to substantially improve the resource efficiency of the European economy and enable the transition from the linear "take-make-use-dispose" model to a regenerative growth model. In a circular economy, the value of products, materials and resources is maintained in the economy for as long as possible, reducing the use of natural resources and the generation of waste. In this context, the constituents of OFMSW are resources that can be transformed into bioproducts by biological processes. On average, OFMSW composition consists of 55.5% carbohydrates, which comprise free sugars, starch and fibers, 17.5% proteins, 17.5% lipids and 9.5% lignin (Campuzano and González-Martínez, 2016). However, OFMSW composition is heterogeneous and highly variable, not only depending on the origin but also on other parameters such as season, or metabolic activities of microbial consortia (Pleissner and Peinemann, 2020). Therefore, OFMSW valorization processes must be flexible and maintain stability regardless of substrate fluctuations (Nizami et al., 2017).

The European project SCALIBUR (Scalable Technologies for Bio-urban Waste Recovery) aims at closing the gap between technological feasibility and industrial application in the field of urban biowaste valorization by enhancing strategic cooperation between sectors. To do so, it proposes improvements across the whole biowaste value chain. At the recovery level, SCALIBUR targets three pilot territories, namely Madrid (Spain), Albano (Italy) and Kozani (Greece), in which the aim is to improve the quality and quantity of urban biowaste selectively collected by directly involving citizens and public authorities, and by technological developments. At the conversion level, three technological lines for the valorization of three urban waste streams are proposed, including the evaluation of the quality and the safety of the final products and a sustainability assessment. Finally, at the stakeholders' level, engagement is promoted by effective communication, involving citizens in "Biowaste clubs", building on

existing social innovation activities and creating new ones, such as The Biowaste Hub (https://thebiowastehub.com/) platform.

The developed technological conversion pathways target three important solid urban waste streams: OFMSW, urban sewage sludge from wastewater treatment plants (WWTP), and HORECA and retail biowaste (**Figure 1.2**). HORECA waste is transformed into proteins, lipids and chitin through insect rearing. Urban sewage sludge is converted into biogas through anaerobic digestion, which is then upgraded to high-value products by bioelectrosynthesis technologies. Also, it is converted to polyhydroxyalkanoates (PHA) polymers. Lastly, OFMSW is subjected to enzymatic hydrolysis followed by liquid fermentation of the sugar extract for the production of bioplastics and solid-state fermentation (SSF) of the residual solids for the production of biopesticides.

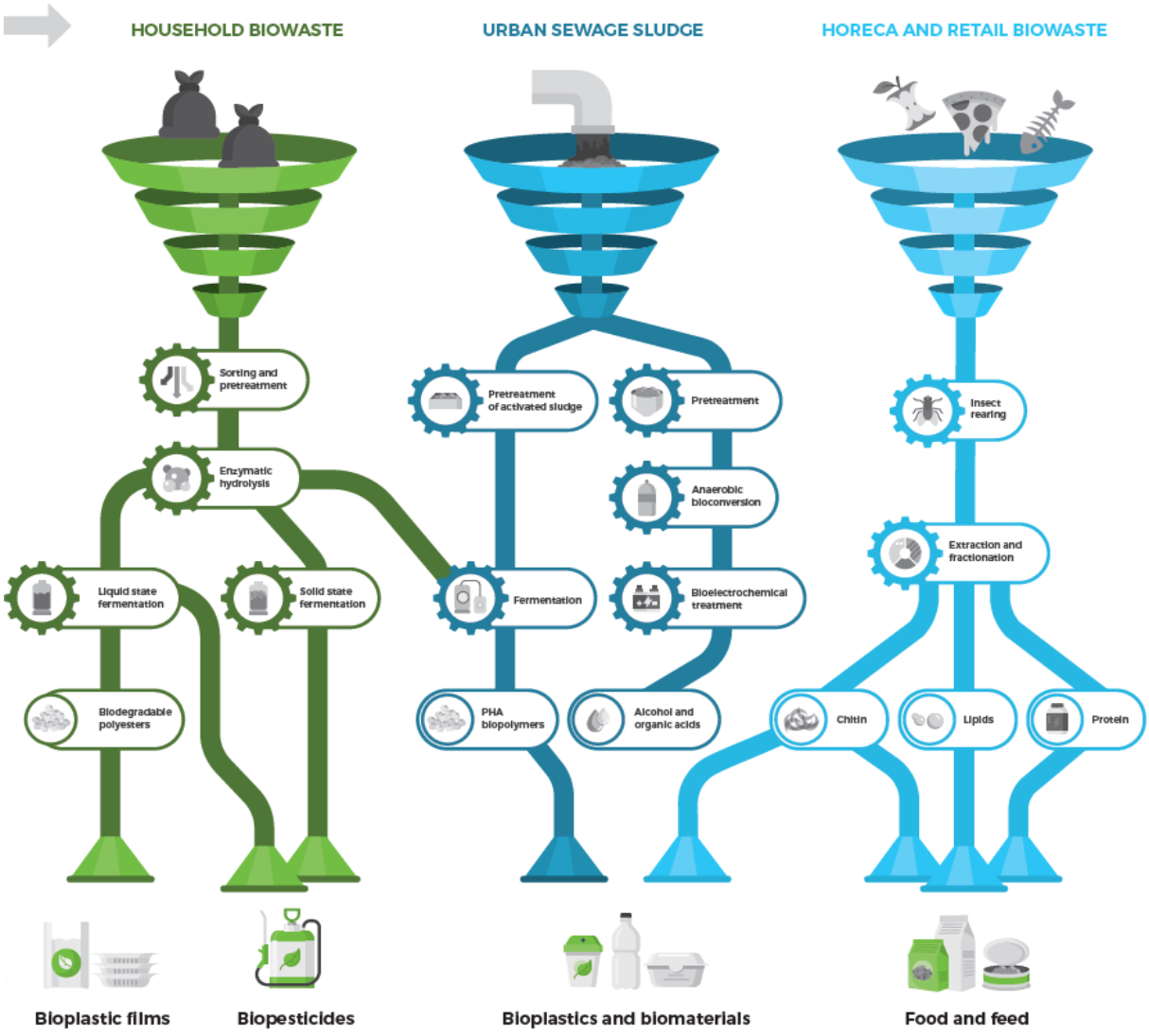

Figure 1.2 SCALIBUR's valorization routes to transform biowaste streams and urban sewage sludge into high added-value bioproducts (from https://scalibur.eu/).

The SCALIBUR project began in November 2018 and run for four years until October 2022. It received €10 million grant from the EU's Horizon 2020 Programme and involved 20 partners from seven countries.

Previously, the partnership GICOM – AERIS participated in the European project DECISIVE ("A decentralized management scheme for innovative valorization of urban biowaste") (http://www.decisive2020.eu/). The goal of this project was to demonstrate the feasibility of reducing the generation of urban biowaste while increasing the recycling rate through a decentralized management strategy. Therefore, biowaste was valorized in short cycles and following a zero-waste strategy, in line with the circular economy concept. To do so, two innovative solutions were proposed and validated, a micro-AD for the production of energy followed by an SSF of the generated digestate for the production of biopesticides (Mejias et al., 2020; Rodríguez et al., 2019).

1.2 Biopesticides

In agriculture, insect pest management is essential to ensure crop cultivation efficiency and quality while preventing food losses. However, the intensive application of chemical pesticides involves negative effects on the environment and human health. The careless overuse of pesticides has resulted in biodiversity loss and pest resistance emergence (Damalas and Koutroubas, 2018). Consequently, international regulations attempt to limit and control their use. For instance, the EU has set a 50% reduction target in the use and risk of chemical and more hazardous pesticides as part of the Farm to Fork Strategy (EC, 2020). However, considering the increasing world population, global pesticide demand is expected to increase to meet future food supply requirements. Therefore, there is an urgent need for sustainable and eco-friendly alternatives for insect pest control.

Biopesticides are natural materials derived from animals, plants, microorganisms, and certain minerals that have pesticide properties (Damalas and Koutroubas, 2018). Biopesticides are mainly classified into three categories depending on the active substance (Chandler et al., 2011). The first category is biochemical pesticides, which include a wide variety of secondary metabolites that are naturally produced by plants to control pests. The second group is referred to as semiochemicals, which are chemical signals produced by one organism that influence the behavior of an individual of the same or different species, such as sex pheromones. Lastly, microbial biopesticides are microorganisms, including bacteria, fungi, oomycetes, viruses and protozoa that entail pest control properties. Despite only accounting for 5-10% of the total crop

protection market globally, the biopesticide market is expected to grow at a 15.1% annual rate in the next years (2022-2027), reaching USD 12.1 billion by 2027 (MordorIntelligence, 2022).

Among microbial biopesticides, those derived from the Gram-positive bacterium *Bacillus thuringiensis* (Bt) are the most widely used worldwide, accounting for over 80% of the biopesticide market (Chandler et al., 2011). Another group of increasing interest is fungal biopesticides. Most entomopathogenic fungi do not require special infection routes and can act as contact pathogens due to their natural parasitic lifestyle (Sala et al., 2019).

1.2.1 *Bacillus thuringiensis*-based biopesticides

Bt toxicity to insect pests derives from crystalline inclusions produced during the sporulation phase that contain one or more toxic proteins known as δ-endotoxins (Adang et al., 2014). The main family of these parasporal crystal proteins is Cry proteins. There are over 300 different Cry protein types that are strain dependent and influence the host-specificity and the shape of the crystal inclusion (**Figure 1.3**) (Reyaz et al., 2021). For instance, the two main commercialized Bt strains as control agents, Bt var *kurstaki* (Btk) and Bt var *israelensis* (Bti), present bipyramidal and cuboidal crystals toxic to Lepidopteran and Coleopteran insects, and spherical crystals toxic to Dipteran insects, respectively (Nair et al., 2018).

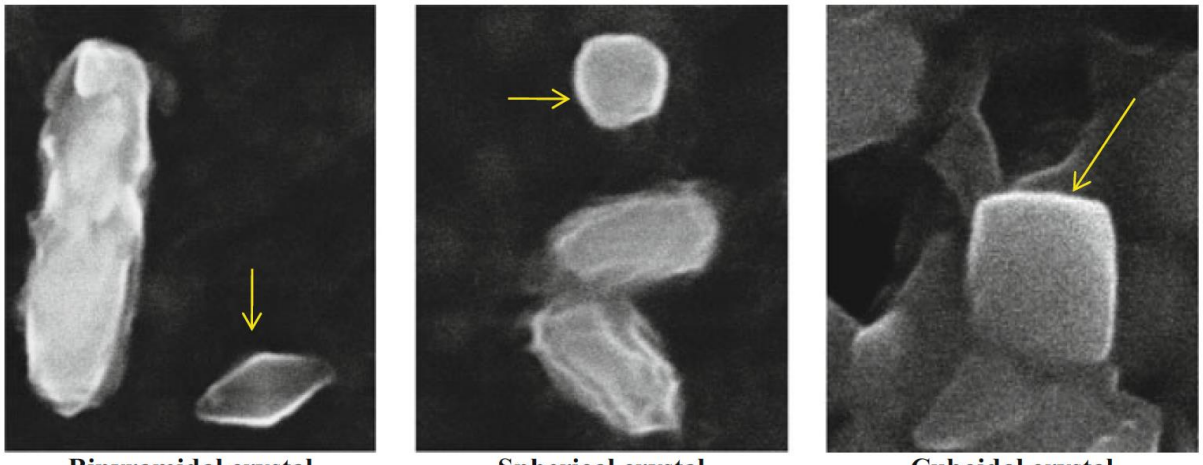

Figure 1.3 Different shapes of Bt crystals (yellow arrows). Image from Reyaz et al. (2021).

For Bt to successfully cause mortality, it first has to be ingested by a susceptible insect. After ingestion of the crystal protein, a multistep process takes place in the larvae midgut, which is summarized in **Figure 1.4**. First, the solubilization of crystal proteins by high alkaline pH causes the release and proteolytic activation of Cry proteins. Then, activated toxins bind to

specific receptors of the midgut epithelial cells membrane, leading to the irreversible insertion of the toxin into the membrane and the formation of pores. This results in epithelial cell lysis and finally death of the intoxicated larvae. Different models to understand the mechanism of pore formation have been proposed (Adang et al., 2014; Reyaz et al., 2021). Once the midgut has been breached, ingested spores can germinate and contribute to larvae septicemia (Raymond et al., 2010).

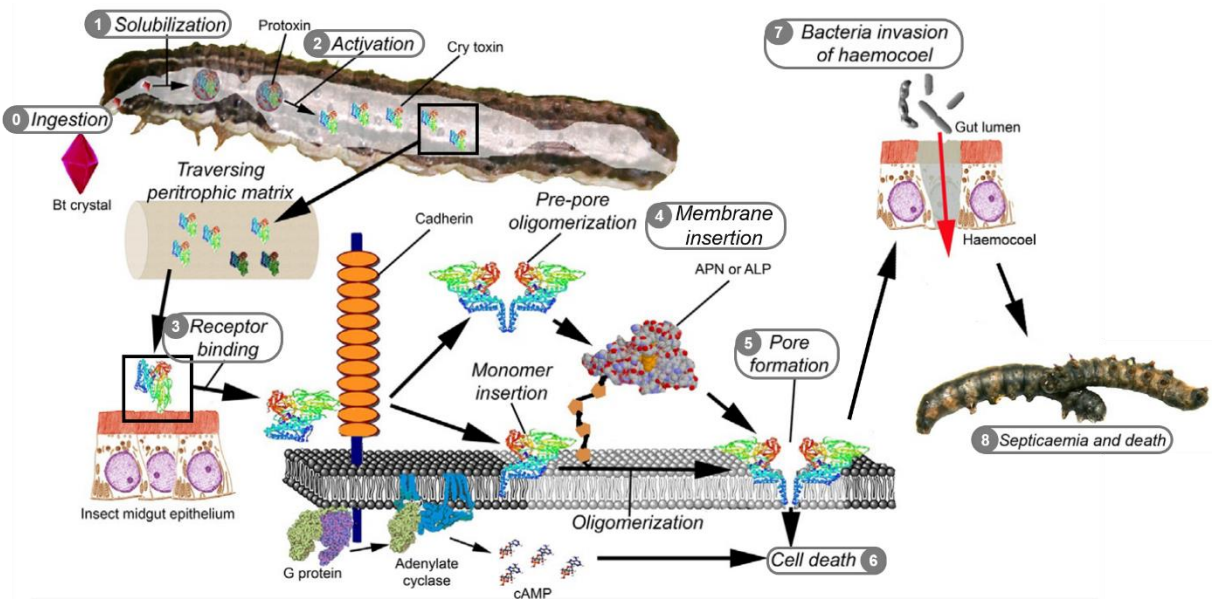

Figure 1.4 Schematic model illustrating the mechanism of action of Bt biopesticides. Image modified from Adang et al. (2014).

Bt-based biopesticides formulations consist of spores and crystal proteins typically produced through liquid fermentation in bioreactors. They offer a number of advantages over chemical pesticides, such as a high level of target selectivity, lack of polluting residues and safety. However, their biodegradability also represents a disadvantage because the crystal proteins are susceptible to natural abiotic factors, such as pH, temperature or sunlight (Damalas and Koutroubas, 2018). In this sense, Bti strains offer some advantages in comparison to Btk because they present longer storage stability, greater temperature tolerance, higher family specificity, and lesser resistance (Bravo et al., 2007). Furthermore, Bti subspecies are toxic against disease-vector mosquitoes, which are a pest of great epidemiological importance (Reyaz et al., 2021).

Besides its use as a pest control agent, Bt has also been gaining interest as a microbial producer of different bioproducts, such as proteases (Kandasamy et al., 2016) or PHA polymers (Odeniyi and Adeola, 2017).

1.3 Enzymatic hydrolysis

Enzymes are biocatalysts that accelerate the biochemical reactions taking place in or by living organisms. They have been used for industrial purposes for many years in purified form, raw as produced or mixed in enzymatic cocktails (Arbige et al., 2019). The size of the global enzymes market was estimated at USD 11.5 billion in 2021 and it is expected to increase at an annual rate of 6.5% until 2030 (Grand View Research, 2021). This trend is expected due to their environmental and economic advantages over chemical usage, such as higher production yields, improved safety in operations and reduced energy usage, byproduct formation and greenhouse gas emissions (Arbige et al., 2019). The main industrial applications for enzymes include food and beverage industries, animal feed, textile processing and detergent applications (Choi et al., 2015). However, one of the most prominent applications is the production of sugars from different types of biomass using hydrolytic enzymes due to the fact that these sugars can be used further in microbial processes, enabling a wide range of sustainable industrial opportunities (Arbige et al., 2019).

Hydrolytic enzymes catalyze the breakdown of natural polymers, such as proteins, starches, lipids or fibers, into their respective monomers. Therefore, enzymatic hydrolysis can be applied to obtain functionalized molecules from complex biomass, such as wood residues, agricultural waste and organic municipal waste. The most frequent hydrolases employed in the conversion of biomass are summarized in **Table 1.1**, alongside other relevant activities such as oxidoreductases for lignin reduction (Escamilla-Alvarado et al., 2017). It should be noted that the process is highly complex, especially when lignin is present in high amounts, and requires an adequate enzyme-substrate balance and, often, the simultaneous action of synergistic catalytic activities (Sweeney and Xu, 2012). This complexity is associated with high operating costs due to the cost of the enzymes and lignocellulosic biomass often requires pretreatments to reduce particle size and enhance enzyme accessibility (Yang et al., 2015).

As enzymatic hydrolysis enables the use of agricultural and urban waste materials to obtain sugars and other functionalized molecules, it opens the door to their management in biorefinery-like scenarios. Currently, the production of energy from OFMSW is being prioritized but, following a direct cascading scheme higher value-added bioproducts could be obtained first. The use of enzymes in an OFMSW biorefinery is reviewed deeply in **Chapter 4** (**Article I**), as well as the potential applications for the obtained liquid and solid fractions.

Table 1.1 Most common enzymes for biomass conversion (Escamilla-Alvarado et al., 2017).

Enzyme type	Main subclasses	Substrate	Product
Cellulases	Endocellulases	Crystalline cellulose	Cellooligosaccharides
	Exocellulases	Extremes of cellulose	Cellobiose
	ß-glucosidases	Cellobiose	Glucose
Hemicelluloses	Endoxylanases	Xylans	Xylooligosaccharides
	Xylosidases	Xylooligosaccharides	Xylose
	Glucuronidases	Hemicelluloses	Arabinose, galactose, …
	Glucanases	Glucans	Glucanoligosaccharides
	Esterases	Hydroxycinnamoyl	Glucuronic, galacturonic, ferulic and coumaric acid
Amylases	α-amylases	Starch	Glucose, maltose, maltotriose
	Glucoamylase	Amylose	Glucose
Pectinases	Polygalacturonase, pectin lyase	Pectin polymers	Galacturonic acids
Lipases		Triglycerides	Diacyl and monoacylglycerols, alkyl esters, glycerol
Proteases		Proteins	Aminoacids
Lignin oxidoreductases	Laccases	Aromatics	Phenolic compounds
	Peroxidases	Lignin	Oxidized products

1.4 Solid-state fermentation

SSF is a biotechnological process that imitates natural habitats for microorganisms to produce marketable products (Kumar et al., 2021). SSF can be defined as the fermentation process that takes place on a moist solid matrix under aerobic conditions (Thomas et al., 2013). The solid material, which has to provide enough moisture content to support microbial activity and allow adequate contact with the gas phase that supplies oxygen, can also serve as a source of nutrients (Chilakamarry et al., 2022). In this sense, SSF emerges as a technology that enables waste recycling by using organic solid waste as substrates for the growth of microorganisms (Soccol et al., 2017). The transformation of waste into added-value bioproducts involves the valorization of solid residues in line with circular economy principles and decreases the need for raw materials, thus favoring the economy of the process.

SSF technology offers many advantages related to the use of solid growth supports. For instance, by providing an environment similar to the natural habits of microorganisms, higher product yields can be achieved (Oiza et al., 2022). This gains particular relevance for fungi, which have been the most studied microorganisms in SSF (Sala et al., 2019), and also for sporulating microorganisms (Chilakamarry et al., 2022; Flores-Tufiño et al., 2021). SSF involves lower energy consumption, water demand and wastewater generation supporting a greener manufacturing industry. Finally, higher product concentrations result in superior product stability and simpler downstream processes but it depends on the final purity required (Kumar et al., 2021; Soccol et al., 2017; Thomas et al., 2013).

SSF has been used for the production of a wide range of bioproducts for different applications. The process is highly versatile in terms of substrate and bioproduct possibilities. For instance, enzymes have been produced from coffee husks (Cerda et al., 2017) or hair waste (Abraham et al., 2014), biopesticides from urban (Ballardo et al., 2016; Cerda et al., 2019; Mejias et al., 2020) and agricultural (Sala et al., 2021) waste, biosurfactants from food waste (Jiménez-Peñalver et al., 2016), or aroma compounds from agricultural byproducts (Martínez-Avila et al., 2019). The performance of SSF processes depends on optimizing a variety of parameters that impact the process behavior in different ways.

1.4.1 Factors affecting solid-state fermentation

- **Substrate**

Typically, SSF processes use agricultural residues due to their high availability and reduced cost. These substrates not only provide structure to the solid matrix but also nutrients and other microelements essential for microbial growth (Sala et al., 2019). Its choice should consider the desired product and the type of microorganism to be employed. Substrate characterization and preparation are essential to ensure an adequate moisture content (MC), pH and porosity for the process. Substrate's biodegradability, which determines the amount of organic matter that can be assimilated by microorganisms (Ponsá et al., 2010), is another relevant characteristic because it determines the content of accessible carbon sources for microbial growth.

Urban organic waste, such as OFMSW, food waste or digested biowaste can be used as SSF substrates (Cerda et al., 2019; Mejias et al., 2020). They present some particularities, such as inherent microbial populations that hamper sterilization processes and complex compositions rich in polysaccharides with low lignin content, which facilitate the accessibility to nutrients. SSF of urban waste is often performed with enriched microbial consortia reproducing the

natural microbiological environment found in processes such as composting or ensiling (Abraham et al., 2014; Ballardo et al., 2017).

- **Inoculum size**

The size of the inoculum is critical to microbial growth during SSF, as low inoculum concentrations cannot initiate growth, while high inoculum concentrations result in reduced metabolism due to mass transfer limitations (Kumar et al., 2021). According to Nigam and Singh (1994), increasing inoculum quantity shortens the time required for substrate utilization, which helps to displace other microbes present in non-sterile conditions.

- **Moisture content**

MC is closely related to the selected substrate and its water-holding capacity, and it is a critical parameter for microbial growth. Low MC implies reduced microbial growth and solubility of nutrients, thus reduced productivity. On the contrary, higher humidity levels lead to particle agglomeration and reduced gas exchange (Kumar et al., 2021). MC does not only depend on substrate and microbial activity but also on the reactor configuration and process scale (Arora et al., 2018). Optimal MC must be ensured during the fermentation and are at least 60-70% for bacteria and 40–80% for filament fungi (Sala et al., 2019). Bacterial cultures are more sensitive to changes in the MC and water activity, a related term that reflects the water available or accessible for the reaction (Chilakamarry et al., 2022). This parameter is affected during the course of the fermentation process, both by microbial growth and evaporation phenomena.

- **Porosity and particle size**

Porosity, i.e. inter-particle space, and particle size are closely related, as well as moisture content. They define the substrate fraction that is initially approachable and therefore, its ability to interchange nutrients with the microorganisms. They also interfere with the exchange of heat and oxygen between air and solid surfaces. Too small particle size lead to poor aeration whereas large particles limit surface area for microbial action (Thomas et al., 2013). It should be highlighted that particle size tends to decrease during the fermentation process and that homogeneous particle size is nearly impossible when working with heterogeneous waste (Krishna, 2008). For those substrates with reduced porosity, a bulking agent can be added to increase surface area and ensure proper aeration and mass transfer as done in composting processes (Ruggieri et al., 2009).

- **pH**

Every microorganism has an optimal pH for growth and metabolic activity, which must be targeted to optimize the fermentation process. Bacteria are known to prefer pH near to neutrality whereas filamentous fungi thrive over a large pH range of 2-9 with an optimum slightly acidic around 4-6. Yeast also prefer acidic pH of 4-5 for their growth (Chilakamarry et al., 2022; Sala et al., 2019). Microorganisms have the ability to adapt to pH changes (Mohd-Zaki et al., 2016), but when the pH of the media goes beyond the desirable range, microbial growth inhibition and even cell death may occur. However, pH monitoring and control during SSF is difficult due to several reasons both substrates-related and technological. The major challenges for pH optimization are the complexity of substrates, the absence of free water, the heterogeneity in the solid matrix, and the lack of online pH measuring devices for solids (Kumar et al., 2021). During aerobic processes, pH changes as a consequence of metabolic activities. First, pH drops due to organic acid production. Then, the assimilation of these organic acids would rise back the pH. The addition of a buffer, such as urea or ammonium salts, to the substrate, can mitigate the effect of pH changes. However, the complex chemical composition of certain waste can also present buffering effects difficult to counteract (Kumar et al., 2021).

- **Temperature**

Temperature is a critical parameter in SSF, also due to the implicit difficulties in controlling it and the fact that microbial growth, enzyme efficiency and synthesis of secondary metabolites depend on temperature (Kumar et al., 2021). There are different factors affecting temperature during SSF. On the one hand, microbial growth involves metabolic heat generation and thus, increases the temperature inside the fermenter. On the other hand, solid substrates generally have low thermal conductivity hindering heat dissipation. These phenomena lead to overheating, which adversely affects product yield and microbial growth. Heat accumulation becomes more challenging when scaling up because it results in a significant loss of moisture and creates humidity and temperature gradients inside the solid matrix (Thomas et al., 2013). Water evaporation leads to condensation, increasing further the heterogeneity of the solid substrate. To overcome this challenge, forced aeration can be increased to remove heat through a gaseous vent. In this sense, humid air prevents the dryness of the solid material to a limited extent (Kumar et al., 2021). Also, the substrate can be cooled from the outer surface (Thomas et al., 2013). In this sense, bioreactors designed at bench and industrial scales are focused on

heat removal or mitigation (Soccol et al., 2017). Lastly, the use of thermo-tolerant microbial strains can enhance the robustness of the process.

- **Aeration rate**

Aeration is essential to provide oxygen for microbial growth, moreover, it eliminates heat, carbon dioxide and other volatile substances (Krishna, 2008). Also, the oxygen content is an important parameter for sporulating microorganisms such as Bt (Boniolo et al., 2012; Méndez-González et al., 2020). Air level must be sufficient to maintain adequate microbial growth rates, but other parameters such as temperature and moisture content have to be considered when manipulating it given the close relationship between them (Mejias et al., 2017; Sala, 2022). An adequate distribution of air inside the packed bed must be favored by bioreactor design and substrate preparation to avoid preferential paths and heterogeneity in the solid matrix (Yazid et al., 2017). To do so, proper porosity must be ensured using bulking agents if necessary (Ruggieri et al., 2009).

- **Mixing or agitation**

In submerged fermentation (SmF) processes, agitation is an important parameter to ensure homogeneity and adequate oxygen gas-liquid transfer. However, in SSF agitation is not straightforward due to the characteristics of the solid substrates, which offer higher resistance toward shear strength. Porosity is greatly affected by agitation because of compaction phenomena (Chilakamarry et al., 2022). In fungal SSF processes, agitation also disrupts mycelium leading to reduced product yields (Sala et al., 2019). Intermittent rather than constant mixing is more common in SSF but the final choice of type and frequency depends on the characteristics of the substrate, microorganism and bioreactor type (Jiménez-Peñalver et al., 2016).

1.4.2 Bioreactor design for solid-state fermentation processes

Bioreactors provide a suitable environment for high biological reaction rates, including growth performance, substrate consumption and product formation (Manan and Webb, 2017). In SSF, the major variables for the design and operating strategies of bioreactors include effective oxygen diffusion and temperature control (heat transfer through bioreactor walls or cooling systems) (Arora et al., 2018). Different bioreactor configurations have been developed

based on the mixing system (static, intermittent or agitated) or the aeration (forced or not). **Figure 1.5** shows different SSF bioreactor designs based on bed type.

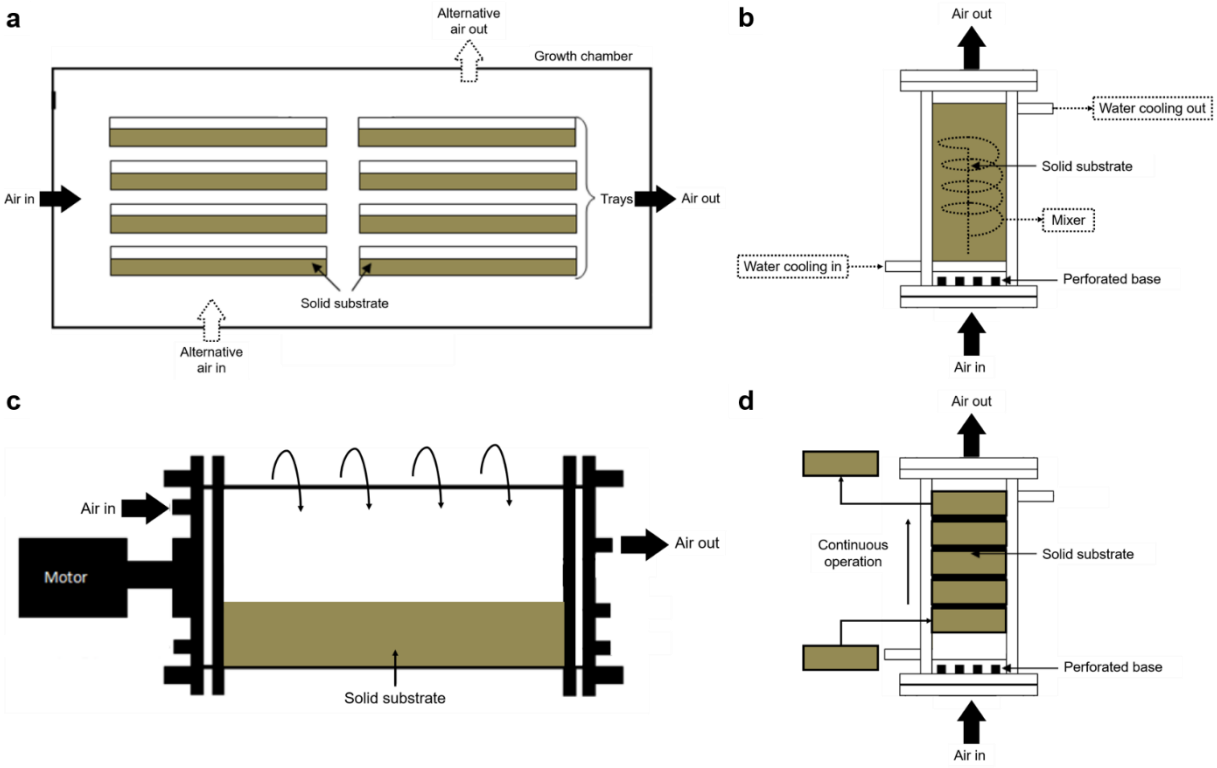

Figure 1.5 Schematic representation of (a) tray-bed bioreactor, (b) packed-bed bioreactor, (c) rotatory-bed bioreactor, and (d) modular-bed bioreactor. Discontinuous lines represent alternative possibilities. Images adapted from Manan and Webb (2017).

Tray bioreactors (**Figure 1.5a**) have been the most used bioreactors in industrial SSF processes, mainly related to food production (Mitchell et al., 2006). The fermentation is done stationary in perforated trays covered with inoculated solid substrate and located in a chamber at a controlled temperature (or not). Bed thickness is an important and limiting parameter to prevent overheating and maintain aerobic conditions. If mixing is required, it is done manually. Tray bioreactors are the most simple and economical, however, their scalability depends on increasing surface area, i.e. number of trays, and therefore, considerable space requirements.

Packed-bed bioreactors (**Figure 1.5b**) have been gaining relevance over tray-bed as they are less labor-intensive and offer more contained environments (Sala et al., 2019). They are column bioreactors filled with solid substrates. The system is closed and forcefully aerated with compressed air. Airflow can be controlled and outlet gases measured, allowing to obtain kinetic parameters (Soccol et al., 2017; Thomas et al., 2013). These bioreactors can be operated statically or under mixing conditions using automatic stirrers. Bed height is a limiting parameter

to prevent heat accumulation and bed compaction. Also, excessive forced aeration can lead to air channeling and bed compaction (Mejias, 2020).

Rolling or rotatory bed bioreactors (**Figure 1.5c**) are generally horizontal cylinders, in which mixing is provided by the rolling motion of the solid substrate. Baffles may be added to the inner walls of the drum to improve mixing. They offer an agitation alternative to those processes that require agitation but are sensitive to shear stress. However, the scale-up cost is a limiting factor as their operating volume is around 30% of the total reactor volume (Mahmoodi et al., 2019).

Lastly, modular-bed bioreactors (**Figure 1.5d**) are an emerging technology based on packed-bed bioreactors that consist on dividing the bed into layers (Rodrigues et al., 2022). This division prevents compaction and allows the movement of layers. For instance, cyclic operations can avoid temperature gradients, enhance air distribution and increase homogenization (Mitchell et al., 2010). This configuration opens the door to continuous operation modes as proposed by Rodrigues et al. (2022).

1.4.3 Solid-state fermentation challenges

The main drawback preventing SSF successful implementation at an industrial scale is the difficulty of process scaling up because of the magnification of the heat and mass transfer issues within the solid matrix (Oiza et al., 2022). Microbial growth produces metabolic heat that tends to accumulate due to the poor thermal conductivity of solid substrates. Accumulated heat is transferred from the solid to the inlet air due to convection phenomena, increasing the air temperature as it moves along the solid matrix, creating a temperature gradient. This gradient depends on the bioreactor configuration and aeration system. At the same time, the evaporation of water increases with the increase in air temperature leading to the progressive drying of the solid matrix. This phenomenon creates moisture gradients that in turn, cause limited oxygen and nutrients diffusion creating concentration gradients. Overall, overheating of the solid matrix increases its heterogeneity and reduces the process efficiency (Arora et al., 2018; Mejias, 2020).

Another drawback related to the use of solids is the lack of control and monitoring in contrast to SmF systems, which implies less control over the process (Kumar et al., 2021). Microbial growth occurs inside the solid matrix and biomass cannot be accurately separated so it needs to be estimated. Generally, outlet oxygen and carbon dioxide content are used to indirectly estimate microbial growth and kinetic parameters. Other methods consist on solid-liquid extraction of cells, spores or growth-related substances, such as proteins, ergosterol, or

nucleic acids (Kumar et al., 2021). However, it should be kept in mind the heterogeneity of the solid matrix and the importance of several sampling points, especially as the scale increases.

As for other fermentation technologies, downstream processing considerations are crucial for the overall process efficiency and cost. However, little research has been published on the topic and is mainly restricted to the recovery of enzymes (Oiza et al., 2022). Recovery of the bioproduct from the solid matrix is performed through extraction methods. Typically extractive solvents are used (Oiza et al., 2022). The type of solvent, solid-to-liquid ratio, time and final degree of purity desired are important considerations when designing a downstream process (Thomas et al., 2013). This methodology involves several disadvantages, such as the high cost and the toxicity, which can prevent the reusability of the exhausted solid waste and the commercialization of the recovered bioproducts in certain markets. Novel alternatives include microwave-assisted extraction, supercritical fluid extraction or ultrasound-assisted extraction (Oiza et al., 2022). To attain a truly sustainable process, the management of the remaining solid waste has to be considered (Marín et al., 2019), which entails additional costs.

1.4.4 *Bacillus thuringiensis* production through solid-state fermentation

At the time of writing, a Scopus-based bibliometric analysis on the topic "*Bacillus thuringiensis*" and "solid state fermentation" limited to scientific articles in English resulted from only 44 documents (November 12, 2022). The GICOM group from UAB was the affiliation with more publications (7 documents, 16%) followed by the National Research Center of Egypt (6 documents, 14%). The different bioproducts produced included biopesticides (Mejias et al., 2020), enzymes, such as chitinases (Chaiharn et al., 2019) or amylases (Abdullah et al., 2018), and PHA (Saeed et al., 2022). However, few papers (9 documents, 20%) dealt with bioproduct "recovery" or "formulation" (El-Bendary et al., 2019; Veloorvalappil Narayanan et al., 2018) and most of them reported using low working volumes. Only half of the documents also included the terms "waste" or "residue" (21 documents, 48%) and reported substrates such as soy waste (Ballardo et al., 2016), shrimp cell waste (Chaiharn et al., 2019), food waste (Zhang et al., 2013) or agricultural waste (El-Bendary et al., 2016). Only publications from the GICOM group dealt with municipal solid waste, such as the OFMSW (Ballardo et al., 2020) and biowaste digestate (Cerda et al., 2019; Mejias et al., 2020; Rodríguez et al., 2019). An overview of the experimental results obtained using municipal and food waste can be seen in **Table 1.2**.

Table 1.2 Published works on SSF for Bt biopesticide production using municipal waste.

Reference	Bt strain	Substrate	Largest scale	Solid load	Spore production (CFU g^{-1} dry matter)	Downstream considerations
Zhang et al., 2013	Btk	Food waste and supplements	15 L	35 kg	9.6 x 10^8	no
Zhang et al., 2015	Btk	Food waste	[a]0.5 L	NM	[b]2.2 x 10^9	Slow-release formulation
Rodríguez et al., 2019	Btk	Digested biowaste	100 L	20 kg	1.7 x 10^9	no
Ballardo et al.,2020	Btk	OFMSW	[c]400 L	40 kg	10^6	Compost-like formulation
Mejias et al., 2020	Btk and Bti	Mixture of digested biowaste and biowaste	22 L	4 kg	1.5 x 10^8 for Btk 4 x 10^8 for Bti	no

[a] Semi-solid. [b] data in CFU mL^{-1}. [c] no SSF bioreactor but home composter. CFU, colony forming units. NM, not mentioned.

Overall, Bt production through SSF appears to be a viable and reproducible process as most studies produced around the same number of spores (**Table 1.2**). However, more research is needed to standardize operational strategies that ease process implementation at larger scales and adaptation to different organic waste while ensuring optimum sporulation and biopesticide production.

Chapter 2 **Objectives**

Briefly, the main objective of this thesis is explained and the specific objectives listed.

The objective of this thesis is related to the activities performed in work package 4 of the SCALIBUR project, which aimed to investigate novel valorization routes for the OFMSW based on the use of enzymatic hydrolysis. The main objective was to develop and optimize an SSF process to produce biopesticides from the solid fraction of the OFMSW enzymatic hydrolysis.

To do so, the following specific objectives were established:

- To study the enzymatic hydrolysis of OFMSW by optimizing key operational parameters for maximizing sugar release and comparing two different enzymatic cocktails to perform this process.

- To evaluate at a laboratory scale the feasibility of producing Bti through SSF from the residual solid fraction after the enzymatic hydrolysis of OFMSW.

- To develop a robust and reproducible SSF operational strategy to modify the pH of the substrate and enhance the production of Bti spores.

- To evaluate the scaling-up effect of the selected strategy for producing Bti biopesticide from the residual solid hydrolysate.

- To assess the integration of the proposed valorization route for OFMSW in a biorefinery framework, in terms of technical feasibility and economic viability.

Chapter 3 **Materials and Methods**

A complete description of the samples processed and methodologies used in this document is given. The solid-state fermentation reactors are shown and the analytic procedures are detailed.

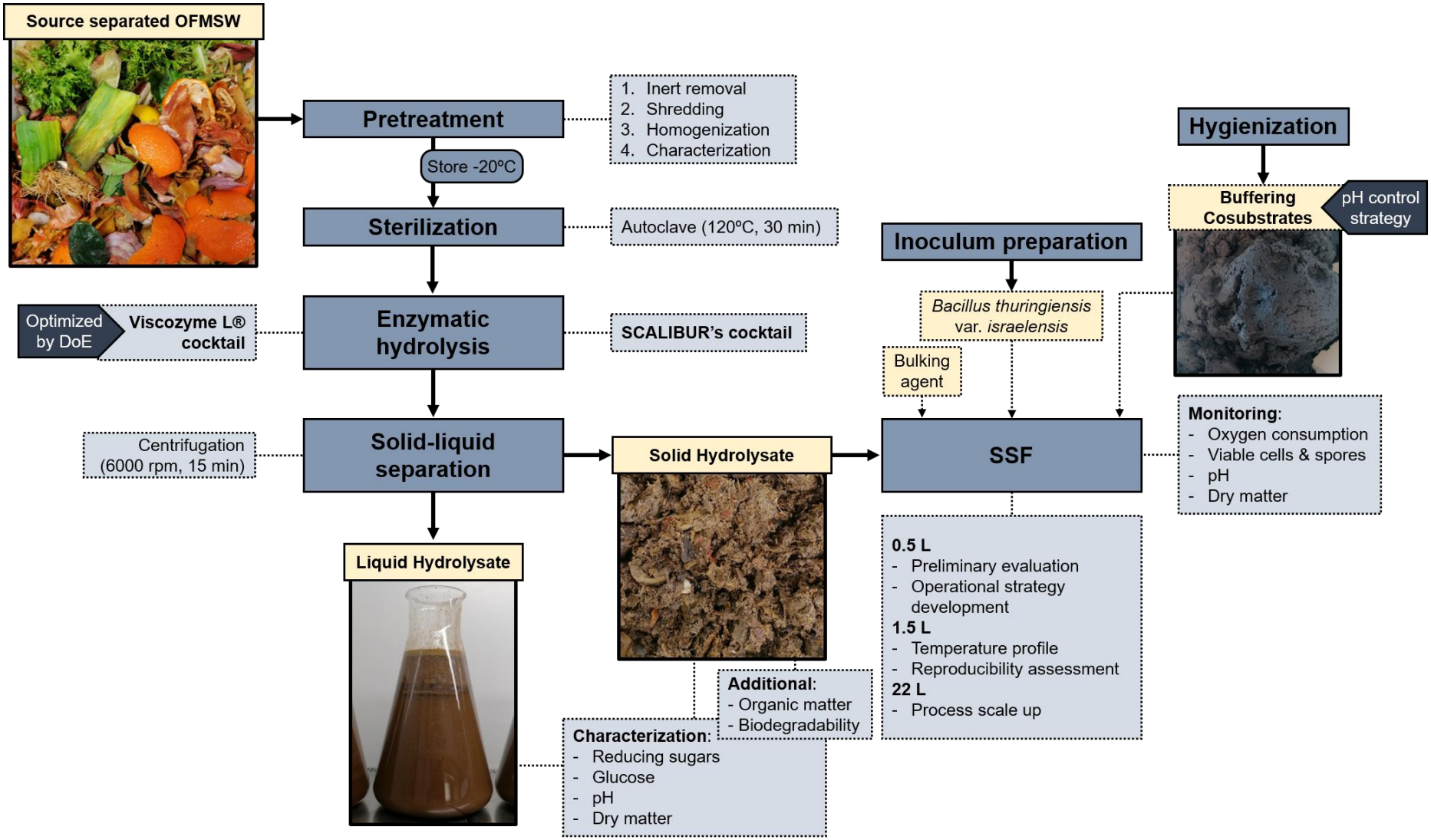

Figure 3.1 Schematic overview of the experimental process developed during this thesis for valorizing high-quality OFMSW.

A summary of the process evaluated in this work is presented in **Figure 3.1**. In the coming section, the different materials and experimental procedure of each step (pretreatment, enzymatic hydrolysis, solid-liquid separation and SSF) are explained in detail.

3.1 Raw materials

The OFMSW samples used throughout this thesis were collected upon arrival at the MSW treatment plant of *Mancomunitat La Plana* (Malla, Barcelona), at the site where the collection trucks unloaded the bags (**Figure 3.2a**). This treatment plant deals with source-separated OFMSW collected by a well-established door-to-door system, resulting in a high-quality organic waste fraction (<1% impurities) (ARC, 2016). First, waste bags were opened manually and screened for the presence of inert materials such as glass, plastics, metals, or textiles. Bones, hard shells, hair, and excess paper were also removed to ease sample manipulation along the process. Then, particle size was reduced mechanically using a home composting shredder (Tecoinsaen SL, Spain) (**Figure 3.2b**), and finally, samples were homogenized, characterized and packed into 1 kg bags for storage at -20ºC. A total of eight samples of around 10-15 kg were collected over the course of this thesis at different times of the year and characterized (data presented in **Chapter 4** and gathered in **Chapter 5**). Images from some samples after manually removing inert materials and after trituration can be seen in **Figure 3.3**.

Figure 3.2 (a) collection point at the MSW treatment plant and (b) shredding machine.

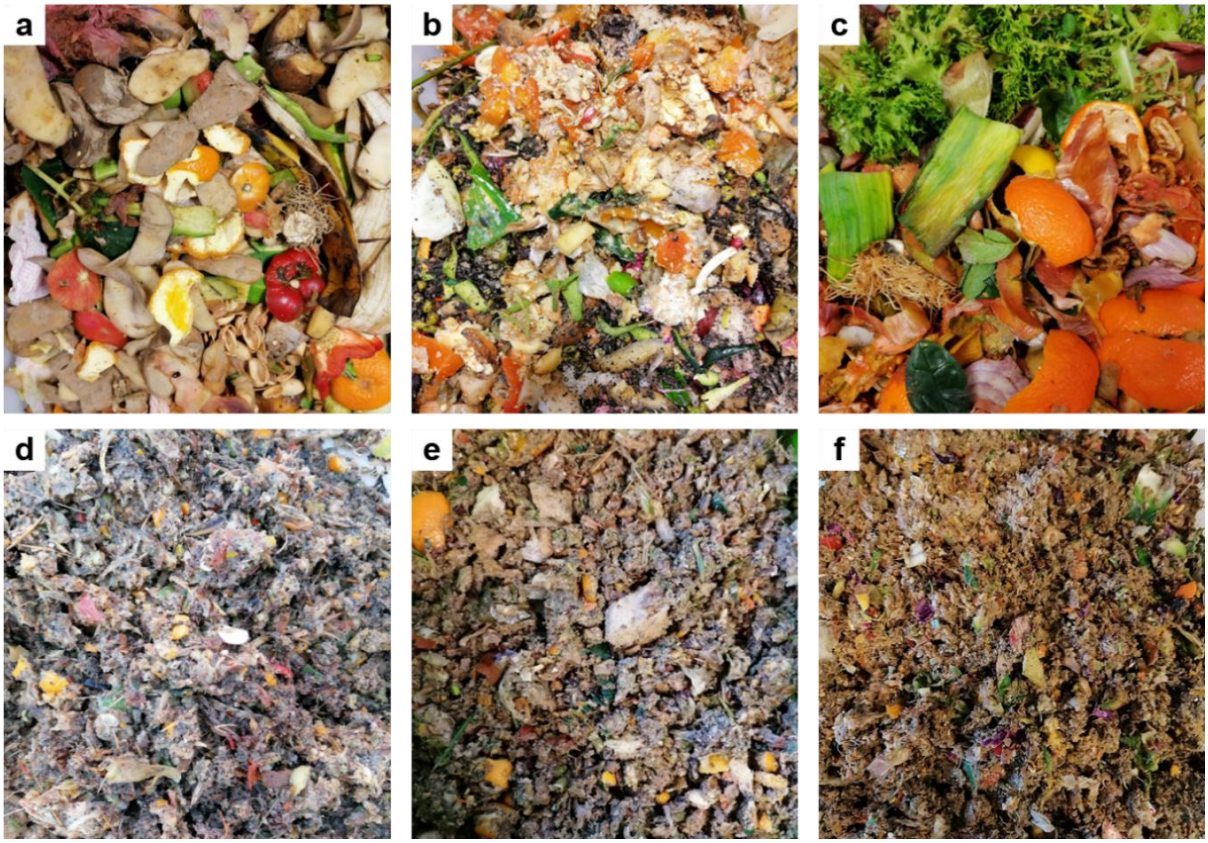

Figure 3.3 (a, b, c) Images of some of the collected OFMSW samples and (c, d, e) after their respective trituration step.

Different materials from the urban waste framework (**Figure 3.4**) were evaluated as cosubstrates for the SSF process:

- Digested and dewatered sewage sludge was collected from the municipal wastewater treatment plant of Sabadell Riu Sec (Barcelona, Spain). It came from a mesophilic wet AD followed by a solid-liquid separation process using a centrifuge.
- Digestate from the AD process of a source selected OFMSW was collected from the municipal solid waste treatment plant of *Consorci per a la Gestió dels Residus del Vallès Oriental* (Barcelona, Spain). It was obtained after a mesophilic wet AD process followed by a solid-liquid separation using a screw press.
- Compost was collected from the composting plant of the source selected OFMSW of *Planta de compostatge de Sant Cugat* (Barcelona, Spain). Samples were directly taken from four weeks old maturation piles.

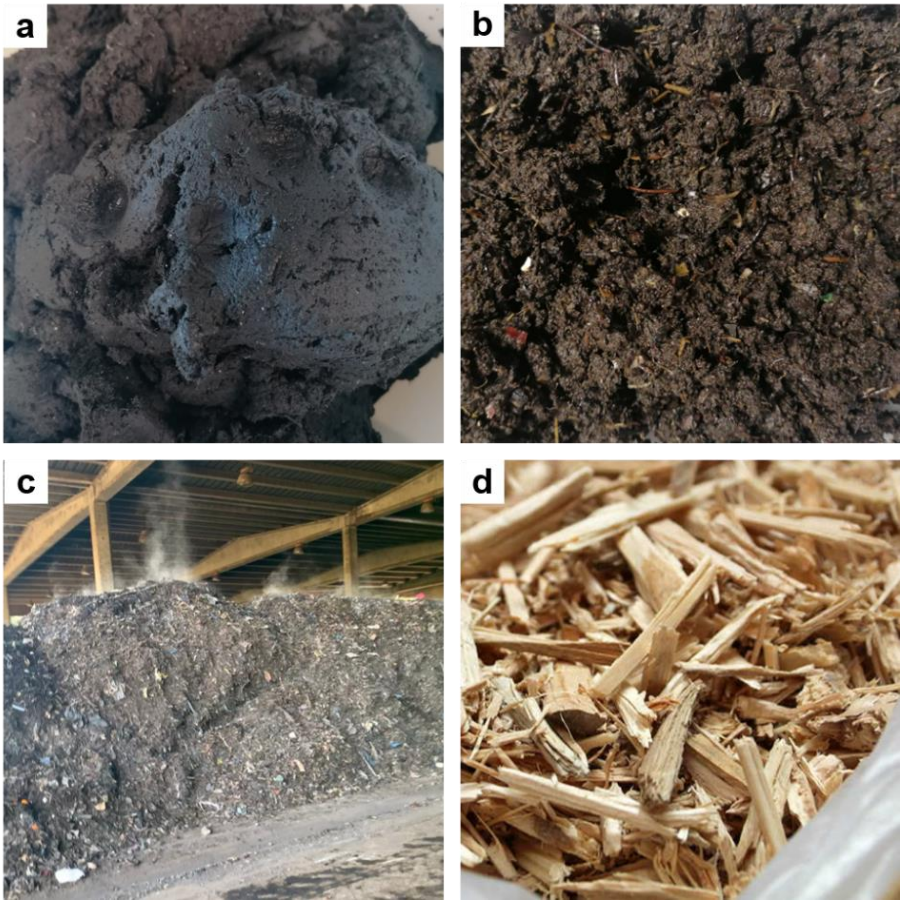

Figure 3.4 Additional materials employed: (a) Digested sewage sludge, (b) digested OFMSW, (c) compost pile, and (d) bulking agent.

All materials, were characterized upon arrival at our laboratory (data presented in **Chapter 4** and **Chapter 5**) and then packed into bags of around 1 kg, and stored at -20ºC until use for a maximum period of three months. Before use, materials were defrosted overnight at 5ºC. Both digested OFMSW and digested sewage sludge were subjected to a hygienization pretreatment to pasteurize them as specified in the European Regulation Nº 142/2011. To do so, materials were kept at 70ºC for 1 h using a previously heated laboratory oven (Binder). The oven set-point temperature was 95ºC to ensure that the solids maintained a 70ºC temperature, which was measured at different points of the trays using a thermometer. To prevent moisture losses, the trays containing the materials were covered with aluminum paper during the whole process. Then, materials were stored in the fridge (5ºC) until their use for a maximum of 24 h.

To provide porosity and structure to the solid matrix, sterile wood chips (**Figure 3.4d**) were used as a bulking agent (Acalora, *Palets Pla d'Urgell*, Lleida, Spain). For laboratory scale bioreactors (0.5 L and 1.5 L), wood chips were sieved with a 3 mm mesh width, whereas for the 22 L and 100 L bioreactors the average width was 7.1 mm.

3.2 Microorganism

Bacillus thuringiensis var *israelensis* (CECT 5904) was obtained from *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain). The stock culture was preserved at -80ºC using a seed lot system to maintain the genetic stability of the strain in cryo-pearls (DeltaLab, Barcelona, Spain). Additional information about the seed lot system and cryopreservation can be found elsewhere (Simione, 2009). For each experiment, a new streak from the cryo-stock was cultivated.

For inoculum preparation, 100 mL of sterile Nutrient Broth nº2 (Oxoid CM0067B, England) were inoculated with one cryo-pearl and incubated for 20 h, at 130 rpm and 30ºC using 0.5 L Erlenmeyers with a SILICOSEN® stopper (Hirschmann Laborgeräte GmbH, Germany). At the end of the incubation period, culture optical density (OD) was around 2.5-3. The culture was centrifuged for 10 min at 3500 rpm and 4ºC, and the supernatant was decanted but not discarded. Then, the obtained pellet was resuspended in 3 mL of the exhausted media (supernatant) and then, diluted 1:10 (v v^{-1}) also with supernatant to reach a concentration of approximately 10^8 CFU mL^{-1}. The inoculum's quality was assessed with OD value, visual inspection, and counting in Petri dishes (**Figure 3.5**). No spores were detected at this point. This protocol was performed under sterile conditions, working in a laminar flow cabin with autoclaved material (121ºC for 30 min). The processed inoculum was covered with aluminum paper to protect it from direct light exposure until use.

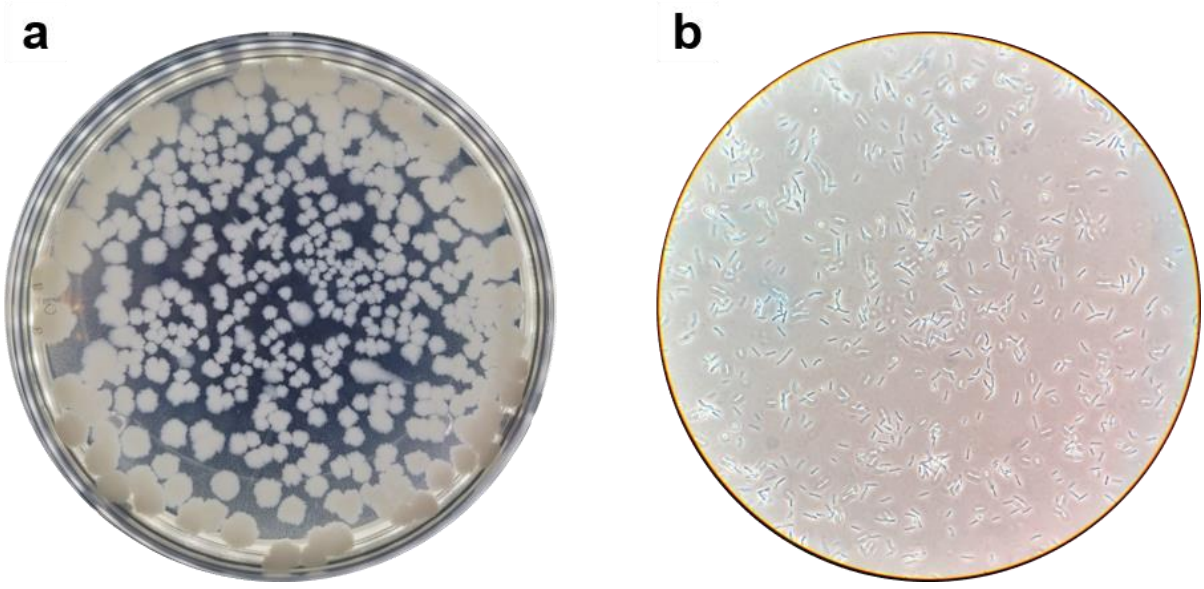

Figure 3.5 (a) Bt colonies in agar plate and (b) Bt cells in an optical microscope (x40 augments).

The tolerance of the Bti strain towards initial pH was assessed by cultivating it for 24 h in sterile Nutrient Broth nº2 with modified pH (from 3 to 7.5) (**Figure 3.6**). Each initial pH value was evaluated in duplicate and acidified to the desired value using citric acid 1M.

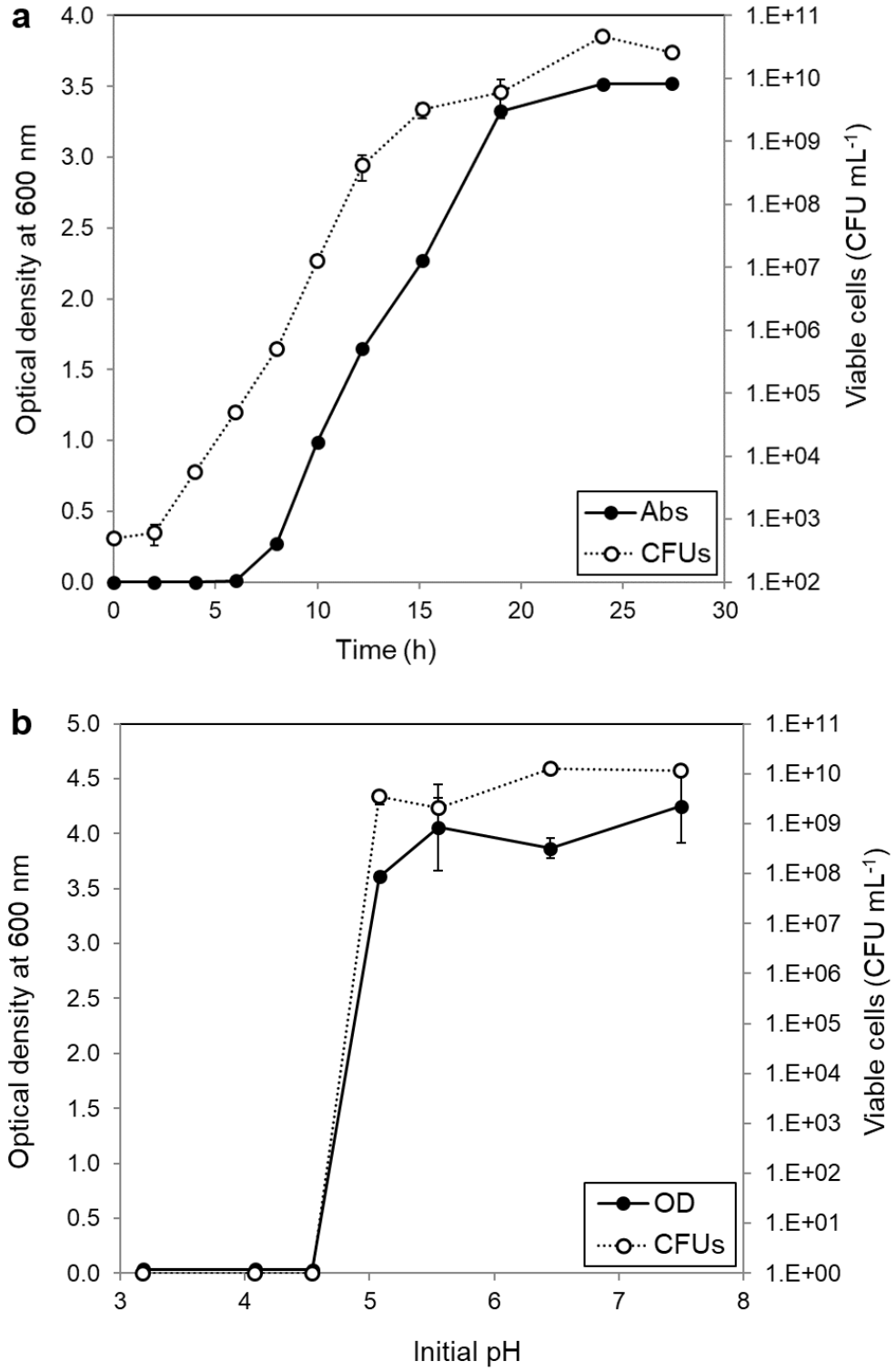

Figure 3.6 (a) Growth curve for *B. thuringiensis* var *israelensis* in liquid media at pH 7.5. (b) Observed growth after 24 h of cultivation at different initial pH.

3.3 Enzymatic hydrolysis

During this thesis, two different enzymatic cocktails have been used for the hydrolysis of OFMSW: one commercially available and the other developed in the SCALIBUR project.

- Viscozyme L® from Novozymes (Denmark) was selected because recently it has gained research interest for the pretreatment of food waste, a major component of OFMSW (Cabas Candama et al., 2020; Chua et al., 2021; Gabiatti Junior et al., 2020). It includes a wide range of enzymatic activities, mostly carbohydrases, such as cellulase, β-glucanase, hemicellulase, xylanase, arabinase, and pectinase. The declared activity by the provider is 100 Fungal Beta Glucanase Units (FBG) per gram with an operating pH range of 3.5–5.5 and temperature range of 25–55ºC. This cocktail was used to develop and optimize a protocol for the enzymatic hydrolysis of high-quality OFMSW (**Chapter 4**, **Article II**).
- The other cocktail was developed by ASA *Spezialenzyme GmbH* (Wolfenbüttel, Germany) in the framework of the SCALIBUR project. This cocktail was tailor-made for the hydrolysis of an OFMSW source selected at Madrid and it is mainly composed of cellulase and pectinase activities. This cocktail was used to prepare the solid hydrolysate for the development and scale-up of the SSF process (**Chapter 4**, **Article III** and **Article IV**).

Before use, shredded OFMSW samples were defrosted overnight at 5ºC and sterilized by autoclaving at 121ºC for 30 min. Enzymatic hydrolysis experiments were always performed under sterile conditions in Erlenmeyer flasks sealed with aluminum paper. Different flask volumes were used depending on the experiment and the desired amount of solid hydrolysate to be produced (0.5 L, 2 L and 3 L). The ratio of flask volume and wet OFMSW loaded was kept constant (0.1 kg, 1 kg and 1.5 kg, respectively). For both enzymatic cocktails, 0.05 M sodium citrate buffer was used to modify the initial pH and adjust the solid content. Also, the same solid-to-liquid ratio, time and agitation speed were set in all the experiments after an initial evaluation using Viscozyme L. The maximum solid-to-liquid ratio that allowed for a proper mixing in Erlenmeyer flasks was 10% (w v^{-1}). The time was set to 24 hours because it was observed that longer times were more prone to contamination, possibly from the inherent activity of the OFMSW and inefficient sterilization due to the poor heat transfer of the solid.

The other parameters considered (temperature, initial pH and enzyme dosage) were optimized for Viscozyme L by an experimental design, as explained later in **Chapter 4** (**Article II**), and adjusted according to the manufacturer's recommendations for ASA's cocktail. In **Figure 3.7** the OFMSW before and after the hydrolysis step can be seen.

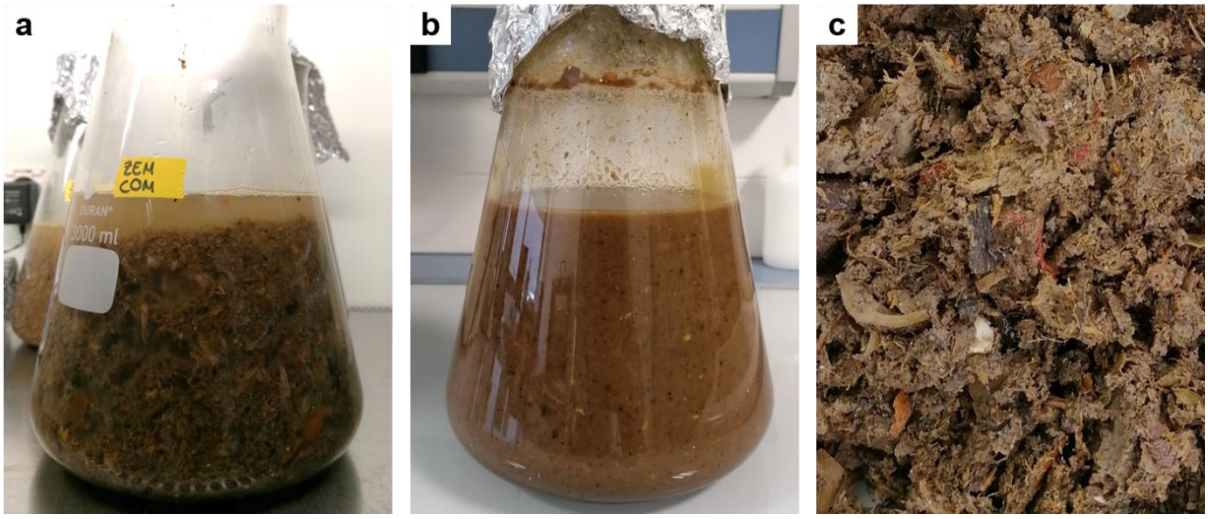

Figure 3.7 Appearance of the OFMSW (a) before the enzymatic hydrolysis, (b) after the enzymatic hydrolysis and (c) after the separation step (centrifuge).

Immediately after the enzymatic hydrolysis, samples were centrifuged at 6000 rpm for 15 min at 4ºC, and both the supernatant (liquid fraction) and the pellet (solid fraction) were collected. For analysis of the sugar content, part of the liquid fraction was centrifuged at 10000 rpm for 20 min, and the supernatant was collected and stored at -20ºC. The solid fraction was collected (without strict sterility) for sugar content determination, routine characterization, and further use as the substrate of SSF processes. The storage of solid hydrolysates was avoided and it was produced as needed for the SSF experiments.

The hydrolysis step was evaluated in terms of solids recovery, measured as the percentage of wet and dry solids recovered in the solid fraction, and of sugars released from the OFMSW into the liquid fraction. Sugar content was expressed as a titer (g L^{-1}) or as a yield (g per g of initial dry matter). In the solid fraction, sugars were also measured and also expressed as a concentration (g DM^{-1}) or as a yield (g per g of initial dry matter).

3.4 SSF experimental set-up

In this work, SSF experiments have been performed at different volumes (0.5 L, 1.5 L, 22 L and 100 L) in packed-bed forced-aerated bioreactors. Despite the scale, bioreactors presented the same configuration, they were tubular bioreactors, with a height twice the diameter, not thermally insulated and forcefully aerated from the bottom part. To improve air diffusion throughout the solid and prevent the material from blocking the air inlet, a stainless-steel net was placed at the bottom parts. The aeration system and oxygen monitoring set-up were also shared among scales and were custom-built at the GICOM group as explained in

previous works (Barrena, 2006; Martínez, 2018). Briefly, compressed air is supplied and specifically controlled by an airflow meter (Mass-Stream D-6311, Bronkhorst, Netherlands). Before entering the reactor, the air is humidified to saturate it with water and prevent the drying of the solid matrix. Exhausted air leaves the reactor from the upper part and goes through a water trap to prevent excess water from reaching the O_2 sensor (O_2-A_2 oxygen sensor, Alphasense, UK). Finally, the measured oxygen content is collected online by a data acquisition system (Arduino ® based), which also controls the air supply (**Figure 3.8**).

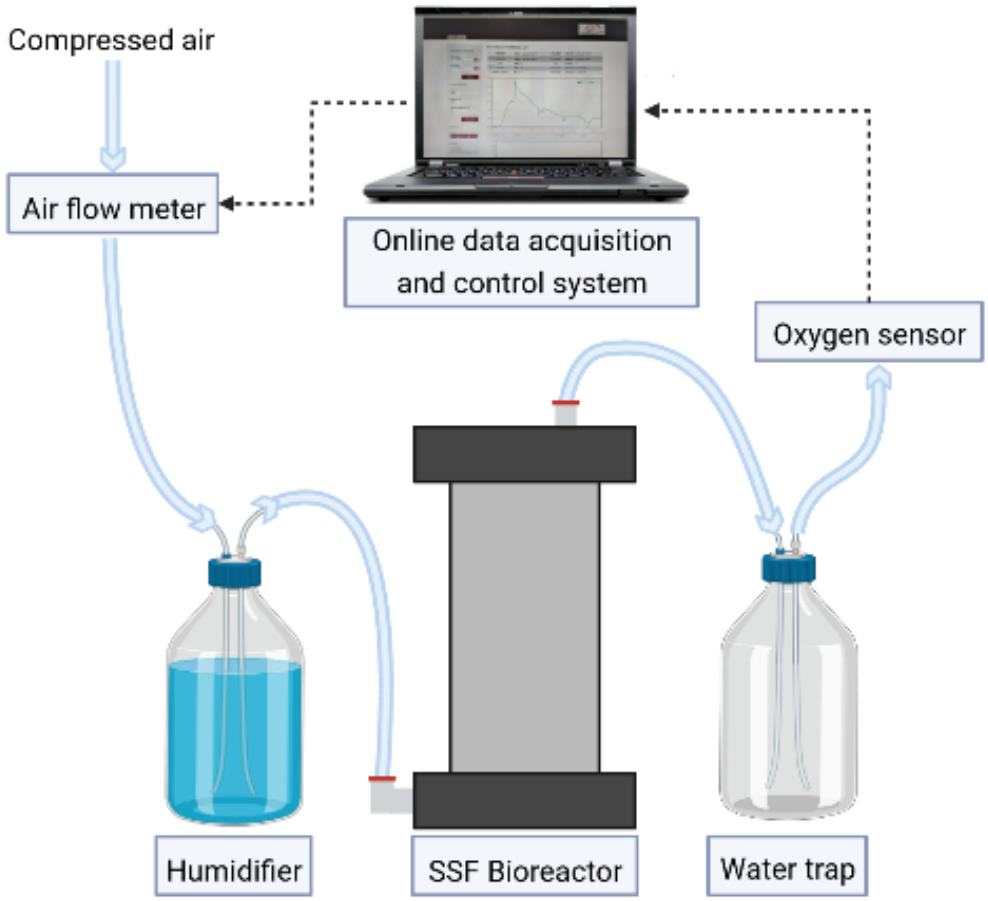

Figure 3.8 General air flow and oxygen monitoring set-up of SSF. Created using BioRender.

All SSF experiments lasted at least 72 h, as it has been established as the required time to maximize the spore count for Bt (Cerda et al., 2019). Samples were always taken at the start and end of the fermentation process, and when necessary, also during the course of it at 24 h intervals. Consistently, these samples were analyzed in terms of DM, pH, viable cells and spores.

3.4.1 SSF bioreactor: 0.5 L scale

These bioreactors were polyvinyl chloride (PVC) cylinders of 13 cm in height and 6 cm in diameter, corresponding to a working volume of 0.370 L, and two air chambers at the top and bottom making a total volume of 0.5 L. For temperature control of the packed bed, reactors were placed in a thermostatic water bath set at 30ºC. In all experiments, reactors were previously cleaned with bleach diluted in water and when more aseptic conditions were required, reactors were loaded inside a laminar flow chamber. A maximum amount of 110 g of wet solids was used including the bulking agent, which was adjusted to a volumetric ratio between 1:1.5 – 1:2 v v^{-1} (corresponding to 15 g and 20 g, respectively). Reactors were inoculated with 0.03 ml g^{-1} of wet solid to reach an initial viable cell count of around 10^7 CFU g^{-1} DM, this parameter was optimized previously by Mejias et al. (2020). As well as the airflow, which was set to 27 mL g^{-1} DM h^{-1} (20 mL min^{-1}) for ensuring aerobic conditions (Mejias et al., 2017). The 0.5 L bioreactors' appearance can be seen in **Figure 3.9a** and **Figure 3.9d**.

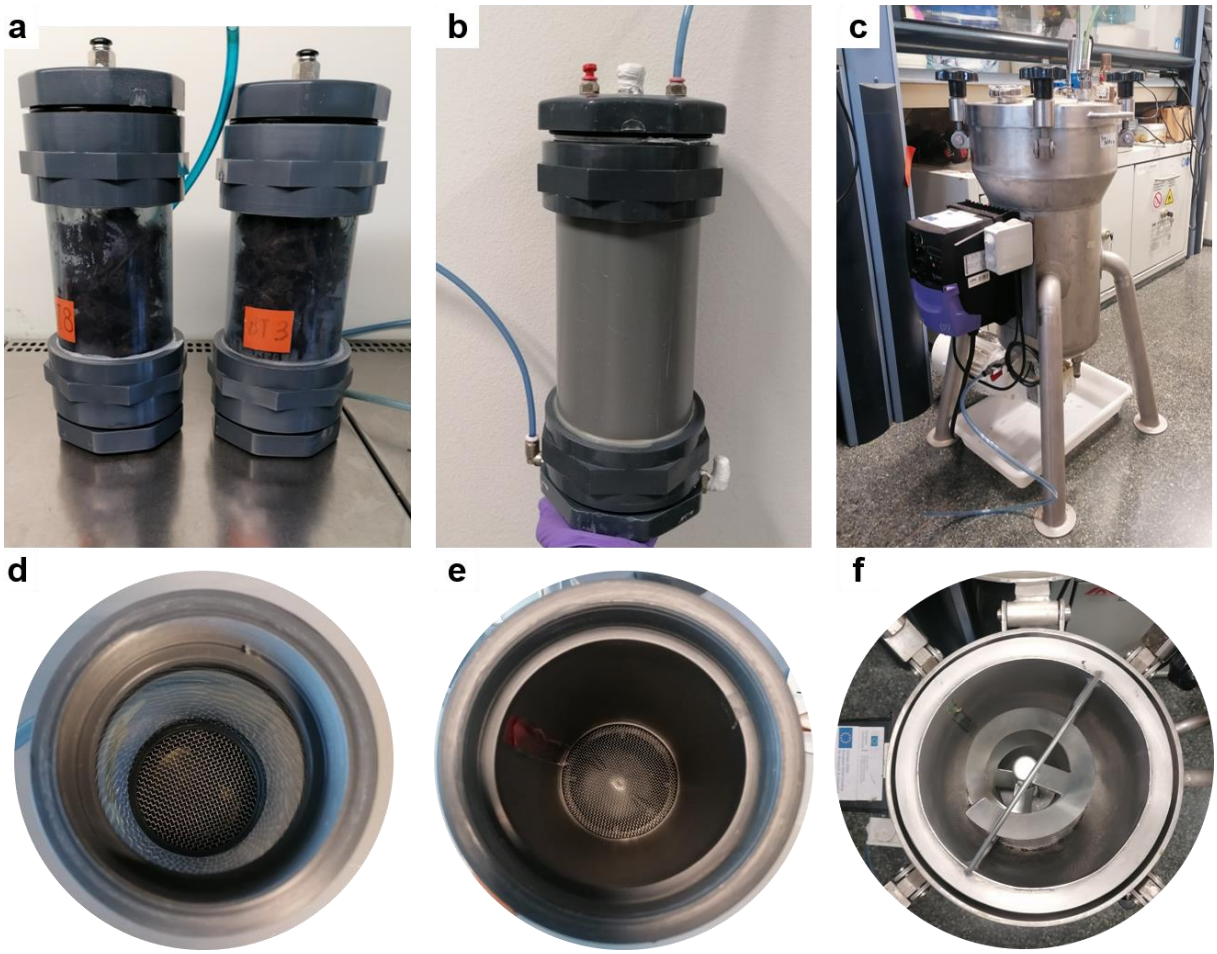

Figure 3.9 SSF bioreactors of 0.5 L, 1.5 L and 22 L scales. (a, b and c, respectively) Frontal views and (d, e and f, respectively) upper views.

3.4.2 SSF bioreactor: 1.5 L scale

These bioreactors were PVC cylinders of 21 cm in height and 9 cm in diameter, corresponding to a working volume of 1.3 L, and two air chambers at the top and bottom making a total volume of 1.6 L. At this scale, experiments were performed with no temperature control (room temperature) to study the effect of a dynamic temperature profile. The temperature was monitored using small wireless temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) to obtain accurate temperature profiles at different reactor heights (5 and 16 cm). A total amount of 440 g of wet solids was loaded into the reactors, corresponding to 360 g of solid substrate and 80 g of bulking agent (1:2 v v^{-1}). The inoculum ratio of the previous scale (0.03 ml g^{-1}) was maintained as well as the airflow (27 mL g^{-1} DM h^{-1} or 80 mL min^{-1}). The 1.5 L bioreactors' appearance can be seen in **Figure 3.9b** and **Figure 3.9e**.

3.4.3 SSF bioreactor: 22 L scale

For a larger scale, a 22 L cylindrical stainless-steel bioreactor was used. This reactor contained a removable inner basket with 2 mm holes at the bottom for air diffusion where the solid substrate was loaded. Inside the basket, there is also an automatic helical ribbon mixer, which is powered by an engine placed at the bottom of the reactor. Temperature is monitored by a temperature probe located between the mixer and the basket wall at 15 cm from the bottom part of the reactor. Also, button sensors were distributed inside the reactor to obtain accurate profiles at different heights inside the packed bed (18 cm and 30 cm) and the basket wall (12 cm, 24 cm and 36 cm). The 22 L bioreactor appearance can be seen in **Figure 3.9c** and **Figure 3.9e**, and a detailed schema is presented in **Figure 3.10**.

The working volume was approximately 85% of the reactor capacity, corresponding to 3.5 kg of the final substrate mixture and 1 kg of sterile wood chips (1:2 v v^{-1}). 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. As in the previous scales, the mixture was inoculated with 30 mL of diluted Bti inoculum per kg and the humidified air flow (730 ml min-1) was provided continuously at the lower part as in the previous setups. Intermittent mixing was automatically set using 12 rpm for 5 min at 24 h intervals before sampling. During the fermentation course, samples were only taken from the upper part of the reactor to minimize process disturbances, but at the end of the process samples from the middle and lower parts were also taken.

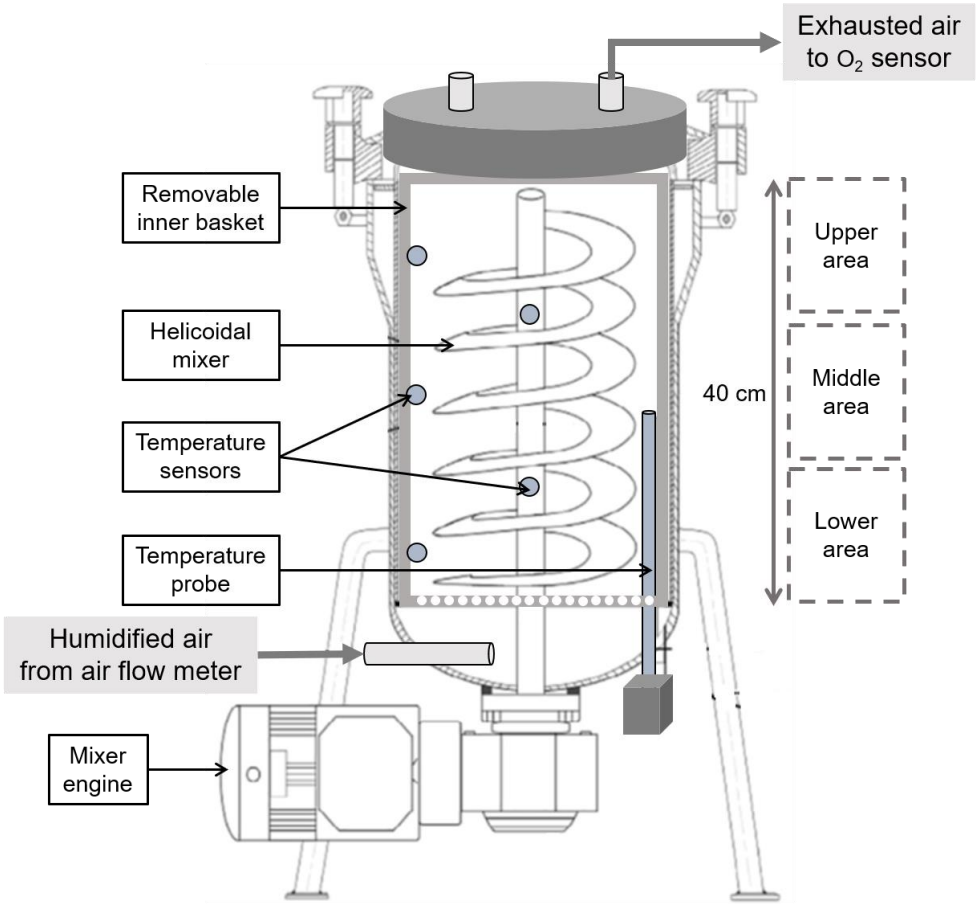

Figure 3.10 22 L bioreactor set-up including parts and sampling areas.

3.4.4 SSF bioreactor: 100 L scale

A validation experiment of the strategy developed in previous scales (**Chapter 4**) for the SCALIBUR project, was performed using a 100 L bioreactor. The configuration was similar to the 22 L bioreactor, a cylindric of stainless steel with 80 cm height and 46 cm diameter. It also contained a stirrer in the center (80 cm height) but this is connected to a rotor situated on the top instead of the bottom of the reactor. On the upper part, there is also a hopper to load the reactor without removing the stirrer and the lower cover can be opened to recover the material once the fermentation is done. The temperature was monitored using button sensors that were distributed inside the reactor attached to the mixer to measure the temperature inside the packed bed (10, 25, 45 and 30 cm) and at the basket wall at the same heights. **Figure 3.11** shows some pictures of the bioreactor.

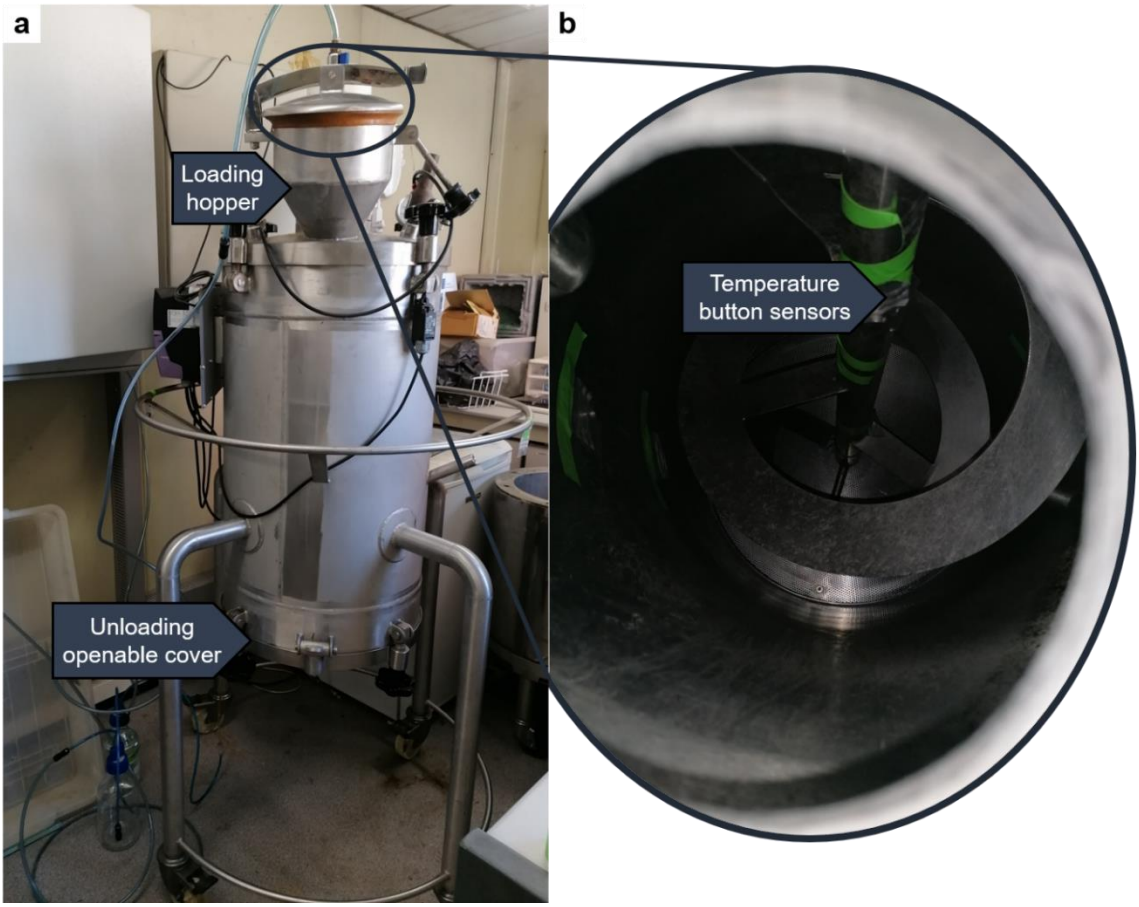

Figure 3.11 100 L SSF bioreactor. (a) Frontal view and (b) inside view from the loading hopper.

3.5 Microbial growth estimation

3.5.1 Specific oxygen uptake rate

With the recorded oxygen concentration by the acquisition system, the specific oxygen uptake rate (sOUR) was calculated as an indicator of the biological activity as stated by Ponsá et al. (2010) (Eq. 1).

$$sOUR = F \times \left(0.209 - y_{O_2}\right) \times \frac{P \times 32 \times 60 \times 1000^{a}}{R \times T \times DM \times 1000^{b}} \quad \text{(Eq. 1)}$$

Where,

sOUR, 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 101325 (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.

3.5.2 Viable cells and spores

Determination of viable cells and spores was performed by counting colony-forming units (CFU) (Ballardo et al., 2016; Mejias, 2020). First, 10 g of solid sample were mixed with 90 mL of Ringer solution and stirred at 180 rpm for 20 min at room temperature. From the liquid extract, serial dilutions were prepared using Ringer solution and 50 µL plated in triplicate onto Petri dishes containing Nutrient agar medium (Oxoid CM0003B, England). For spore estimation, 20 mL of the previous extract were submitted to a thermal shock by incubating them at 80ºC for 10 min and then immediately into ice for 5 min. Then, samples were serially diluted and plated. All plates were incubated at 30ºC for 20 h and counted in a range between 25 and 250 colonies. Viable cells or spores were estimated in terms of colony-forming units (CFUs) and related to the DM of the sample (Eq. 2):

$$Viable\ cells\ or\ spores\ concentration\ (\text{CFU}\ g^{-1}\text{DM}) = \frac{No\ CFUs \times D \times Ex}{0.05 \times DM} \quad \text{(Eq. 2)}$$

Where,

No CFUs, number of colonies counted by Petri dish (25 - 250)

D, dilution factor of the sample

Ex, extraction factor (9 mL g^{-1})

0.05, mL of diluted extract plated

DM, dry weight per g of wet solid.

The sporulation ratio at a certain time was calculated considering that the viable cell count included both vegetative cells and spores according to the (Eq. 3):

$$Sporulation\ ratio\ (\%) = \frac{spores\ g^{-1}DM}{viable\ cells\ g^{-1}DM} \times 100 \quad \text{(Eq. 3)}$$

The sporulation yield that expresses the spores produced per initial viable cell inoculated is calculated using the viable cell count at time 0 h, and it enables a comparison between processes at different scales and conditions (Eq. 4).

$$Sporulation\ yield = \frac{spores\ g^{-1}DM}{initial\ viable\ cells\ g^{-1}DM} \quad \text{(Eq. 4)}$$

3.6 Routine analysis for the characterization of solids

3.6.1 Moisture and dry matter

For moisture content (MC) and dry matter content (DM) determination, a specific amount of solid sample (30-100 g) was placed in a previously weighed crucible and dried in an air oven at 105ºC for 24 h. Then, the crucible containing the dry sample was weighed again and the MC was calculated as the quantity of water evaporated as stated in Eq. 5.

$$MC\ (\%) = \frac{(W_i - W_f)}{(W_i - W_0)} \times 100 \quad \text{(Eq. 5)}$$

$$DM\ (\%) = 100 - MC \quad \text{(Eq. 6)}$$

Where,

W_i, initial weight of the crucible and the wet sample (g)

W_f, final weight of the crucible and the dry sample (g)

W_0, weight of the empty crucible (g)

3.6.2 Organic matter

For organic matter (OM) analysis (equivalent to volatile solids, VS), a specific amount of the dried solid sample (1-3 g) from **Section 3.6.1** was placed in a previously weighed dry crucible and ignited at 550ºC for 3h. After cooling, the crucible containing the remaining ashes was weighed and the OM was calculated as the loss of weight of the sample as stated in Eq. 7.

$$OM\ (\%) = \frac{(W_d - W_a)}{(W_d - W_0)} \times 100 \quad \text{(Eq. 7)}$$

Where,

W_d, initial weight of the crucible and the dry sample (g)

W_a, final weight of the crucible and the remaining ashes (g)

W_0, weight of the empty crucible (g)

Results for DM and OM content are presented as the mean of three technical replicates ± standard deviation.

3.6.3 pH and conductivity

For pH and conductivity determination, an aqueous extract was obtained by mixing the sample and distilled water in a ratio of 1:5 (w v^{-1}). At least 10 g of the solid sample was used if possible. The sample was shaken at room temperature for 30 min to allow the salts to solubilize into the liquid phase. Then, the supernatant was used to measure the pH in an electronic pH meter (Crison®, micropH2001) and the conductivity in an electrical conductivity meter (XS Cond 8).

3.6.4 Bulk density and air-filled porosity estimation

Bulk density (BD) refers to the wet weight per volume unit of a sample, calculated as expressed in Eq. 8. Air-filled porosity (AFP) refers to the volume fraction of air in a solid porous sample (Richard et al., 2004) and is estimated according to Eq. 9.

$$BD\ (kg\ L^{-1}) = \frac{W_s}{V_s} \quad \text{(Eq. 8)}$$

$$AFP\ (\%) = 1 - BD\left(\left(\frac{1-DM}{D_W}\right) + \left(\frac{DM \times OM}{PD_{OM}}\right) + \left(\frac{DM(1-OM)}{PD_{ash}}\right)\right) \quad \text{(Eq. 9)}$$

Where,

W_s, weight of the wet sample (kg)

V_s, volume of the sample (m^3)

BD, wet bulk density (kg m^{-3})

DM, dry matter on a wet basis

D_W, water density (1000 kg m^{-3})

OM, organic matter on a dry basis

PD_{OM}, organic matter particle density (1600 kg m^{-3})

PD_{ash}, ash particle density (2500 kg m^{-3})

3.6.5 Biodegradability

For biodegradability evaluation, which measures the biological stability of a solid sample, two respiration indices were used: the dynamic respiration index (DRI_{24}) (Eq. 10), which represents the average oxygen uptake rate during the 24 h of maximum activity, and the cumulative oxygen consumption index (AT_4) (Eq. 11), which is the cumulative oxygen consumption of the four days after the lag phase. More details can be found in Ponsá et al., (2010). The analysis is performed using the bioreactors presented in **Section 3.4.1**, with 100 g of solid samples including bulking agent if required at 37ºC for around 7 days. All measurements were conducted in triplicate.

$$DRI_t\ (mg\ O_2\ h^{-1}DM^{-1}) = \frac{(O_{2,i}-O_{2,o})\times F\times 31.98\times 60\times 1000^{a}}{1000^{b}\times 22.4\times DM} \quad \text{(Eq. 10)}$$

Where,

$(O_{2,i} – O_{2,o})$, difference in oxygen content between airflow inlet and outlet

F, volumetric airflow measured under normal conditions (mL min^{-1})

31.98, oxygen molecular weight (g mo^{-1})

60, conversion factor (min h^{-1})

1000^a, conversion factor (mg g^{-1})

1000^b, conversion factor (mL L^{-1})

22.4, volume occupied by one mol of an ideal gas under normal conditions (L)

DM, dry mass of the sample (g)

$$AT_4\ (mg\ O_2\ h^{-1}) = \int_{t1}^{t2} DRI_t\ dt \quad \text{(Eq. 11)}$$

Where,

t1, lag phase time (h) measured as the time that takes to reach 25% of the maximum activity value

t2, 96 hours after the lag phase (h)

3.7 Analysis of sugars

3.7.1 Reducing sugars content

Reducing sugars (RS) were quantified using the DNS method (Miller, 1959). For solid samples, it was first performed a solid-liquid extraction using distilled water in a 1:10 (w v-1) ratio for 30 min at 50ºC and 150 rpm. Then, samples were centrifuged at 10000 rpm for 20 min at 4 ºC and the supernatant was collected and stored at -20ºC. For liquid samples, the same centrifugation step was applied. The supernatants were filtered through a 0.45 µm membrane filter and, when needed, diluted with water to obtain a concentration in the range of the calibration curve (glucose, 0.2–3.3 g L^{-1}) (**Figure 3.12**). Briefly, the method consisted of adding 3 mL of DNS reagent to 1 mL of filtered supernatant in a 25 mL glass test tube. Then, the mixture was placed in a boiling water bath for 5 min. After cooling, 20 mL of distilled water were added, and the sample was mixed by inversion. For the blank, one mL of distilled water, instead of the sample was used. The absorbance was measured at 540 nm in a spectrophotometer (Cary 50 Bio, UV-Visible Spectrophotometer). Analyses were performed in triplicate. The reducing sugar content in solid samples was expressed as grams of glucose equivalent per gram of dry matter (DM).

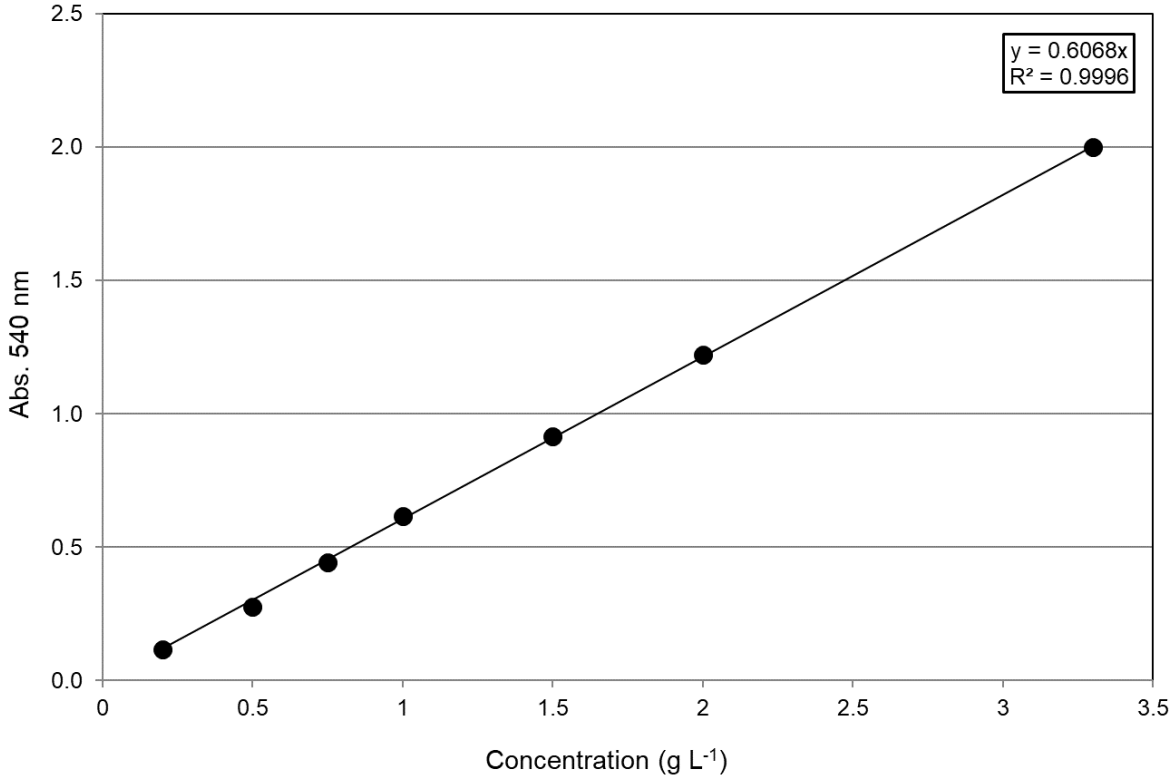

Figure 3.12 Example of glucose calibration curve for reducing sugar analysis.

3.7.2 Glucose content

Glucose was quantified from the sugar extracts using a biochemistry analyzer (YSI 2950D, YSI Inc. / Xylem Inc., USA). Samples were diluted within the equipment range for glucose (0.05 - 25 g L^{-1}).

3.7.3 Sugar profile (HPLC)

Specific sugar profile in the liquid fractions was quantified by high-performance liquid chromatography (HPLC) (Ultimate 300, Thermo Fisher Scientific) using a Rezex™ column for monosaccharides of 300 mm length x 7.8mm diameter with 8 µm particle size (reference: 00H-0135-K0, Phenomenex Inc.). A constant flow rate of ultrapure water at 0.6 mL min^{-1} was used for 30 min. The temperature was set at 75ºC and a refractive index-based sensor was used for detection (Water 2410). Liquid samples were filtrated using 0.45 µm filters and diluted accordingly before injection. For the identification and quantification of peaks, a calibration curve for nine different mono- and disaccharides (0 – 20 g L^{-1}) were prepared (**Figure 3.13**). Disaccharides could not be properly separated in this method and therefore, considered a group for quantification purposes.

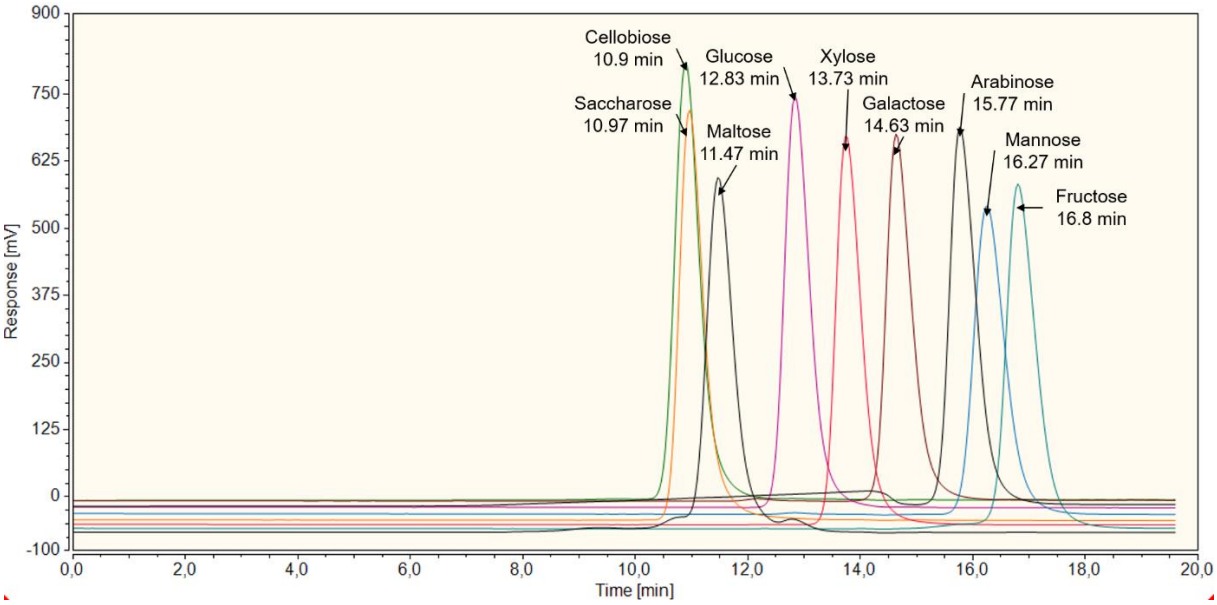

Figure 3.13 HPLC chromatograms of the six monosaccharides (glucose, xylose, galactose, arabinose, mannose and fructose) and three disaccharides (saccharose, cellobiose and maltose) evaluated (20 g L^{-1}).

3.8 Specific analysis

3.8.1 Elemental analysis

Elemental determination was carried out by the *Servei d'Anàlisi Química UAB* using a CHNS elemental analyzer Flash 2000 (Thermo Scientific). A dry sample was combusted at 1200°C with air excess and quantification was performed by gas chromatography.

3.8.2 Fiber content

The fiber content, including cellulose, hemicellulose, and lignin, was determined by the *Departament de Ciència Animal i dels Aliments* of UAB using an Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY).

3.8.3 SEM imaging

Scanning Electron Microscope (SEM) (Zeiss EVO, Germany) from the *Servei de Microscòpia* of UAB was used to visualize Bti cells, spores and crystals. Pure culture and samples of fermented material were used for analysis. For solid samples, a solid-liquid extraction was performed with Ringer solution (1:10 w v^{-1}) for 20 min. For spores and crystal visualization, samples were sonicated by 10 rounds of 1 min in an ultrasonic bath (J.P. Selecta, Spain) followed by 30 s of ice each. All samples were fixed on adhesive paper and dried for further sample metallization with gold.

The dimensions of spores and crystals were determined using the software ImageJ®.

3.8.4 DNA extraction and 16S rRNA sequencing

Sequencing was performed by the *Genomic Service* of UAB. Solid samples were processed for DNA extraction using the Soil DNA Isolation Plus Kit (Norgen Biotek, Canada) and following the manufacturer's instructions. The DNA extracts (stored at -20ºC) were tested for concentration and quality using a NanoDrop spectrophotometer (NanoDrop 1000, Thermo Fischer Scientific) and used to construct the corresponding genomic libraries for microbial community analysis.

3.9 Statistical analysis

Statistical difference between different samples was analyzed using one-way ANOVA ($p < 0.05$ confidence) with the Tukey test in SigmaPlot 12.5 software.

Chapter 4 **Results**

This chapter consists of four sections, three of them published as a scientific paper in international indexed journals. Therefore, each section includes its specific introduction, materials and methods, result presentation and discussion, conclusions and references. Accordingly, each section is presented in the corresponding format of the journal. Figures and tables are numbered independently in each article.

Article I

Organic municipal waste as feedstock for biorefineries: bioconversion technologies integration and challenges

Esther Molina-Peñate, Adriana Artola, Antoni Sánchez

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European policies presented in Chapter 1 promote the sustainable use of OFMSW. Currently, composting and anaerobic digestion are the most consolidated and implemented technologies but considerable research efforts are being undertaken to produce higher value-added products. This section presents a bibliographic review on the use of enzymatic hydrolysis as a method for obtaining organic functionalized molecules from OFMSW in a biorefinery context. It includes potential uses for the resultant liquid and solid fractions

Organic municipal waste as feedstock for biorefineries – Bioconversion technologies integration and challenges

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Abstract

The need for ensuring resources and energy supplies has stimulated the use of renewable feedstocks for biorefineries. Among organic wastes, the organic fraction of municipal solid waste (OFMSW) outstands because of its increasing amounts and management requirements. Unlike other homogeneous organic waste from food and other industries, OFMSW is characterized by high instability, complexity, and heterogeneity. This review aims to unfold the potential of the OFMSW as feedstock for biorefineries through a discussion on recent valorization alternatives to the commonly employed anaerobic digestion for biogas production. Enzymatic hydrolysis has been identified as a key to unlock the capabilities of OFMSW through the fractioning of structural components into functionalized molecules. In addition, multiple scenarios for the subsequent utilization of such molecules are also presented, together with suitable configurations for process integration. Lastly, challenges for the OFMSW biorefinery implementation have been identified.

Keywords

Biorefinery; Biowaste; OFMSW; Enzymatic hydrolysis; Bioconversion; Valorization

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1. Introduction

The transition from the current linear economy to a circular economy has been attracting widespread interest in recent years. One major driver of this socioeconomic shift paradigm is the expected depletion of material resources(Jowitt et al. 2020). Efficient management of resources becomes, therefore, essential to prevent scarcity. Moreover, resource recovery from the current take-make-waste economic models secures their supply providing a competitive advantage in the future global economy (Ellen MacArthur Foundation 2021; Tonini et al. 2013). Another major driver is the increasing municipal solid waste (MSW) generation, which represents an environmental burden and a high cost to society (Kaza et al. 2018). Comprehensively addressing MSW as a source of resources and not as a residue to be managed opens a door towards a more sustainable society (Sánchez et al. 2015).

The dramatic increase of MSW generation from 1.3 billion tonnes in 2012 to 2.01 billion tonnes in 2018 has been related to population growth rate, rapid urbanization, and overflowed waste streams in high-income countries (Kaza et al. 2018). The global MSW generation is predicted to reach 3.4 billion tonnes by 2050. Nearly half of this amount are biodegradable materials, known as the organic fraction of municipal solid waste (OFMSW) (Al Seadi et al. 2013; Kaza et al. 2018). This fraction comprises two major streams: green waste from parks and gardens, and food waste from households, cafeterias, restaurants, lunch-rooms, and markets (Al Seadi et al. 2013). It is characterized by high moisture and organic matter content, a rather acidic pH, and containing metals and macro/micronutrients (Campuzano and González-Martínez 2016; Barampouti et al. 2019). Therefore, OFMSW is an abundant, carbon-rich, and, so far, free-of-cost resource.

The need for ensuring materials and energy supply as well as minimizing dependency on fossil fuels led to the concept of biorefineries, which is analogous to petroleum-based refineries but uses biomass instead of petroleum as raw material. The major goal of a biorefinery is to maximize the value derived from biomass constituents and intermediates by converting them into a palette of valuable bioproducts and bioenergy (Kamm and Kamm 2004). Several classification systems have been proposed according to different elements of the biorefineries, i.e. platforms, feedstocks, processes, and products (Budzianowski and Postawa 2016). The platform system has been reported as the most significant because it describes intermediates that can be reached via different conversions processes or feedstocks and act as building blocks of different products. Examples of platforms are biogas, C6 sugars, C5 sugars, or H_2 (Venkata Mohan et al. 2016). In terms of feedstock, it has been distinguished between dedicated

crops and residues. After raising concerns about food and land-use competition from whole crops, research efforts were shifted towards lignocellulosic biomass-based biorefineries. Recently, different types of waste have been proposed as feedstock as a way of transitioning towards a circular economy, in which waste generation is minimized to a larger extent (Venkata Mohan et al. 2016; Alibardi et al. 2020). To date, the focus of waste biorefineries research has been centered on homogeneous waste streams of specific industries (Mirabella et al. 2014; Carmona-Cabello et al. 2018). However, biorefineries based on OFMSW have been reported to offer larger climate benefits due to the avoidance of conventional management, and of land and fertilizers use for the cultivation of agricultural biomasses (Tonini et al. 2016; Vea et al. 2018). Ongoing advances in OFMSW valorization technologies may unlock its potential as a feedstock for biorefineries.

This review aims to discuss the feasibility of the integration of OFMSW valorization technologies into the biorefinery concept. In this context, enzymatic hydrolysis is presented as a way to obtain functionalized molecules from OFMSW that serve as platforms for bioconversion processes. The current technological status of enzymatic hydrolysis is presented and the most promising valorization routes for the resulting fractions are evaluated. Available examples of OFMSW biorefineries configurations are summarized. The major bottlenecks for ensuring the viability of the OFMSW biorefinery are also discussed.

2. OFMSW valorization state of the art

Resources recovery from waste is not a recent phenomenon (Velis et al. 2009), but it was public health and environmental concerns that brought proper waste management to the political agenda of high-income countries (Wilson 2007). The high biodegradability and moisture content of OFMSW convert this fraction into the major contributor to the environmental impact of landfilled MSW (Wilson 2007). Natural biodegradation of organic waste implies the uncontrolled release of methane and other greenhouse gases, production of leachates that contaminate soil and groundwater, unpleasant odors, and spread of pathogenic microorganisms (Kaza et al. 2018). Consequently, significant national and regional efforts have been done to prevent OFMSW landfilling. For instance, in the European Union, the policy efforts related to waste management are the Landfill Directive 1999/31/EC (DIRECTIVE 2000) and the Waste Framework Directive 2008/98/EC (DIRECTIVE 2008). According to these, waste management should follow a 5-step hierarchy, in which waste prevention becomes the priority followed by waste reuse, recycling, recovery, and disposal. As a result, the landfilling rate dropped from 64% in 1995 to

23% in 2018 according to Eurostat statistics (Eurostat 2021). On top of that, the European Commission launched in 2015 the Circular Economy Action Plan, which aims to reduce landfilled waste to a maximum of 10% (Union 2014). In this context, it is clear that ambitious solutions are needed to ensure that the not-landfilled OFMSW serves a better and more sustainable purpose.

2.1 Current management technologies

To redirect the OFMSW away from landfilling, it was necessary to develop and promote tailored technologies for its treatment. The most implemented technologies were incineration, anaerobic digestion (AD) for biogas production, and composting. Reviews of the benefits and drawbacks from these well-established technologies have already been published (Cerda et al. 2018; Angelidaki et al. 2018; Makarichi et al. 2018), and they will not be discussed here nor thermochemical processes, such as gasification or pyrolysis (Matsakas et al. 2017). Shortly, incineration allows energy recovery from OFMSW, at the expense of high capital and operating costs and the possibility of recovering valuable nutrients (Makarichi et al. 2018). AD for biogas production has been proven to be a robust, efficient, and relatively low-cost process (Scoma et al. 2016; Mayer et al. 2020), but biogas upgrading is necessary for its effective utilization as higher fuel standard (Angelidaki et al. 2018). When applied together, AD and composting treatments allow energy recovery alongside nutrient recycling as a soil amendment. However, AD does not ensure full intrinsic energy exploitation, and high-quality compost is difficult to attain (Cerda et al. 2018).

The shift from landfilling towards more specialized technologies was also fostered by an increase in source-separated collection systems of MSW, which has been described as the first condition for OFMSW valorization (Sisto et al. 2017; Mayer et al. 2020). Source-separated collection facilitates the reuse of waste by reducing pretreatment needs and easing quality verification (Velis et al. 2009; Paes et al. 2019). In contrast to mechanical selection from mixed collection systems, the selection is carried out directly at generating properties or at communal collection points. The OFMSW derived from unsorted or poor source-separated collection systems results in low efficient AD systems and bad quality, non-marketable compost (Cerda et al. 2018; Mayer et al. 2020). For example, Moreno et al. (2021) evaluated the effect of source-separating on the production of bioethanol and biogas. The maximum ethanol concentration achieved for the source-separated OFMSW was double than for the non-separated. However, building robust and high-quality source-separated collection systems involve a significant

137 economic investment (Kaza et al. 2018; Mayer et al. 2020). Therefore, efforts need to be directed to
138 finding more profitable processes to justify the economic investment.

139 **2.2 OFMSW composition**

140 Compared with organic waste streams from the agriculture and food processing industry, which
141 are mostly homogeneous and constant in composition, OFMSW composition is heterogeneous and highly
142 variable (Fava et al. 2015; Barampouti et al. 2019). Hence, its characterization becomes essential in the
143 selection of the appropriate valorization route. .The physical and chemical characteristics generally
144 measured are presence of impurities, humidity and solids content, elemental composition (C, H, N, S),
145 pH, and organic matter (biodegradable or not), depending on the objective of the study (Campuzano and
146 González-Martínez 2016). Pleissner and Peinemann (2020) suggested that OFMSW composition should
147 be evaluated in terms of its main constituents, i.e. carbohydrates, proteins, lipids and lignin, and not in
148 further detail. Campuzano and González-Martínez (2016) gathered the characteristics of the OFMSW
149 from 43 cities in 22 countries and obtained an average composition (w/w) of 55.5 % carbohydrates, 17.7
150 % protein, 17.5 % lipids, and 9.7 % lignin. Carbohydrates are the mayor fraction and are composed of
151 free sugars, starch and fibers (cellulose, hemicellulose and pectin). Sugars and starch are more easily-
152 biodegradable than fibers, and therefore highly influenced by the activity of the indigenous microbial
153 consortium during storage and transportation (Campuzano and González-Martínez 2016; Pleissner and
154 Peinemann 2020). While high xylan is associated with more stable composition, it also increases the
155 chemical complexity of the sample hindering its biodegradability and increasing pretreatment
156 requirements (Yang et al. 2015). OFMSW composition has a significant influence on the efficiency of
157 biological processes and their final products as the type of organics and nutrients available influence the
158 kinetics, the efficiency of the process and the bioproducts production potential (Dogan and Demirer 2009;
159 Alibardi and Cossu 2016; Tyagi et al. 2018).

160 OFMSW characterization is influenced by the continuously changing composition because of
161 seasonal, regional, technological, and socio-economic (Pleissner and Peinemann 2020). Therefore,
162 characterization should be carried out carefully and as site-specific as possible (Tyagi et al. 2018).
163 Straightforward methodologies, such as biodegradability measurement (Ponsá et al. 2010a) and chemical
164 oxygen demand (COD) (Yang et al. 2015), are also relevant for regular quality verifications.
165
166

2.3 One waste, multiple names

During the elaboration of this review, an evident confusion with the terminology employed to designate the organic fraction of municipal solid waste was observed. Table 1 compiles different terms that have been employed interchangeably throughout literature, some of them not accurately enough. The term "biomass", i.e mass of living organisms (Houghton 2008), has been employed to designate all natural carbonaceous resources that can be used to generate fuels (Pang 2016). Thus, it is an unspecific and widely overused term. In Table 1 it can be seen how only 10% of the published works related to the term "biomass", are also related to the term "waste". Terms such as "organic waste", "biowaste" or "food waste" fail to describe the origin of the residue, i.e. industrial, agricultural, or municipal. For them, less than 30% of the published works are related to municipal (or urban) wastes (Table 1). Contrary, the terms "municipal waste" or "household waste" fail to describe the type of residue, i.e. plastic, metal, electronic, or organic, and again, less than 30% of the published works actually discuss organic wastes. The most accurate term "organic fraction of municipal solid waste", or its acronym "OFMSW", is the less used one with only 925 papers published in the Web of Science (Table 1). It was also observed that the term "OFMSW" is used indiscriminately to refer to fresh food used to simulate real waste or food waste from university cafeterias. However, these types of waste may not be representative of the complexity of the OFMSW coming from municipal treatment facilities (Zhou et al. 2013; Alibardi and Cossu 2015). The authors of this review would like to emphasize that the term used to designate the organic fraction of the waste separately collected from municipalities should be standardized to not increase its inherent variability and to facilitate the comparison of results reported in the literature.

188 **Table 1** Number of published works for the keyword search in the Web of Science (June 2021)

Word Search	Article	Review	Total Papers	% Total
"Biomass"	313014	14451	327465	
AND "Waste"	32133	2633	34766	10.6%
AND "Municipal" OR "Urban"	10328	732	11060	3.4%
"Organic waste"	4418	410	4828	
AND "Municipal" OR "Urban"	1145	133	1278	26.5%
"Biowaste"	1658	100	1758	
AND "Municipal" OR "Urban"	404	27	431	24.5%
"Food waste"	7647	807	8545	
AND "Municipal" OR "Urban"	1612	229	1841	21.8%
"Municipal waste"	5455	430	5885	
AND "Organic"	1460	146	1606	27.3%
"Household waste"	1597	64	1661	
AND "Organic"	430	23	453	27.3%
"Organic fraction municipal solid waste" OR "OFMSW"	862	63	925	

189

190 **3. The OFMSW biorefinery**

191 Biorefineries design is not a straightforward task and it should be tailored to the specifics and
192 quantity of the feedstock, the location constraints, and regional policies (Kamm and Kamm 2004;
193 Moncada B et al. 2016). A common principle when designing a biorefinery is that the decomposition of
194 the feedstock should be conducted hierarchically following a flexible and logical sequence, this is known
195 as the cascading principle (Fava et al. 2015; Moncada B et al. 2016; Alibardi et al. 2020). The logic of the
196 sequence might be adapted to the goal of each biorefinery but commonly, selling price and purity
197 restrictions establish the first steps (Moncada B et al. 2016). Another common principle is the integration
198 of feedstocks, technologies, and products to maximize the use of resources and optimize the overall
199 performance within the biorefinery (Moncada B et al. 2016; Alibardi et al. 2020). For such a
200 heterogeneous substrate as the OFMSW, i.e. with unpredictable quality, the integration of different
201 conversion processes also reduces the inherent risk of failure (de Sousa et al. 2021).

This intrinsic complexity of municipal organic wastes entails a wide spectrum of potentially marketable products in a waste biorefinery (Moretto et al. 2019a; Pleissner and Peinemann 2020). So far, most of the proposed OFMSW biorefinery-like configurations have been centered around AD technology for biogas production (Dogan and Demirer 2009; Escamilla-Alvarado et al. 2017b; Tyagi et al. 2018; Khoshnevisan et al. 2018b). However, authors in the literature do not seem to agree whether an OFMSW biorefinery should employ AD technology for biogas production as the main platform (Sisto et al. 2017; Elyasi et al. 2021) or as a complementary platform to handle intermediates (Mahmoodi et al. 2018a; Valentino et al. 2018). For a biorefinery to be economically robust and minimize financial risks it has to be able to decide among different bioproducts thus pushing towards multi-platform designs that would also enhance the recovery of resources (Moncada B et al. 2016; Alibardi et al. 2020; Tonini et al. 2013) Furthermore, Barampouti et al. (2019) and Mahmoodi et al. (2018a) highlighted that biogas might be outcompeted by other liquid biofuels, i.e. bioethanol or biodiesel. Finally, to reach environmental sustainability, the substitution of complex chemical routes and petroleum-based precursors in the production of commodities or high-value products would tip the balance in favor of bioproducts (Laurent et al. 2014; Venkata Mohan et al. 2016; Escamilla-Alvarado et al. 2017b). Therefore, future efforts should be focused on integrating other valorization technologies alongside energy production.

As of traditional refineries, the configuration of biorefineries involves several conversion pathways or platforms with their corresponding upstream and downstream operations. To summarize the latest advancements in the valorization processes of OFMSW, technologies have been classified according to the step of the conversion process they belong to: upstream, midstream, and downstream. A summary of all the discussed technologies can be seen in Figure 1.

3.1 Upstream

Upstream steps comprise all activities that occur before the bioconversion, ranging from the generation and collection of OFMSW to the pretreatments required to prepare the material for the subsequent steps. The control of waste generation is beyond the reach of biorefineries and it is associated with seasonality, population dietary patterns, and income levels (Kaza et al. 2018).

As for many other organic wastes, pretreatment technologies have been evaluated for OFMSW. Generally, pretreatment aims to remove unsorted materials, reduce particle size, increase stability or enhance accessibility to simpler components and its configuration depends on the objective of the subsequent bioconversion process (Yang et al. 2015; Liu et al. 2021). Pretreatment methods for biowaste

have been extensively reviewed and traditionally classified in different categories: physical or mechanical, chemical, and biological (Romero-Cedillo et al. 2017; Mahmoodi et al. 2018a; Barampouti et al. 2019; Cesaro et al. 2020), which are non-exclusive but rather complementary. The final choice of pretreatment layout highly influences the efficiency of subsequent bioconversion and downstream processes, the cost and benefit, the energy demand, and the environmental impact (Yang et al. 2015; Alibardi et al. 2020).

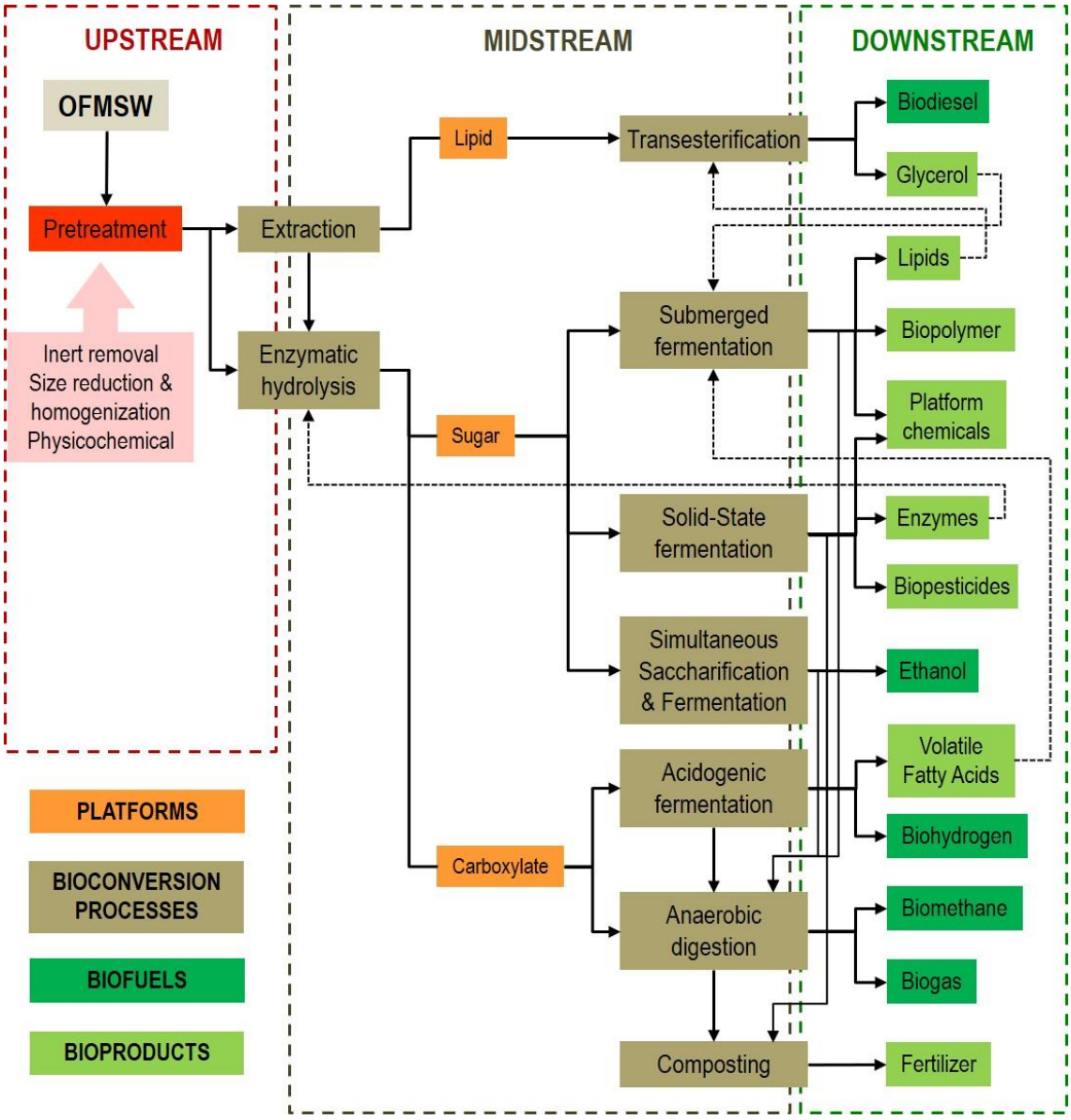

Fig. 1 Possible configuration options and products for a multi-platform OFMSW biorefinery

Physical pretreatments are applied for size reduction through milling, chipping, or grinding (Barampouti et al. 2019) to enhance the surface area accessible to enzymes or microorganisms (Romero-Cedillo et al. 2017). Physical pretreatments are also applied to reduce the degree of polymerization of the insoluble fraction through exposure to high temperatures (Barampouti et al. 2019). The application of high temperature for a certain period also acts as a pasteurization step reducing the activity of the inherent microbial consortium of the OFMSW (Barampouti et al. 2019).

Chemical pretreatments employ chemical agents, alkaline or acid, to modify the structure of the substrate, typically with the combination of temperature (physicochemical) (Romero-Cedillo et al. 2017). Dilute acid pretreatments have been widely applied because they also act as a hydrolysis step (Barampouti et al. 2019). Favorable results in terms of sugar solubilization have been reported in the literature when employing acids on kitchen waste (Vavouraki et al. 2013).

3.2 Midstream: the role of enzymatic hydrolysis

The integration of two or more platforms can counteract the fact that biorefineries are highly capital intensive (Escamilla-Alvarado et al. 2017a). As proposed by Alibardi et al. (2020), to construct different platforms, it is crucial to first fractionate, separate, or isolate individual components that act as intermediates for specific conversion processes. These authors proposed four different technologies for this purpose, washing, solid-liquid extraction, enzyme, or membrane technologies (Alibardi et al. 2020). A summary of the main advantages and disadvantages of these processes can be seen in Table 2. Washing, solid-liquid, and membrane separation processes have gained more relevance for homogeneous food waste streams to separate abundant components with high market prices, such as antioxidants, pigments, or polyphenols (Ng et al. 2020; Sharma et al. 2021). When working with OFMSW, extraction techniques are mainly used for oil extraction in the production of biodiesel (Barampouti et al. 2019; Liu et al. 2021; Ischia et al. 2021). Conversely, filtration techniques using membranes are used for the separation of high-value products obtained at further steps of the processing cascade (Huang and Ramaswamy 2013; López-Gómez et al. 2020). Of special interest are the enzyme-based separation technologies, the only ones that allow a fractioning of the complex OFMSW structures into functionalized molecules. These molecules become building blocks for the subsequent steps of the processing cascade (Escamilla-Alvarado et al. 2017a; Pleissner and Peinemann 2020).

Table 2 Advantages and disadvantages of the main technologies for initial separation of OFMSW components

Technology	Objective	Advantages	Disadvantages	Ref
Washing	Solubilization of organic matter	Cost-effective; eco-friendly (water)	Inefficient; limited accessibility to the substrate	(Ao et al. 2020)
Solid-liquid extraction	Targeted group of compounds (i.e. lipids)	Simple; wide adaptability	Environmental and economic cost of organic solvents; chemical transformation of the matrix; long processing time; limited efficiency; temperature requirement	(Alvira et al. 2009, Naviglio et al. 2019)
Membrane separation	Separation of specific compounds	Highly selective; energy efficient; eco-friendly	Cost; fouling; not efficient for complex streams	(Arbige et al. 2019, Matharu et al 2016)
Enzymatic hydrolysis	Fractioning of structural components	Possibility to integrate with bioprocesses; no inhibitory compounds; energy efficient; eco-friendly	Long processing time; high cost; limited by accessibility to the substrate	(Escamilla-Alvarado et al. 2017a)

Enzymes are proteins that catalyze chemical reactions of the metabolism of all living organisms with great specificity and efficacy. They withhold a great potential as biocatalysts in many industrial sectors, including biorefineries (Chaplin and Bucke 1990; Escamilla-Alvarado et al. 2017a). Indeed, the industrial market of enzyme technologies has increased from US $600 million to US $7 billion in revenues in the last 20 years (Arbige et al. 2019). They have been essential for the development of 2nd generation biorefineries that convert lignocellulosic biomass, such as agricultural feedstock, to bioethanol and other bioproducts (Alvira et al. 2010). So, it can be expected that they also hold the key to unlock the potential of OFMSW despite the challenge of continuous adaptation to its variable composition (Pleissner and Peinemann 2020). The pathway for the use of enzymes on renewable feedstocks is being facilitated by developments in enzyme formulations through protein engineering, i.e. greater stability and substrate specificity or lower operating temperatures (Chapman et al. 2018), alongside dropping enzyme prices, which are a limiting factor for its application (Arbige et al. 2019).

The main goal of enzymatic hydrolysis is the breakdown of macromolecules into their functional units. Considering that carbohydrates and fibers represent up to 85% of the OFMSW composition (Campuzano and González-Martínez 2016), a great number of fermentable sugars could be obtained upon the fractioning of this waste (Yang et al. 2015). The rate of this conversion process is influenced by several factors, such as lignin content and distribution, cellulose crystallinity and degree of polymerization, accessible surface area and particle size of the substrate, or chemical and structural changes during the conversion (Alvira et al. 2010; Liu et al. 2021). Additionally, the types of enzymes employed and their synergistic effects also impact the outcome (Escamilla-Alvarado et al. 2017a; Hu et al. 2018). Escamilla-Alvarado et al. (Escamilla-Alvarado et al. 2017a) reviewed the most common enzymes applied in biorefineries and their applications.

297 **Table 3** Summarized valorization routes based on enzymatic hydrolysis of real OFMSW

Enzymes[a]	Enzymatic hydrolysis conditions	Pretreatment	Total sugars concentration	Valorization liquid hydrolysate		Valorization solid hydrolysate	Ref
				Microorganism used	Bioproduct obtained		
Tailor-made cocktail (cellulases and amylases)	50ºC; pH 5; 150 rpm; 72 h; 20% (w/v)	Remove impurities, milling, autoclaving	80 g/L from ssOFMSW 66 g/L from mcOFMSW	*Bacillus coagulans*	Lactic acid 230 g/kg dry OFMSW	-	(López-Gómez et al. 2019)
Tailor-made cocktail	50ºC; pH 5; 150 rpm; 72 h; 20% (w/v)	Remove impurities, milling, autoclaving	31.2-107.3 g/L	*Actinobacillus succinogenes*	Succinic acid 300.4 g/kg dry OFMSW[b]	-	(Stylianou et al. 2020)
Cellulase (Celluclast 1.5 L) β-Glucosidase (Novozym188)	50ºC; pH 7; 150 rpm; 72 h; 10% (w/v)	Remove impurities, milling, dilute-acid	96.1 g/L in total: 65.6 g/L (acid hydrolysis) 30.5 g/L (enzymatic hydrolysis)	*Cryptococcus aerius*:	Lipids 39.6 g/kg dry OFMSW	-	(Ghanavati et al. 2015)
Xilanase (Pentopan 500 BG) Cellulase (Celluclast BG) Glucoamylase	50ºC; pH 4.8; 170 rpm; 48 h; 13.5% (w/v)	Remove impurities, lyophilize, milling, dilute-acid	25.3 g/L	*Burkholderia sacchari*:	Poly(3-hydroxybutyrate) 20.6 g/kg dry OFMSW[b]	-	(Izaguirre et al. 2019)

(NS 22035)							
Cellulases (Cellic CTec2) Hemicelluloses (Cellic HTec2)	45ºC; pH 4.8; 120 rpm; 72 h; 5% (w/v)	Remove impurities, milling, autoclave, dilute-acid	70.8 g/L in total: 46.7 g/L (acid hydrolysis) 24.1 g/L (enzymatic hydrolysis)	*Mucor indicus*	Ethanol 194 g/kg dry OFMSW	Biomethane 145 L/kg dry OFMSW	(Mahmoodi et al. 2018b)
Cellulases (Cellic CTec2) α-amylase Glucoamylase	45ºC; pH 4.8;120 rpm; 72 h; 5% (w/v) Followed by: 90ºC; pH 6; 2 h Followed by: 65ºC; pH 4.5;24 h	Remove impurities, drying, milling, autoclave, organosolv (ethanol and acetic acid)	40.9 g/L	*Enterobacter aerogenes*	Hydrogen 71.4 L/kg dry OFMSW 2,3-butanediol 139.1 g/kg dry OFMSW Ethanol 98.3 g/kg	Biomethane 23 L /kg dry OFMSW	(Ebrahimian et al. 2020)
Viscozyme L	55ºC; pH 3.5; 24 h; 10% (w/v) 25ºC; pH 4.5; 24 h; 10% (w/v)	Remove impurities, milling, autoclaving	47 g/L (50ºC) 42 g/L (25ºC)	-	-	*Bacillus thuringiensis* spores $1.3x10^8$ spores/ g dry OFMSW	(Molina-Peñate et al. 2022)

ssOFMSW: source-separated OFMSW; mcOFMSW: mixed collected OFMSW

[a] In all the references enzymes were provided by Novozymes A/S.

[b] Calculated based on the data provided by the paper.

Enzymatic hydrolysis of OFMSW has been applied successfully as pretreatment to increase the biogas yield during AD processes, as it facilitates the hydrolysis step of complex components, which is the first and rate limiting step followed by acidogenesis, acetogenesis and methanogenesis (Romero-Güiza et al. 2016; Mlaik et al. 2020). However, its application for releasing and recovering sugars from waste is scarce in literature. Table 3 summarizes the retrieved publications dealing with enzymatic hydrolysis as a sugar releasing step for further valorization processes. All publications dealt with "real" OFMSW collected from different treatment facilities (Spain, Iran, and Germany), therefore, besides the inherent variability of this waste, variabilities related to the specifics of each region should be expected. The selection of enzymes varies among authors with cellulases as a shared type. Cellulases are a family of enzymes including endoglucanase, exoglucanase, and β-glucosidase activities that depolymerize cellulose into cellobiose and ultimately glucose (Lynd et al. 2002; Escamilla-Alvarado et al. 2017a). Amylases are the second employed enzymes and degrade starch into oligosaccharides and ultimately glucose or maltose (Escamilla-Alvarado et al. 2017a). Remarkably, all authors employed enzymatic cocktails, therefore benefiting from synergistic effects of enzymatic activities. It has been shown how cellulases benefit from the removal of xylan by xylanases, which leads to enhanced fiber swelling and accessibility (de la Torre et al. 2017; Hu et al. 2018). Despite the variety of enzymatic activities, the conditions employed for the enzymatic hydrolysis are very similar and characterized by high temperatures and a rather acidic pH. Molina-Peñate et al. (2022) explored the use of milder conditions, in terms of lower temperature and higher pH, achieving a 93% of the hydrolysis performance at high temperature. Enzyme dosage is the most inconsistent condition, sometimes not even addressed (López-Gómez et al. 2019; Stylianou et al. 2020), because of the lack of standardized units for enzymatic cocktails that contain several enzymatic activities. All authors performed mechanical pretreatments of the waste, mainly for inert materials removal and particle size reduction, whereas some also performed a dilute acid pretreatment previous to the enzymatic hydrolysis of the OFMSW (Ghanavati et al. 2015; Mahmoodi et al. 2018a; Izaguirre et al. 2019; Ebrahimian et al. 2020). The improvement in the released sugars after the dilute acid pretreatment calculated based on the data provided by the authors was 28% for Izaguirre et al.(2019), 49% for Mahmoodi et al. (2018a), and 17% for Ebrahimian et al. (2020). Ghanavati et al. (2015) did not provide the data of the enzymatic hydrolysis of untreated OFMSW. However, when comparing the total sugars concentration attained, these improvements do not excel the enzymatic hydrolysis performed without a preliminary chemical pretreatment (Table 3). In fact, the highest sugar concentration declared was

331 achieved by Stylianou et al. (2020) without chemical pretreatment using a tailor-made enzymatic cocktail
332 and was of 107.3 g/L, equivalent to 75% glucan and 12.5% xylan conversion. It should be highlighted
333 that in all cases glucose was the major sugar representing more than 80% of the final composition of the
334 hydrolysates. López-Gómez et al. (2019) made a comparison between separately and non-separately
335 collected OFMSW obtaining 18% more sugars in the separately collected (80 g/L). It seems that tailor-
336 made enzyme cocktails could overcome the need for physicochemical pretreatments that might have
337 negative impacts in later processing steps because of the release of inhibitors of fermentative processes
338 (Ghanavati et al. 2015). Mahmoodi et al. (2018a) were the only researchers assessing the influence of the
339 pretreatment on the resulting solid fraction after the enzymatic hydrolysis. These authors declared a
340 complete removal of xylan and a significant removal of starch after treatment with 1% acid at 160ºC for
341 60 minutes, hence proving a reduction in the degree of depolymerization beneficial for the enzymatic
342 hydrolysis (Alvira et al. 2010). These sugars concentrations are generally lower than those recently
343 obtained in enzymatic hydrolysis of food waste (164 – 204 g/L), which are homogeneous streams with
344 lesser inert content (Kwan et al. 2018).
345 After the enzymatic hydrolysis two fractions can be differentiated, a liquid fraction containing
346 solubilized compounds and a solid fraction containing partially digested and undigested components,
347 which are substrates for subsequent steps. An overview of the main advantages and disadvantages of the
348 bioconversion processes shown in Figure 1 can be seen in Table 4.
349

Table 4 Advantages and disadvantages of the main technologies of bioconversion processes in an OFMSW biorefinery

Technology	Objective	Advantages	Disadvantages	Ref
Transesterification	Bioconversion of lipids to biodiesel	Energy-efficient, simplified downstream	Time consuming, cost of biocatalyst	(Escamilla-Alvarado et al. 2017a)
Submerged fermentation	Bioconversion of liquids to bioproducts	High process control and versatility, facilitated downstream	Large wastewater, limited soluble oxygen	(Sala et al. 2019)
Solid-state fermentation	Bioconversion of solids to bioproducts	Reduced wastewater, increased yield, low-energy	Mass-heat transfer problems, difficult process control and scale-up, complex downstream	(Sala et al. 2019)
Simultaneous fermentation and saccharification	Bioconversion of liquids and solids to bioproducts	Process simplification in time and cost, reduced substrate inhibition	Reduced yields by compromised optimum conditions	(Chacón et al. 2021)
Acidogenic fermentation	Bioconversion of liquids and solids to VFAs	Complex and variable substrates	Difficult process control	(Agler et al. 2011)
Anaerobic digestion	Bioconversion of liquid and solid wastes to biogas	Robust, energy-efficient, relatively low-cost	Need of further processing of solids, high cost of biogas upgrade	(Mayer et al. 2020)
Composting	Bioconversion of solid organic wastes to compost	Simple; robust	Quality requirements, low value of compost, odors	(Cerda et al. 2018)

VFAs, volatile fatty acids

3.2.1 Valorization of sugar concentrated hydrolysate

The liquid hydrolysates obtained after enzymatic hydrolysis processes are not only high-concentrated in sugars (carbon source) but also contain other functionalized molecules, i.e. proteins, amino acids, organic acids, and minerals. Therefore, these liquors are a suitable substrate for fermentative processes to produce bioproducts with higher market value (Pleissner and Peinemann 2020). In Table 3, the valorization routes proposed include commodities, such as acetic acid, succinic acid, and 2,3-butanediol, homopolymer for bioplastic applications, and biofuels, such as ethanol, hydrogen, and lipids for biodiesel. Thus, a wide range of bioproducts and applications can be obtained from OFMSW.

Processes simplification is an important goal for biorefineries. From Table 3 only López-Gómez et al. (2019) employed the hydrolysate without the need of supplementation of other carbon sources or nutrients nor an additional autoclave step. These authors selected a low nutritional requirement and thermophilic strain, which provided competitive advantages to unavoidable contaminants originally present in the waste. Furthermore, they achieved the highest production per kg of OFMSW (230 g lactic acid/ kg of dry OFMSW). However, in some instances, the addition of supplements can be justified by the increase in product yield. For instance, Izaguirre et al.(2019) showed the inability of *Burkholderia sacchari* to produce Poly(3-hydroxybutyrate) in the hydrolysate related to a lower C/N ratio than the metabolically required and they recommended further studies on limiting nutrient selection. The use of physicochemical pretreatments is decisive for the performance of fermentative processes because of the release of inhibitory compounds. Ghanavati et al. (2015) required a detoxification step before the use of the hydrolysate from the dilute acid pretreatment because of the high content in inhibitors, such as furfural or phenolic compounds. A cost-benefit evaluation should be performed when designing a biorefinery as a higher sugar yield might not compensate for the cost derived from additional detoxification steps after harsh pretreatments. A better selection of the enzyme cocktail or a proper valorization of the remaining solid fraction, which only 2 authors in Table 3 considered, can lead to greater overall performance.

3.2.2 Valorization of solids remaining after enzymatic hydrolysis

There are two valorization routes for the solid fraction remaining after the enzymatic hydrolysis of OFMSW presented in Table 3, the anaerobic digestion to biogas, or ultimately biomethane, and solid fermentation with a specific microorganism. The biomethane amount per gram of dry matter is 6-times higher for Mahmoodi et al. (2018a) than for Ebrahimian et al. (2020). This might be explained by the different configurations proposed. Mahmoodi et al. (2018a) achieved a better integration of processes by reducing residues. These authors used both liquid fractions, from the acid pretreatment and enzymatic hydrolysis, for the production of bioethanol and both solid fractions, from the enzymatic hydrolysis and the ethanolic fermentation (suspension remaining after evaporation of ethanol), for biogas production. In this sense, Ebrahimian et al. (2020) did not use the liquid fraction from the ethanol pretreatment nor the solids after the anaerobic fermentation of *Enterobacter aerogenes*. However, the latter obtained a wider spectrum of bioproducts from a more versatile fermentative process. Despite the clear benefits of AD to biogas, other technologies might bring new opportunities in a biorefinery scenario.

Solid-state fermentation (SSF), described as the fermentation of solids in the absence or near absence of free water (Pandey 2003), is a technology that has gained relevance for the valorization of organic wastes (Yazid et al. 2017; Sadh et al. 2018; Martínez-Avila et al. 2021). In contrast to submerged fermentation, SSF is a simpler process that has lower energy requirements and operational cost but also reduced options for process control and monitorization, hindering the scale-up (Sala et al. 2019). Molina-Peñate et al. (2022) performed a preliminary evaluation of the resulting solid fraction after enzymatic hydrolysis as a substrate for a SSF process producing spores of the widespread bacterial biopesticide *Bacillus thuringiensis*. Ballardo et al. (2017) also evaluated the growth of this microorganism on untreated OFMSW, even though they showed a promising valorization pathway to a compost-like material with enriched biopesticide properties, the use of enzymatic hydrolysis opens a multi-platform scenario. OFMSW has been also evaluated as a substrate for SSF processes after other pretreatments. Estrada-Martinez et al. (2019) evaluated on a pilot scale (18 kg) the use of the fruit and vegetable fraction of the OFMSW after a mild thermal pretreatment as the substrate for a mixed yeast culture SSF. These authors reached an ethanol production of 186.4-193.5 g/g dry OFMSW at pilot scale. SSF can also be complementary to the AD process, for instance, digestate has been evaluated as the substrate of SSF (Rodríguez et al. 2019; Mejias et al. 2020). One of the most relevant applications of SSF from a biorefinery perspective is for the production of enzymes. The integration of enzyme production from OFMSW within the biorefinery will increase cost-efficiency and reduce dependency on third parties (Vea et al. 2018; Marín et al. 2019). For this purpose, fungi outstand as the preferred microorganism because of their inherent enzymatic battery for biomass degradation (Payne et al. 2015). Crude enzymes have been produced using OFMSW as substrate in SSF for *Trichoderma reseei* growth and evaluated for enzymatic hydrolysis showing a similar efficiency to that of a commercial enzyme preparation. (J. Abdullah and Greetham 2016). The use of homogeneous streams, richer in lignocellulosic materials and porosity can lead to higher enzyme yields production (Bansal et al. 2012).

Simultaneous saccharification and fermentation (SSCF) consist in the integration of enzymatic hydrolysis and fermentation processes (Barampouti et al. 2019). The main advantages of SSCF are the simplification of production steps, which results in time and costs reduction, and the attenuation of product inhibition in enzymatic hydrolysis. However, it is hampered by the incompatibility of optimum pH and temperature for the different processes (Chacón et al. 2021). Chacón et al. (2021) recently reported a production of 255 g of lactic acid per kg of OFMSW (not clear if on a dry or wet basis) using

mechanically separated OFMSW and a thermophilic strain. This value is slightly higher than that reported for the fermentation of the liquid hydrolysate (Table 3).

All the mentioned processes are based on sugars conversion (sugar platform), yet another bioconversion platform has been proposed, the carboxylate platform (Agler et al. 2011). It comprises the conversion of organic feedstocks to short-chain carboxylates, such as volatile fatty acids (VFAs). Carboxylate platform is generally based on anaerobic fermentation with mixed culture, which can effectively cope with the variability of municipal substrates because of the interaction among the metabolism of the microbial community (Agler et al. 2011). Basically, it consists in promoting the first stages of AD, hydrolysis, and acidogenesis (Demirel and Yenigün 2002). VFAs levels that can be achieved from OFMSW can reach 770 g COD_{VFA}/ g volatile solids (VS). It has been shown that this process benefits from microaerobic conditions, which can enhance productivity and VFAs chain length (den Boer et al. 2016). The VFAs are building blocks of many chemical and biological processes. For instance, polyhydroxyalkanoate (PHA) production within the same reactor can be attained by applying a feast-famine regime (Korkakaki et al. 2016).

3.3 Downstream technologies

Few studies have focused on the downstream of OFMSW valorization processes. In part, because the substantial efforts required to develop them and advancements are rather slow, except for AD-related ones, but also because of the arduousness of the task. The repeatedly mentioned complexity and heterogeneity of the OFMSW difficult the purification of desired products as undesired by-products complicate the downstream processes (Bonk et al. 2015). Theoretical approximations with models and techno-economic analysis have been made to provide better insights into the critical requirements and milestones (Demichelis et al. 2020; Elyasi et al. 2021). It is undoubtedly that more technical and economical efforts in the downstream sections are required for the manufacturing of the bioproducts and implementation of an OFMSW biorefinery (Liu et al. 2021).

The majority of advancements are related to biogas upgrade (Sun et al. 2015) and bioethanol production (Demichelis et al. 2020), as they have been the most studied and implemented technologies. However, tentative steps are also being taken for the separation of chemical building blocks. López-Gómez et al. (2020) attempted a downstream configuration for the proposed lactic acid production in Table 3. These authors performed a purification of a pilot-scale fermentation of OFMSW, highlighting the importance of large volumes of fermented broth for separation processes to succeed technically and

452 economically. The lactic acid recovery involved several membrane steps based on electrodialysis and
453 implied a reduction by half of the yield, from 220 to 110 g/kg of dry OFMSW.

454 **3.4 Configuration options for the integration of OFMSW valorization technologies**

455 This section reviews a collection of OFMSW biorefineries proposed in the literature that explore
456 valorization routes beyond biogas and compost production. A comparison of the bioconversion
457 technologies used and of the state of development, in terms of product recovery consideration,
458 economical assessment, and energy balance for each biorefinery is summarized in Table 5. These
459 proposals are initial implementation stages mostly based on technologies already developed at pilot-scale,
460 yet lacking a complete implementation assessment.

461 The presented approaches of OFMSW biorefineries (Table 5) include two at pilot scale (200-380
462 L) located within the facility of a municipal full-scale waste water treatment plant (WWTP) in northeast
463 Italy. Another approach combines pilot and laboratory facilities located in an experimental biorefinery of
464 organic solid wastes within a Brazilian university campus. These pilot approaches take advantage of
465 already existing waste management facilities, which offer logistical advantages, i.e. no mechanical
466 pretreatments or transportation requirements. The last two are a laboratory-scale proposal, the only one
467 not working with source-separated OFMSW, and a theoretic approach based on literature.
468

469 **Table 5** Reviewed biorefinery configurations for OFMSW

Feedstock	**Scale**	**Conversion technologies**	**Product recovery**	**Economic evaluation**	**Mass balance** Products (Yields[a])	**Energy balance** Produced	Consumed	Net	**Ref**
ssOFMSW + Biological sludge from WWTP	Pilot	Acidogenic fermentation; Aerobic SmF for PHA production; co-AD	No	Preliminary	Yes PHA (76 g/kg VS); Biogas (0.42 m^3/kg VS)	6.8 MJ/kg VS	6.1 MJ/kg VS	0.7 MJ/kg VS	(Moretto et al. 2020)
ssOFMSW	Pilot	Acidogenic fermentation; Aerobic SmF to PHA; AD	No	Yes	Yes PHA (37 g/kg VS); Biogas (0.68 m^3/kg VS)	7.8 MJ/kg VS; 93.4 MWh/d	6.4 MJ/kg VS; 7.7 MWh/d	1.4 MJ/kg VS; 85.7 MWh/d	(Valentino et al. 2018)
ssOFMSW + cooking oil	Pilot & Bench	Transesterification; Glycerol SmF to 1,3-Propanediol; AD; Composting	Yes, for biodiesel	Preliminary	No Biodiesel; 1,3-Propanediol; Biogas (0.58 m^3/kg VS); Compost	NM	NM	NM	(de Sousa et al. 2021)
mcOFMSW	Bench	Enzymatic hydrolysis; Ethanolic SmF; AD	Yes, for bioethanol	No	Yes Bioethanol (199 g/kg VS); Biogas (0.16 m^3/kg VS)	11.2 MJ/kg VS	NM	NM	(Mahmoodi et al. 2018a)

ssOFMSW	Theory	Oil extraction; Transesterification; Enzymatic hydrolysis; Fermentation; AD	Yes	No	Yes Biodiesel (0.1 L/kg VS); Glycerol (0.01 L/kg VS); Bioethanol (078 g/kg VS); Biomethane (0.08 m^3/kg VS); Biofertilizer (100 g/kg DM)	8.6 MJ/kg VS	NM	NM	(Barampouti et al. 2019)

ssOFMSW: source-separated OFMSW; mcOFMSW: mixed collected OFMSW; WWTP: wastewater treatment plant; SmF: submerged fermentation; PHA: polyhydroxyalkanoates; AD: anaerobic digestion; VS: volatile solids

[a] Yields are expressed per kg of initial substrate; NM: Not mentioned

In terms of conversion technologies applied, all the proposals have in common the use of AD for waste minimization and energy production. Moretto et al. (2020) and Valentino et al. (2018) employed a similar configuration scheme, first, they performed an acidogenic fermentation to obtain a fermented stream rich in VFAs that was sent to a solid-liquid separation unit. The filtered stream was sent to an aerobic line composed of a sequencing batch reactor (SBR) for biomass selection and a batch reactor for PHA production. The solid stream was converted to biogas via AD. Following this scheme with an AD conducted at thermophilic conditions (55ºC), Valentino et al. (2018) obtained a yield of 37 g of PHA/kg VS and 0.42 m^3 of biogas/kg VS. In a later study using biological sludge from the municipal WWTP as co-substrate, Moretto et al. (2020) evaluated the application of a thermal pretreatment (72ºC, 48h) on the performance of the acidogenic fermentation. The enhancement in organic matter solubilization led to a yield improvement from 0.37 to 0.65 g COD_{VFA}/g VS so the authors decided that the implementation of the thermal pretreatment was crucial for the process. These authors also evaluated the addition of an ultrafiltration membrane to the centrifuge for the solid-liquid separation step and two temperatures (37ºC and 55ºC) for the anaerobic co-digestion (co-AD) step. The thermophilic operation of the co-AD led to higher yields in terms of specific gas production, 0.51 m^3 of biogas/kg VS compared with 0.44 m^3 of biogas/kg VS for the mesophilic. However, the energy balance showed the mesophilic operation as more beneficial because of lower thermal energy consumption that allowed the anaerobic line to be self-sustainable. This fact highlights the importance of performing mass and energy balances when designing potential biorefineries (Moncada B et al. 2016). Comparing the two described proposals in terms of mass balance and energy, it can be seen how for similar energy consumptions, the net profit was half for the biorefinery proposed by Moretto et al. (2020). Yet, the PHA production was doubled, which leads to higher economic benefits from the sale of this product. Both papers performed a preliminary economic assessment on top of the energy and mass balances, which is essential to evaluate the dichotomy between energy and bioproducts for each specific case scenario.

The approach presented by Sousa et al. (2021) was an experimental biorefinery to treat the wastes generated in a university campus (40 L/d cooking oil, 2500 kg/d pruning waste, and 750 kg/d food waste) and provide a model for municipal managers of small towns. The configuration consisted of a pilot transesterification reactor (40 L) for the conversion of oil waste into biodiesel and glycerol with a 93% conversion yield. Then, the produced glycerol was evaluated at a lab-scale fermentation to produce 1,3-Propanediol. Additionally, a traditional biogas-composting configuration was set to process the food and

pruning wastes, it included a 9.6 m^3 low-cost biodigester with a 4 m^3 gasometer and a 450 m^2 compost yard. Since mass and energy balances are not presented it is difficult to evaluate the performance of the biorefinery. The declared biogas yield (0.58 m^3 of biogas/kg VS) was relatively low considering that the other pilot-scale biorefineries partition the carbon line into PHA and achieved similar values. However, this proposal offers a small-scale and low-cost point of view that might ease the implementation pathway of OFMSW biorefineries.

Finally, the last two proposals from Table 5 are configured to produce bioethanol and biogas. On a lab scale, Mahmoodi et al. (2018a) proposed a hydrothermal pretreatment to solubilize starch and increase surface area followed by a separation step, amylase hydrolysis of the liquid fraction, cellulose hydrolysis of the solid fraction to release the remaining sugars, an ethanolic fermentation for the resulting liquid fractions and an AD process for the solid fractions. The final yields attained were 199 g ethanol/kg VS and 0.16 m^3 of biogas/kg VS. Biogas yield is considerably smaller than the presented at pilot scales, which might be related to more exhaustive operations upstream AD. In the publication of Mahmoodi presented in Table 3 (Mahmoodi et al. 2018b), this same author proposed a different configuration using acid hydrolysis to substitute the need for amylase hydrolysis. The results from both configurations were similar but slightly lower for the acid pretreatment with 194 g ethanol/kg VS and 0.15 m^3 of biogas/kg VS. Before the scale-up of these processes, an economic evaluation to study the impact of the cost of enzymes and an environmental evaluation to study the impact of the acid pretreatment are necessary to ensure the best possible configuration is selected.

4. Implementation challenges of OFMSW biorefineries

The implementation of biorefinery frameworks for the management, treatment, and valorization of organic municipal wastes has to overcome several challenges besides the previously highlighted technical aspects, i.e. handling the impact of waste composition, energy and chemicals demand of pretreatments, the efficacy of enzymatic cocktails, technological readiness at larger scales, and difficulties to recover bioproducts. Close collaboration among research actors, municipalities, and industries is necessary to achieve a fruitful implementation with its associated environmental benefits.

4.1 Composition

As repeatedly mentioned in this review, OFMSW is a variable and heterogeneous stream. The main factors affecting the composition of OFMSW are seasonality, weather, urban density, and regional

nutritional habits, economic activities, and sorting instructions (Puig-Ventosa et al. 2013; Campuzano and González-Martínez 2016; Cerda et al. 2018). On top of that the citizen engagement in waste sorting, the disposal bags employed, and the collection system highly influence the quality of the waste in terms of non-organic, or inert, components (Al Seadi et al. 2013; Sisto et al. 2017). These factors can also influence the purity of final bioproducts as for compost (Cerda et al. 2018). Campuzano et al. (2016) observed that the characteristic of OFMSW with higher variability were nutrients such as phosphorous, sulfur, free sugars, or raw fiber. To mitigate the effect of such variations in subsequent microbial processes, mechanical pretreatments for the removal of non-organic materials are practically mandatory (Alibardi and Cossu 2016; Cerda et al. 2018). For instance, the VFA production from organic wastes has been reported to be more influenced by the feedstock characteristics than by the fermentation conditions (Moretto et al. 2019b).

Another compositional challenge is the low calorific value and high moisture content of OFMSW. This results in higher volume and weight, hampering transportation, and microbial activity, which leads to biodegradation and lactic acid production (Matsakas et al. 2017; Alibardi et al. 2020; Stylianou et al. 2020). The establishment of an organized and efficient value chain is required to reduce collection, transportation, and storage period to a minimum.

4.2 Economic investment

Biorefineries are associated with high costs of construction, maintenance, and operation of the conversion plants (Lee et al. 2019). To justify such an investment biorefineries need to be economically profitable and, ideally, rely on multiple income sources. An additional income source to the revenues from the sale of the obtained bioproducts or energy is a gate fee for waste acceptance and treatment (Sadhukhan and Martinez-Hernandez 2017). Gate fees are particularly interesting at initial implementation stages as they represent an incentive for waste managers to deviate from landfills and implement waste valorization technologies and also provide a stable income until the complete establishment of the biorefinery (Alibardi et al. 2020). Budzianowski and Postawa (2016) recently stated that for a biorefinery to be truly economically viable, a total chain integration is required to ensure the optimization of energy and resources and reduce capital and operating costs. Economical sustainability is also benefited from flexibility towards diverse feedstocks and products (Kamm and Kamm 2004). The ability to shift between energy and commodity chemicals production endows the biorefinery to assimilate fluctuations in value-added products prices and market demands (Duan et al. 2020). Finally, size is

another relevant factor influencing the economy of biorefineries. Larger sizes benefit from the economy of scale (Ragauskas et al. 2006), yet smaller sizes can lead to more specialized systems. Reduction of size by decentralization also reduces the cost associated with long-distance transportation and approaches valorization technologies to low-volume generation points (Matsakas et al. 2017).

4.3 Stakeholders interest

The implication of actors throughout the value chain of the OFMSW conversion process is necessary to develop long-term strategies and move forward from the current waste management model towards a more sustainable scenario. These stakeholders range from political figures to local citizens (Sisto et al. 2017). Local governments and decision-makers shape the policies required to favor bio-based products over chemical-based. Khoshnevisan et al. (2018a) define future policies as a source of uncertainty when assessing the environmental profile of source selected OFMSW. This lack of guarantees on long-term political interest might discourage investors from accepting bioconversion technologies and resonate with technological advances. The main producers of OFMSW are households, therefore citizens' engagement in waste sorting and acceptance of waste-derived bioproducts is also essential. A recent survey evaluates the marketability of bio-based products showing a general consumer acceptance in specific markets and a willingness to pay related to ideology (Moretto et al. 2020).

5 Conclusions

The need for new municipal solid waste management scenarios that ensure the continuity of materials within production cycles calls upon holistic and sustainable solutions. OFMSW has shown remarkable potential for its management in a biorefinery-like environment, where it can be initially fractionated to attain multi-platform configurations. Research studies in enzymatic hydrolysis have displayed promising perspectives for it to be used as an initial separation step of OFMSW’s components. Meanwhile, the traditional waste management technologies, i.e. AD for biogas production and composting, will remain as powerful tools for the integration of secondary waste streams, already depleted of high-interest components. However, before the implementation of the OFMSW biorefineries, valorization technologies need to step from laboratory scale to industrial scale and final products formulations need to be addressed in cost-effective downstream processes. Ultimately government regulations promoting bioeconomy strategies and cooperation among the different parties involved have the ability to increase industrial interest and foster technological advances.

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Article II

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

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In biorefineries, enzymatic hydrolysis is generally applied as a pretreatment technology to decrease the chemical complexity of feedstocks. During this process, monomeric components, such as sugars, are released. This section includes the development and optimization of an enzymatic hydrolysis process to extract sugars from OFMSW. Valuable insights on different operational possibilities that led to a sugar production of around 50 g L^{-1} are presented. This section also includes a preliminary assessment of the resulting solid fraction as a substrate to grow the microbial biopesticide *Bacillus thuringiensis*.

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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

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ABSTRACT

To reach a more sustainable society, the implementation of a circular economy perspective in municipal waste management becomes essential. In this work, the enzymatic hydrolysis of source-separated organic fraction of municipal solid waste (OFMSW) has been optimized as a sugar-releasing step. A liquid sugar concentrate, with a maximum reducing sugar concentration of 50.56 g L^{-1}, and a solid hydrolyzed fraction were obtained. The effect of the harshness of the hydrolysis conditions was evaluated on the performance of the resulting solid fraction as a substrate for *Bacillus thuringiensis* biopesticide production through solid-state fermentation. A production of 3.9 $\times$ 10^8 viable cells g^{-1} dry matter with a 33% sporulation ratio was achieved for milder hydrolysis conditions, highlighting the potential of the solid fraction of hydrolysis as a substrate of SSF processes. The proposed valorization pathway for the OFMSW results in a sugar concentrate with potential for fermentative processes and a fermented solid containing biopesticides from *Bacillus thuringiensis*.

1. Introduction

The organic fraction represents nearly half of the globally generated municipal solid waste (MSW), which is produced at a rate of 2.01 billion tonnes per year (Kaza et al., 2018). This organic fraction of municipal solid waste (OFMSW) comprises green and food waste from parks, households, commerce, and restaurants (Al Seadi et al., 2013). It is an abundant organic waste, with high moisture content and complex composition in polysaccharides, lignocellulose, sugars proteins, lipids, and macro/micronutrients (Campuzano and González-Martínez, 2016). From a circular economy perspective, in which organic waste as a source for new bioproducts must be kept within the production cycles as long as possible (Sánchez et al., 2015), the OFMSW becomes a potential feedstock for biotechnological processes.

To date, the valorization of the OFMSW has been focused mostly on composting and biogas production through anaerobic digestion. Pretreatments have appeared as a tool to reduce the chemical complexity of the OFMSW and to increase its biodegradability by enhancing access to individual components (Romero-Cedillo et al., 2017; Romero-Güiza et al., 2016). In this sense, enzymatic hydrolysis has been applied successfully as a pretreatment for enhancing biogas yield (Mlaik et al., 2019). Recently, the focus has been shifted to the production of higher market value products and enzymatic hydrolysis has emerged as a tool for extracting functionalized compounds, such as sugars, fatty acids, or proteins (Pleissner and Peinemann, 2020). Sugars are of special interest as they can be the platform for the production of bioproducts through fermentation processes (Cabas Candama et al., 2020; Zhang et al., 2020). Cellulases are the most commonly used enzymes for the hydrolysis of OFMSW but, considering its complex composition, the use of complementary enzymatic activities, such as xylanases or amylases, is advisable to increase the rate and yield of fibers conversion (de la Torre et al., 2017; Hu et al., 2013).

Most studies dealing with enzymatic hydrolysis as a step to release sugars from the OFMSW have been focused on the use of the liquid fraction of the hydrolysis. Different valorization scenarios have been considered with relative success, for example, the production of succinic acid (Stylianou et al., 2020), acetic acid (López-Gómez et al., 2019), or lipids (Ghanavati et al., 2015). Conversely, few studies have also taken into consideration the valorization of the solid fraction, which has been either diluted and fermented together with the liquid fraction

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(Ebrahimian and Karimi, 2020) or simply directed to anaerobic digestion processes (Mahmoodi et al., 2018). To attain the implementation of enzymatic hydrolysis as an extractive step and provide a zero-waste alternative to the current model, it is necessary to ensure the full exploitation of the OFMSW potential. In this regard, solid-state fermentation (SSF) appears as a potential technology for the utilization of unhydrolyzed solids.

SSF, defined as a process that occurs in the absence or near absence of free water (Pandey, 2003), has been established as an efficient and environmentally friendly tool for the valorization of various solid organic waste (Yazid et al., 2017) to produce different marketable products, such as aroma compounds (Martínez-Avila et al., 2021), biosurfactants (Jiménez-Peñalver et al., 2016), or biopesticides (Mejias et al., 2020; Sala et al., 2020). Among them, biopesticides derived from *Bacillus thuringiensis sp*. (Bt), the most widely used microbial biopesticide, have shown a robust production on SSF using OFMSW or similar wastes because of their content in easily biodegradable organic matter and a great variety of macro and micronutrients (Ballardo et al., 2017; Rodríguez et al., 2019; Zhang et al., 2013; Zou et al., 2016). During the sporulation phase, Bt species produce crystal inclusions containing toxic proteins (Cry or Cyt protein) (Bravo et al., 2011). These proteins are selectively toxic for a wide spectrum of hosts including insects of Coleoptera, Lepidoptera, Diptera, Hymenoptera, Hemiptera, and Orthoptera orders, as well as phytopathogenic nematodes and terrestrial gastropods (Malovichko et al., 2019). The toxicity is produced by ingestion causing gut cell lysis. Bt-derived biopesticides are already available in the market commercialized under different names and forms, such as DiPel®or XenTari® (Kenogard), Agree®(Bioamvac), or Deliver®(Certis), and produced through submerged fermentation employing defined synthetic media.

This paper aims to evaluate the enzymatic hydrolysis of OFMSW as a sugar-releasing step to obtain a sugar-rich solution, and the subsequent use of the unhydrolyzed solid fraction to produce Bt-derived biopesticide. First, an optimization of the hydrolysis to increase the amount of reducing sugars (RS) is presented, providing two optimum scenarios for the Viscozyme L® enzymatic cocktail. Then, the suitability of the resulting solid fraction to be used as a substrate of a SSF process producing Bt spores is analyzed. To the best of our knowledge, this is the first work using SSF technology to valorize the solid fraction resulting from enzymatic hydrolysis of the OFMSW.

2. Materials and methods

2.1. OFMSW collection and preparation

Source separated OFMSW (ssOFMSW) samples, kindly provided by Mancomunitat La Plana (Malla, Barcelona), were collected upon arrival at the MSW treatment plant. First, samples were screened manually for the presence of inert materials such as glass, plastics, metals, or textiles. Bones, hard shells, hair, and excess paper were also removed. Then, samples were homogenized mechanically using a home composting shredder (Tecoinsaen SL, Spain), packed into 1 kg bags, and stored at −20°C for a maximum of two months. Initial characterization of the two homogenized OFMSW batches collected for this study (September and November 2020) was performed. Before use, samples were defrosted overnight at 5°C and sterilized by autoclaving at 121°C for 30 min.

2.2. Enzymatic hydrolysis

For the enzymatic hydrolysis, the commercial cocktail Viscozyme L® from Novozymes (Denmark) was selected. This cocktail has been gaining interest in the pretreatment of food waste, a major component of OFMSW, (Cabas Candama et al., 2020; Chua et al., 2021; Gabiatti Junior et al., 2020) because of its wide range of carbohydrases, including cellulase, β-glucanase, hemicellulase, xylanase, arabinase, and pectinase. The declared activity by the provider is 100 Fungal Beta Glucanase Units (FBGU) per gram with an operating pH range of 3.5–5.5 and a wide operating temperature range of 25–55°C.

Experiments were conducted in sterile conditions using 500 mL Erlenmeyer flasks containing 100 g of sterile ssOFMSW diluted with 0.05 M sodium citrate buffer to reach a 10% (w v^{-1}) solid to liquid ratio. Experiments were conducted for 24 h at 180 rpm and temperature, initial pH, and enzyme load were adjusted according to the experimental design (Table 2). The adjustment of the pH was done by changing the pH of the sodium citrate buffer. Immediately after the enzymatic hydrolysis, samples were centrifuged at 6000 rpm for 15 min at 4°C, both the supernatant (liquid fraction) and the pellet (solid fraction) were collected. The liquid fraction was centrifuged at 8000 rpm for 10 min, and the supernatant was collected and stored at −20°C for RS determination. The solid fraction was collected for RS determination, characterization, and further use in the SSF process.

2.2.1. Optimization of the enzymatic hydrolysis procedure

Enzymatic hydrolysis conditions were evaluated and optimized by RSM using a Box-Behnken design. Temperature, initial pH, and enzyme load were chosen as the three independent variables of the design and were tested at three different levels (low, medium, and high). For temperature (25°C, 40°C, 55°C) and pH (3.5, 4.5, 5.5), the selected levels were based on the operating ranges specified by the enzyme cocktail supplier (Novozymes, Denmark). The enzyme load levels (0.01, 0.05, 0.1 mL g^{-1} DM) were equivalent to 1.2, 6.6, 12 FBGU g^{-1} dry matter (DM) according to the supplier and were based on the range proposed by Arapoglou et al. (2010) and Cabas Candama et al. (2020) and assessed on preliminary experiments (data not shown). Time was fixed in 24 h, agitation in 180 rpm, and solid load in 10% (w v^{-1}) because it was found as the maximum percentage ensuring a proper mixing under the experimental set-up. The final design consisted of 15 runs with a triplicate in the central point to allow for the estimation of the pure error (Box and Behnken, 1960). Two responses were considered, RS concentration in the liquid fraction (mg g_i^{-1} DM) and RS concentration in the solid fraction per initial gram of DM (mg g_i^{-1} DM). Calculations were done considering the 18 g of initial dry OFMSW in all the experiments and the % of recovered wet solids after centrifugation (around 70%). A second-order polynomial model, as presented in Equation (1), was fitted for the results of each response.

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \quad (1)$$

where Y is the predicted response; β_0 is model constant and β_i, β_{ii}, β_{ij} are regression coefficients of linear, quadratic, and cross-product terms; X_i and X_j are coded independent variables. The quality of fit of the polynomial model equations was expressed by the coefficient of determination (R^2) and their prediction capability by the predicted R^2.

To verify the obtained models, the enzymatic hydrolysis was performed in triplicate under the optimum conditions predicted. The experimental and predicted response values were compared and the predictive capability of the model was assessed. The efficiency of the hydrolysis is reported through a performance index computing the increase of RS in both fractions after the hydrolysis with respect to the RS initially present in the substrate.

2.2.2. Statistical analysis

Results were analyzed using the Design-Expert® statistical software (Stat-Ease, Inc, United States). With the aid of the program, multiple regression analysis for the construction of the model, and statistical analysis of variance (ANOVA) for evaluating its significance, were conducted. Linear and quadratic effects of the variables and their interactions on the release of RS were calculated and plotted into three-dimensional and contour plots. Maximum RS concentration in each fraction was estimated using numerical optimization.

2.3. Solid-state fermentation of the solid fraction of hydrolysis

2.3.1. Microorganism, inoculum preparation, and growth assessment

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, Spain).

Inoculum preparation was performed according to the methodology presented by Mejias et al. (2020). Shortly, one cryo-pearl was inoculated in 100 mL of sterilized Nutrient Broth N°2 (Oxoid CM0067B, England) and incubated for 20 h, at 130 rpm and 30°C until it reached an optical density of 2.5–3. Afterward, the culture was centrifuged for 10 min at 3500 rpm and 4°C. The obtained pellet was resuspended in 3 mL of supernatant. To reach an inoculum concentration on the solid substrate of approximately 10^7 CFU g^{-1} DM, the resuspended inoculum was diluted at 1:10 (v v^{-1}) with supernatant. The final inoculum contained around 10^8 CFU mL^{-1} and no spores were detected.

Bti growth was assessed in terms of viable cells and spores. The procedure used is as described by Mejias et al., 2020. First, a solid–liquid extraction was performed using Ringer solution in a 1:10 (w v^{-1}) ratio at 150 rpm for 20 min. For spore determination, 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. Serial dilution banks of both extracts were prepared using Ringer and plated in triplicates onto Petri dishes containing Nutrient agar medium (Oxoid CM0003B, England). The plates were incubated for 20 h at 30°C and viable cells or spores were estimated in terms of colony-forming units (CFUs). The sporulation ratio at a certain time was calculated considering that the viable cell count includes vegetative cells and spores.

2.3.2. Substrate preparation

The solid fractions resulting from the selected as optimal conditions of the enzymatic hydrolysis were collected sterile and mixed with sterile wood chips of particle size between 0.5 and 5 cm (Acalora, Palets Pla d'Urgell). Wood chips act as a bulking agent and are necessary to provide porosity to the solid matrix. The resulting SSF substrate for each reactor consisted of 95 g of solid fraction and 15 g of wood chips manually mixed and inoculated with 2.6 mL of diluted Bti inoculum. Triplicates for each condition studied were performed.

2.3.3. Experimental SSF set up

SSF experiments were conducted in 0.5 L packed bed reactors under aseptic conditions. Filled reactors were placed in a temperature-controlled water bath at 30°C and connected to a mass flow meter (Bronkhorst, Netherlands), which continuously supplied a specific airflow saturated through a humidifier to prevent drying in the solid matrix. Airflow was set to 37 mL h^{-1} g^{-1} DM in all the experiments for ensuring aerobic conditions (Mejias et al., 2017). Exhaust gases exited from the top of each reactor, went through a water trap, and reached an oxygen sensor (Alphasense, UK) connected to a custom-built acquisition system (Arduino® based). With the recorded oxygen concentration, the specific oxygen uptake rate (sOUR) was calculated as an indicator of the biological activity as stated by Ponsá et al. (2010). Experiments lasted 72 h, which has been established as the maximum spore count time for Bt (Cerda et al., 2019). At this time, the final pH, viable cells, and spores were determined.

A summary of the experimental process described can be seen in Fig. 1.

2.4. Analytical methods

RS were measured using the DNS method (Miller, 1959) both in the liquid and solid fractions of the enzymatic hydrolysis. For the solid fraction analysis, a solid–liquid extraction was performed using distilled water in a 1:10 (w v^{-1}) ratio for 30 min at 50°C and 150 rpm. The liquid phase from the extraction, together with the supernatant obtained from the centrifugation of the liquid hydrolysate were filtered through a 0.45 μm membrane filter and diluted with water to obtain a concentration in the range of the calibration curve (glucose, 0.2–3.3 g L^{-1}). RS in the solid fraction is always expressed per initial gram of DM before the hydrolysis was performed.

Solid fractions were characterized physiochemically in terms of moisture content (MC), dry matter (DM), organic matter (OM), ashes, and pH according to standard procedures (Thompson et al., 2001). Cellulose, hemicellulose, and lignin content were determined using an Ankom200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY). C/N ratio was determined using a CHNS elemental analyzer Flash 2000 (Thermo Scientific). Biodegradability was assessed through the dynamic respiration index (DRI), which represents the average oxygen uptake rate during the 24 h of maximum activity observed, as described elsewhere (Ponsá et al., 2010; Sala et al., 2020). DRI is expressed in mg of oxygen consumed per g of dry matter per hour. All measurements were conducted in triplicates.

3. Results and discussion

Table 1 summarizes the characteristics of the two batches of ssOFMSW used in this study. The majority of the physicochemical values were in close agreement with the average values from 43 cities in 22 countries presented by Campuzano and González-Martínez (2016). Except for the reducing sugars content in batch 1 ($p = 0.008$), the lignin ($p = 0.049$), and nitrogen ($p = 0.041$) content in batch 2, which showed significant differences (Table S.1). These parameters were reported as highly variable by the same authors. Sugars are a very easily degradable compound and their amount fluctuates depending on the time from waste generation to analysis (Hansen et al., 2007). This higher RS value can also explain the higher biodegradability of batch 1, measured by the DRI, which is a measure of biological activity in terms of oxygen consumption. It is important to highlight that the OFMSW is an inherently variable material that does not only variates among countries or cities,

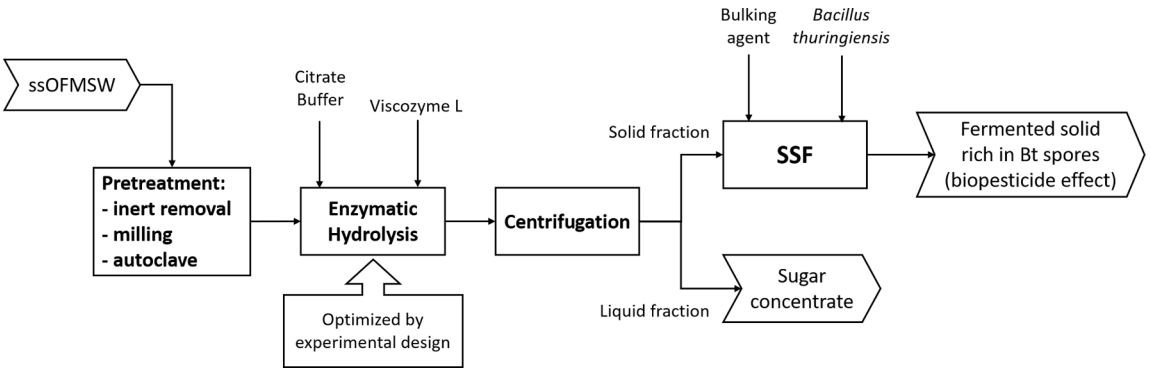

Fig. 1. Scheme of the experimental process conducted for the valorization of source-separated OFMSW (ssOFMSW).

Table 1
Characterization of the two batches of source-separated OFMSW used in this study together with average values from 43 cities in 22 countries reported by Campuzano and González-Martínez, 2016.

Parameter	Batch 1	Batch 2	(Campuzano and González-Martínez, 2016)
Moisture content (%)	81.0 ± 0.1	72.9 ± 1.0	72.8 ± 7.6
DM (%)	19.1 ± 0.1	27.1 ± 1.0	27.2 ± 7.6
OM (%*)	90.1 ± 1.0	89.7 ± 1.7	84.6 ± 9.9
RS (%*)	24.1 ± 0.5[a]	15.7 ± 1.3	10.5 ± 6
C (%*)	45.4 ± 0.3	45.5 ± 1.5	46.6 ± 4.4
H (%*)	6.5 ± 0.03	6.4 ± 0.2	6.6 ± 0.62
N (%*)	2.1 ± 0.7	1.9 ± 0.3[a]	2.9 ± 0.6
S (%*)	0.1 ± 0.04	<0.1	0.3 ± 0.26
C/N ratio	17	24	16
Cellulose (%*)	15.9 ± 0.2	7.4 ± 0.1	18.6 ± 15
Hemicellulose (%*)	10.0 ± 0.1	6.8 ± 0.1	8.6 ± 4.6
Lignin (%*)	7.8 ± 0.3	2.1 ± 0.2[a]	9.7 ± 5.3
pH	5.25 ± 0.05	5.31 ± 0.04	5.02 ± 0.95
DRI (mg O_2 g^{-1} DM h^{-1})	7.5 ± 0.2	4.2 ± 0.3	NA

* Dry basis. NA, not available. Data presented as mean values ± standard deviation (n = 3). [a] significantly different parameter ($p < 0.05$) based on the Tukey test analysis.

but also within the same region by other factors such as weather conditions, seasonal changes, nutritional habits, or recollection system (Campuzano and González-Martínez, 2016; Hansen et al., 2007).

3.1. Enzymatic hydrolysis as sugar releasing step

The evaluation of the enzymatic hydrolysis as a sugar-releasing step was performed using batch 1 of ssOFMSW and based on three operational parameters for two response variables. The operational parameters studied were temperature, pH, and enzyme load, previously identified as significant factors affecting the enzymatic hydrolysis of organic wastes (Guan and Yao, 2008; Yan et al., 2011). These parameters were evaluated based on their effect on the release of RS in the liquid fraction and the solid fraction (Table 2).

The highest value of RS concentration in the solid fraction was obtained in run 13, 133.2 mg g_i^{-1} DM, corresponding to temperature 40°C, initial pH 4.5, and enzyme load 0.05 mL g^{-1} DM. By contrast, the minimum value was obtained in run 9, 60.8 mg g_i^{-1} DM, corresponding to temperature 40°C, initial pH 3.5, and enzyme load 0.01 mL g^{-1} DM. Comparison with literature values is difficult because, among the few publications dealing with the solid fraction of enzymatic hydrolysis, none of them measured the RS concentration in the solid (Ghanavati et al., 2015; Zhang et al., 2020). As detailed in Table 2, the highest value of RS concentration in the liquid fraction was obtained in run 4, 365.1 mg g_i^{-1} DM, corresponding to temperature 25°C, initial pH 4.5, and enzyme load 0.1 mL g^{-1} DM. By contrast, the minimum value was obtained in run 3, 167.4 mg$_i$ g^{-1} DM, corresponding to temperature 25°C, initial pH 4.5, and enzyme load 0.01 mL g^{-1} DM. Comparing both of them, the effect of enzyme concentration is evident as at equal conditions the amount of liberated RS in the liquid fraction doubled. In contrast to the solid fraction, the maximum release of RS occurred at the maximum enzyme concentration. The maximum obtained RS concentration in the liquid fraction, 50.56 mg mL^{-1} is comparable to other studies performing enzymatic hydrolysis of OFMSW. For example, López-Gómez et al. (2019) obtained 55.41 mg mL^{-1} of glucose from a source selected waste, and Ebrahimian and Karimi (2020) a maximum of 35 mg mL^{-1} of glucose. Generally, the achieved RS concentration is lower than those experiments that employed selected food waste, which reached between 130 and 170 mg mL^{-1} (Yan et al., 2011; Zhang et al., 2020), because this type of waste is lesser in complexity and amount of impurities (Alibardi and Cossu, 2015). An exception was found in Cabas Candama et al. (2020), who reached 25 mg mL^{-1} of glucose using waste from fruit and vegetables and the same enzymatic cocktail as the employed in this paper. In every run the concentration of RS obtained in the solid fraction was lower than that initially present in batch 1 (241 mg g^{-1} DM), indicating the solubilization of the sugars towards the liquid fraction. The total amount of released RS increased almost two-fold in runs 4 (467.2 mg g_i^{-1} DM) and 8 (466.9 mg g_i^{-1} DM) suggesting gradual hydrolysis of the fibers present in the OFMSW. Those runs (3, 7, 9, 12) without enzyme addition did not show an increase in RS after the enzymatic hydrolysis as displayed by the performance index of 1, proving the hydrolysis effect of the cocktail to a greater or lesser extent depending on the conditions of the hydrolysis.

In general, low enzymatic load and high pH lead to lower RS concentrations in both fractions whereas medium and high enzyme loads and medium and low pHs lead to larger amounts of RS released. Reaching an agreement between the sugar release in both fractions, as seen in runs 1, 5, and 8 in Table 2, would maximize the potential of

Table 2
Design matrix of the Box-Behnken model and observed responses: reducing sugars in the solid and liquid fractions. The performance of the hydrolysis is expressed as fold increase of RS.

Run	Temp (°C)	pH	Enzymatic load (ml g^1 DM)	RS solid fraction (mg g_i^{-1} DM)	RS liquid fraction		Hydrolysis performance*
					(mg mL^{-1})	(mg g_i^{-1} DM)	
1	25	3.5	0.05	121.8	43.5	314.3	1.8
2	25	5.5	0.05	121.6	38.0	274.8	1.6
3	25	4.5	0.01	80.2	23.2	167.4	1.0
4	25	4.5	0.1	102.1	50.6	365.1	1.9
5	55	3.5	0.05	130.8	45.2	326.1	1.8
6	55	5.5	0.05	94.4	31.5	227.3	1.3
7	55	4.5	0.01	66.8	25.2	182.0	1.0
8	55	4.5	0.1	128.5	46.9	338.5	1.9
9	40	3.5	0.01	60.8	23.7	171.5	1.0
10	40	3.5	0.1	124.5	40.8	295.0	1.7
11	40	5.5	0.01	69.2	25.1	181.2	1.0
12	40	5.5	0.1	82.2	41.6	300.8	1.6
13	40	4.5	0.05	133.2	33.2	239.5	1.3
14	40	4.5	0.05	119.7	35.4	256.0	1.6
15	40	4.5	0.05	122.3	30.7	221.4	1.4

* Calculated as the total amount of RS (liquid and solid fraction) with respect to the initially measured in batch 1 (241 mg g^{-1} DM).

OFMSW hydrolysis as a pretreatment of fermentative steps.

3.2. Process optimization and verification

The RSM Box-Behnken design was implemented to optimize the sugar release from OFMSW, both in the liquid and solid fractions of hydrolysis. The variables were analyzed by a multiple regression analysis to obtain a regression equation that could predict the response within the specified range (Guan and Yao, 2008). Analysis of variance (ANOVA) was used to investigate the significance of fit for the model equations. As the goal of this paper is the valorization of the solid fraction of hydrolysis the following discussion is focused on it but the model and ANOVA for the liquid fraction can be found in the supplementary material (Table S.2).

The experimental results were fitted with a quadratic equation. The ANOVA analysis presented in Table 3 resulted in a significant regression model ($p < 0.05$) and a not-significant lack of adjustment ($p > 0.05$). Consequently, the model presents a good adjustment with the experimental data reported. A logarithmic transformation of the response for analyzing the data was performed to achieve a better adjustment in the quadratic model (Joglekar and May, 1987). The parameters enzyme load and pH showed significant effects, specifically, the linear effect of the enzyme load was the most significant as the smaller the p-value, the more significant the corresponding coefficient (Haber and Runyon, 1973). According to the R^2, which is a measure of the degree of fit (Haber and Runyon, 1973), the model could explain 98.76% of the variability in the response. The model also has the capacity to explain 88.40% of the variations in new observations according to the predicted R^2, which is in reasonable agreement (<0.2) with the adjusted R^2 (96.51%) (Haber and Runyon, 1973; Joglekar and May 1987). In addition, it shows a low coefficient of variation (1.11%), being indicative of the reliability of the experimental design. The resulting regression equation for the response of RS concentration in the solid fraction for the conditions studied is presented in Equation (2).

$$\log_{10}RS(mgg^{-1}DM) = +2.10 - 0.007X_1 - 0.033X_2 + 0.10X_3 - 0.035X_1X_2 + 0.045X_1X_3 - 0.059X_2X_3 + 0.011X_1^2 - 0.042X_2^2 - 0.146X_3^2 \quad (2)$$

where X_1, X_2 and X_3 represent the temperature (°C), initial pH, and enzyme load (mL g^{-1} DM), respectively.

The interaction effects of the three variables were corroborated in three-dimensional response surface plots (Fig. 2). Based on the ANOVA analysis (Table 3), the temperature was not a significant parameter as illustrated in plots showing the interaction of temperature with enzyme load and pH (Fig. 2.B and Fig. 2.C, respectively). Therefore, the Viscozyme L cocktail was suitable for the hydrolysis of OFMSW through the whole temperature range described by the provider. This observation has also been recently reported by Cabas Candama et al. (2020) for waste of fruits and vegetables. The effect of the pH, reported as a parameter with significant effects in Table 3, can be seen in Fig. 2.A and Fig. 2.C. The RS concentration increases when pH decreases, reaching the maximum at pH 3.5. However, as shown in Fig. 2.C, for low temperature (25°C) the maximum RS concentration is reached at pH around 4.5. The adequate performance on milder conditions, i.e. lower temperature and not so acidic pH, makes Viscozyme L a notable cocktail for OFMSW hydrolysis as a reduction in energy and chemical requirements highly influences the process economic cost (Alvira et al., 2010). Lastly, enzyme load was found the most significant parameter (Table 3) as evident in the steeper surfaces observed in Fig. 2.A and Fig. 2.B compared with Fig. 2.C. It can be seen that RS concentration increases as enzyme load does until a certain value after which decreases. This observation suggests that the enzymes had limited access to the solid fraction so all attachment sites in the solid were occupied reaching an enzyme saturation effect, which has also been previously reported by Cabas Candama et al. (2020) for waste of fruits and vegetables. At the same time, it is important to highlight that the hydrolysis of the complex composition of OFMSW requires several enzymatic activities. For food waste, the major contributor to OFMSW, it has been shown that polysaccharides such as xylan and pectin, can interfere in the hydrolysis of cellulose and hemicellulose by masking them (de la Torre et al., 2017; Van Dyk et al., 2013). Viscozyme L contains a wide range of carbohydrases addressing these fractions, however, the design of tailor-made cocktails with ratios of enzymatic activities specific for OFMSW or with increased substrate specificity by protein engineering, might bring further the release of sugars from OFMSW (Chapman et al., 2018).

Finally, optimization of the RS concentration in the solid fraction was conducted by a numerical optimization method using the Design-Expert® software. The target was to maximize the RS concentration while keeping temperature, initial pH, and enzyme load within the study range. The optimum operating conditions were close to temperature 55°C, initial pH 3.5, and enzyme load 0.08 mL g^{-1} DM, and correspond to 157.0 mg g_i^{-1} DM. Considering that milder conditions would benefit the economy and energy balance of the process, another optimum that minimizes temperature and enzyme load was also selected. Mild conditions were temperature 25°C, enzyme load 0.06 mL g^{-1} DM, and initial pH 4.5, which correspond to a prediction of 131.9 mg g_i^{-1} DM. Both conditions, extreme and mild, were verified experimentally. Considering the inherent variability of the OFMSW, it was decided to verify the optimum conditions using batch 2 of ssOFMSW to also assess the reproducibility of the process. The experimental results obtained were 122.6 ± 13.4 mg g_i^{-1} DM for extreme conditions and 105.2 ± 9 mg g_i^{-1} DM for mild conditions. The experimental RS concentrations observed using conditions predicted by the model were lower than expected but within the 95% confidence interval for the extreme and the 99% for the mild. It should be noted that batch 2 contained considerably fewer sugars (157.7 mg g^{-1} DM) than batch 1, which might explain the lower RS concentrations achieved. These results are quite satisfactory considering the complexity of working with such a variable substrate as OFMSW (Hansen et al., 2007). This lower initial amount of sugars resulted in greater hydrolysis performance, 2.7 for mild conditions and 2.9 for the extreme.

These conditions were selected for maximizing the RS concentration in the solid fraction, however, they also led to high values in the liquid fraction (304.2 ± 9.5 mg g_i^{-1} DM for mild conditions and 339.4 ± 7.2 mg g_i^{-1} DM for extreme conditions, equivalent to concentrations of 42 and 47 g L^{-1}respectively). This can be explained because the hydrolysis of the solid fibers is a gradual process, and partially hydrolyzed fibers in the solid fraction might be solubilized during the extraction required for RS analysis. The partial hydrolyzation of fibers results in easily accessible sugars during the SSF.

Table 3
ANOVA for the response surface quadratic model when RS concentration in the solid fraction was used as a response.

Source	DF	Mean Square	*p*-value
Model	9	0.1154	0.0003*
X_1 – Temperature (°C)	1	0.0023	0.3908
X_2 – pH	1	0.0474	0.0081*
X_3 – Enzyme load (mL mg^{-1} DM)	1	0.3978	0.0001*
$X_1 \cdot X_2$	1	0.0264	0.0247*
$X_1 \cdot X_3$	1	0.0429	0.0099*
$X_2 \cdot X_3$	1	0.0739	0.0032*
X_1^2	1	0.0024	0.3867
X_2^2	1	0.0347	0.0149*
X_3^2	1	0.4175	0.0001*
Residual	5	0.0026	
Lack of Fit	3	0.0022	0.6315
Pure Error	2	0.0033	

R^2 = 98.76%, R^2 (adj) = 96.51%, R^2 (pred) = 88.40%, and C.V. = 1.11%.
*Significant parameters ($p < 0.05$).

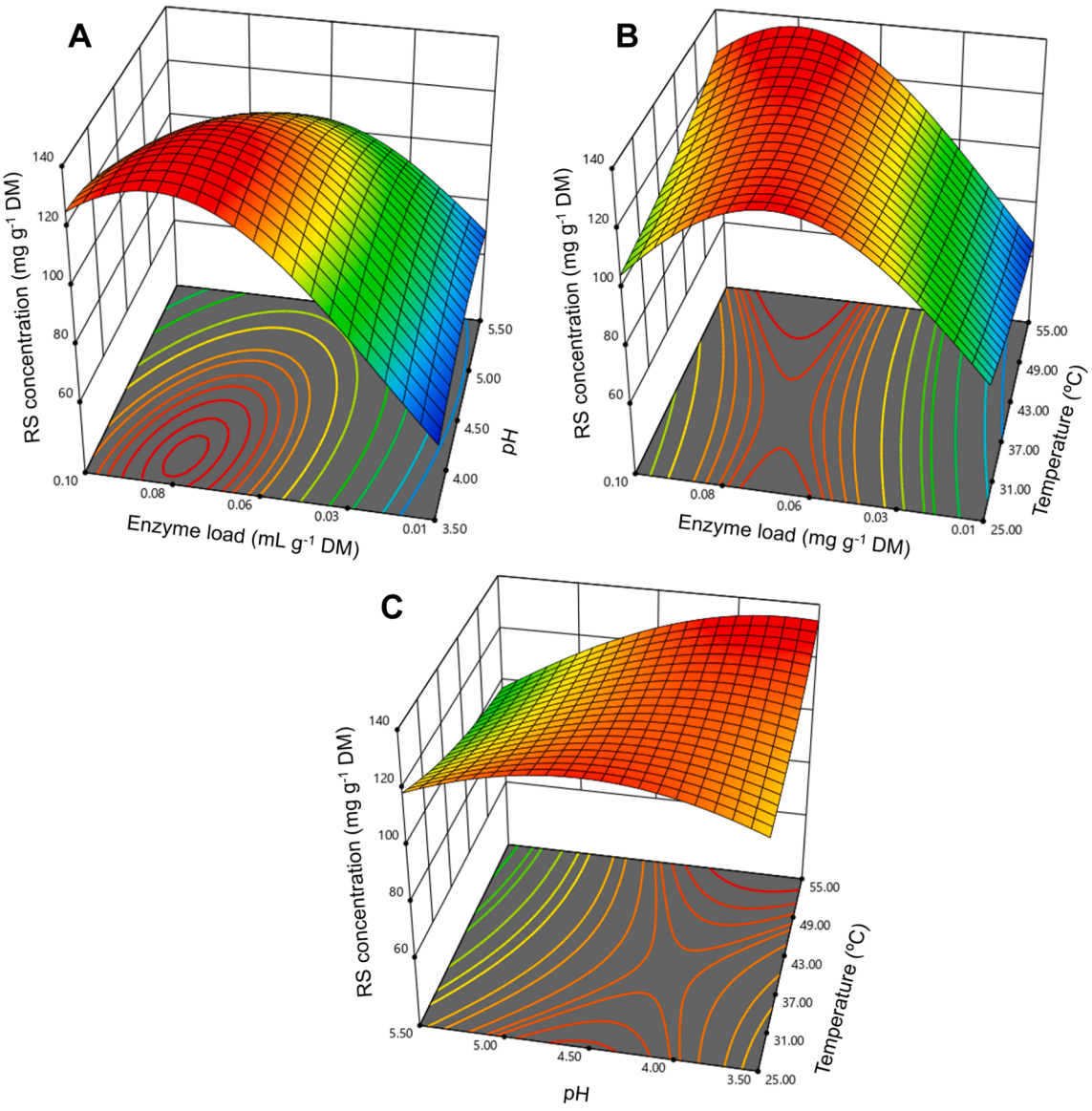

Fig. 2. Combined effect of (A) enzyme load and pH; (B) enzyme load and temperature; and (C) pH and temperature in RS concentration in the solid fraction. Other factors were at medium levels.

3.3. Assessment of the solid fraction of hydrolysis as a substrate for biopesticide production through SSF

A preliminary assessment has been performed to evaluate the capability of the obtained solid hydrolysate to support Bti growth and sporulation. Even though spore count and endotoxicity are not always proportional, spore count can be considered an indirect estimation method because during sporulation each Bt cell produces a spore that might contain toxic proteins (De Lourdes Tirado Montiel et al., 2001). The SSF operation parameters were based on the optimization performed by Mejias et al. (2020) using biowaste mixed with digestate as substrate. The performance of the solid hydrolysate fractions from extreme and mild pretreatment conditions of batch 2 was evaluated on a triplicate after 72 h. The solid fractions were characterized before the SSF in terms of DM and pH, resulting in 23.2 ± 0.9 and pH 6.3 for the mild hydrolysis and 23.9 ± 1.1 and pH 5.2 for the extreme hydrolysis conditions.

The fermentation started with no spore presence in the solid matrix and, as seen in Fig. 3, the solid hydrolysate derived from the mild conditions reached an average concentration of 1.3×10^8 CFU g^{-1} DM for spore count and 3.9×10^8 CFU g^{-1} DM for viable cell count. Viable cell value was in close agreement with those obtained by Mejias et al. (2020), but there still was potential for improvement in the sporulation ratio (33%). Conversely, the solid hydrolysate derived from the extreme conditions did not support Bti growth but resulted in a 12-fold decrease. This could be related to the low initial pH which was 5.2 compared with the 6.3 of the mild treatment. This pH value was lower than the optimum reported for Bti sporulation (7.0) and close to inhibitory (5.5) (De Lourdes Tirado Montiel et al., 2001; Özkan et al., 2003). Therefore, an additional step of pH adjustment using 1 M NaOH to neutral values after the enzymatic hydrolysis was evaluated in the SSF. Results from this SSF experiment can also be seen in Fig. 3. It is illustrated how the growth of Bti was favored by the change of pH, reaching values of viable cells of 2.5×10^7 CFU g^{-1} DM and spores of 1.6×10^6 CFU g^{-1} DM, with a 6% of sporulation ratio. Therefore, after a pH adjustment, the solid hydrolysate derived from extreme conditions also appeared as a suitable substrate for Bti growth but not as promising as the one derived from mild pretreatment conditions. Regarding the RS concentration, values at 72 h were below the detection levels of the method, which indicates consumption of over 90% of the RS. Compared to a previous work using

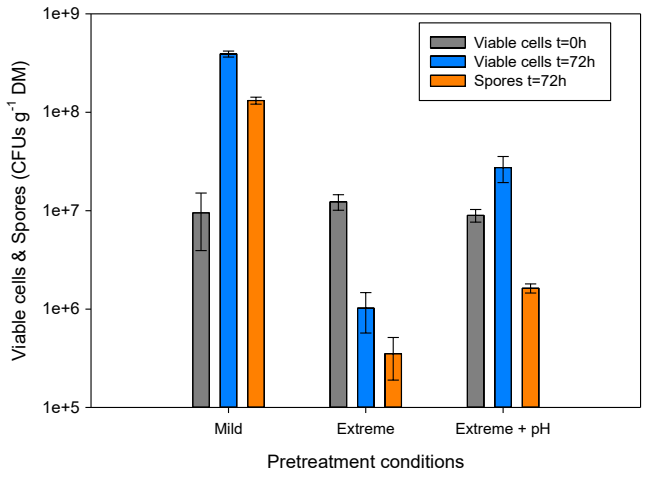

Fig. 3. Bti initial viable cells, final viable cells, and final spore concentration depending on the enzymatic treatment conditions of the solid hydrolysate. The initial spore count was 0.

non-hydrolyzed sterile OFMSW under different operation strategies, which resulted in spore counts between $3.5 \times 10^6 - 2.1 \times 10^7$ CFU g^{-1} (Ballardo et al., 2017), the spore count achieved in this first approach was one order of magnitude higher for mild conditions. Taking this into account and the fact that milder conditions are also related to less energy consumption, the economy of the process favors the hydrolysate derived from mild treatment conditions. A comprehensive material and economic balance of the process, including the utilization of the liquid fraction, would be necessary for achieving maximum profitability from all fractions derived from the enzymatic hydrolysis of OFMSW.

Overall, the OFMSW has been valorized to a sugar concentrate with great potential for fermentative processes and a solid rich in Bt spores, hence providing an alternative pathway for closing the organic matter cycle. This work contributes to the ongoing paradigm shift in waste management, fostered by the Circular Economy Action Plan of the European Commission, which aims to reduce landfilled waste to a maximum of 10% (Union, 2014). Future studies have to validate these results at larger scales and field test the pesticide action of the final product so it can be introduced in the growing global market of biopesticides, projected to reach USD 11,438.1 million in 2026 (Mordor Intelligence, 2018).

4. Conclusions

Enzymatic hydrolysis of source-separated OFMSW has been optimized reaching reducing sugars values of 365.1 mg g_i^{-1} DM in the liquid fraction and 184.11 mg g_i^{-1} DM in the solid fraction, and almost a 2-fold increase in total reducing sugars. Two optimum operational conditions were selected to evaluate the effect of the harshness of the enzymatic hydrolysis on the SSF of the resulting solid fractions. *Bacillus thuringiensis var israelensis* has been grown successfully on the solid fraction deriving from milder conditions. This finding is relevant looking towards process development and its economy. This work provides an alternative scenario for the valorization of organic municipal solid waste, producing a sugar-rich liquid with a concentration of reducing sugars of 50.56 mg mL^{-1} and a solid containing biopesticide from *Bacillus thuringiensis*.

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 material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2021.11.014.

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Supplementary Material

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.

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Table S.1 Significance based on the Tukey test of the characterization parameters between the three groups evaluated (batch 4, batch 5 and literature) in Table 1 in the main document.

Parameter	*p*-value	Statistical difference
Moisture content (%)	0.106	No
DM (%)	0.106	No
OM (%*)	0.484	No
RS (%*)	0.008	Yes
C (%*)	0.835	No
H (%*)	0.785	No
N (%*)	0.041	Yes
S (%*)	0.127	No
C/N ratio	0.326	No
Cellulose (%*)	0.399	No
Hemicellulose (%*)	0.049	Yes
Lignin (%*)	0.827	No
pH	0.001	Yes

Table S.2 ANOVA for the response surface linear model when RS concentration in the liquid fraction was used as a response.

Source	DF	Sum of Squares	Mean Square	*p*-value
Model	3	896.68	298.89	0.0005*
X_1 – Temperature (ºC)	1	5.48	5.48	0.6255
X_2 – pH (pH units)	1	36.08	36.08	0.2240
X_3 – Enzyme load (mL mg^{-1} DM)	1	855.12	855.12	< 0.0001*
Residual	11	239.03	21.73	
Lack of Fit	9	227.55	25.28	0.1987
Pure Error	2	11.48	5.74	

R^2 =78.95%, R^2 (adj)= 73.21%, R^2 (pred)= 57.64%, and C.V.= 13.08%.

**Significant parameters*

When the RS in the liquid fraction was used as the response variable of the optimization, the experimental results did not fit with a quadratic equation but a linear one. The ANOVA analysis for the resulting model reports a significant regression model ($p < 0.05$) governed by the enzyme load as the only significant parameter. However, in this instance, the R^2 (78.95%) indicated less relevance of the dependent parameters to illustrate the performance variation.

Table S.3 Significance based on the Tukey test of the different scenarios evaluated in the SSF process (mild, extreme and extreme +pH) in Figure 3 in the main document.

Parameter	*p*-value	Statistical difference
Viable cells (t=0h)	0.506	No
Viable cells (t=72h)	<0.001	Yes
Spores (t=72h)	0.001	Yes

Article III

Bacillus thuringiensis biopesticide production through solid-state fermentation using organic fraction of municipal solid waste (OFMSW) enzymatic hydrolysate

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The development of SSF processes is highly dependent on the substrate characteristics, which can be modified to a certain extent. This section presents an operational strategy to overcome the difficulties encountered in the valorization of the exhausted solid hydrolysate via SSF. The use of cosubstrates emerges as an alternative to chemical modifications for maintaining pH under control during SSF at a laboratory scale. Growth and sporulation of *Bacillus thuringiensis* is achieved.

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ORIGINAL PAPER

Bacillus thuringiensis Production Through Solid-State Fermentation Using Organic Fraction of Municipal Solid Waste (OFMSW) Enzymatic Hydrolysate

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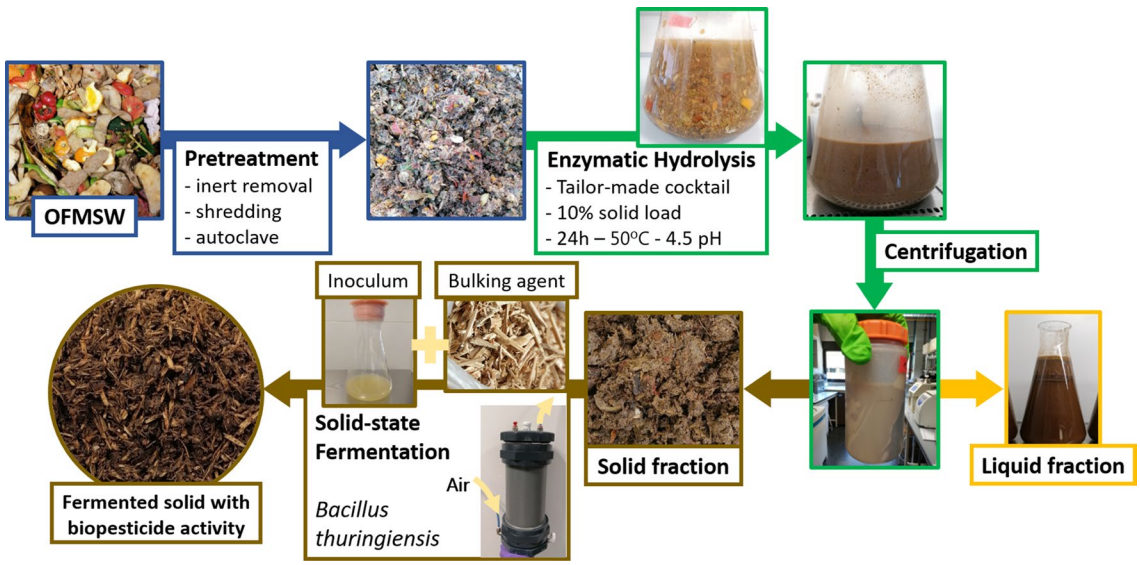
Abstract

This paper aims to explore an alternative pathway to valorize the organic fraction of municipal solid waste. It is based on the use of enzymatic hydrolysis to obtain a sugar-rich fraction with the potential for liquid fermentative processes and a partially hydrolyzed solid that is evaluated as a substrate for solid-state fermentation. Different strategies to modify the pH of the solid substrate to reach a neutral pH, suitable for the growth of biopesticide producer *Bacillus thuringiensis*, have been explored. The use of alkaline cosubstrates was evaluated on two different scales and temperature was assessed as a preliminary indicator of the scale-up viability of the process strategy. By ensuring a proper pH throughout the process, the growth and sporulation of *Bacillus thuringiensis* were achieved. The best cosubstrates and mixing ratios were 50% of digested sewage sludge and 25% of digested organic fraction of municipal solid waste, which led to a spore concentration of 1.1×10^9 spores g^{-1} of dry matter and 6.4×10^8 spores g^{-1} of dry matter, respectively. Overall, a reproducible and flexible solid-state fermentation process has been achieved for hydrolyzed organic municipal waste based on the use of alkaline urban wastes as cosubstrates. This valorization pathway fits with the concept of urban biorefineries.

Graphical Abstract



Keywords Enzymatic hydrolysis · Solid-state fermentation · Biowaste · Biopesticide · *Bacillus thuringiensis*

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Statement of Novelty

The present research provides a novel, flexible and reproducible solid-state fermentation process for the valorization of solid hydrolysates from the organic fraction of municipal solid waste into biopesticides in the context of an urban waste biorefinery. The results obtained will aid in the search and implementation of alternative valorization pathways for specific byproducts of urban waste with large environmental impacts associated.

Introduction

With the increasing municipal solid waste (MSW) generation over the years, significant political and social efforts have been made to introduce selective collection systems that allow for its recycling. In the case of the organic fraction (OFMSW), which represents nearly half of the total MSW [1], selective collection systems prevent its natural biodegradation associated with the release of greenhouse gases and other major environmental impacts [2]. Besides, it improves the quality of OFMSW and facilitates its recycling, which is commonly performed through composting or anaerobic digestion (AD) [3, 4]. Considering the complex composition of the OFMSW, rich in polysaccharides, lignocellulose, proteins, lipids and macro/micronutrients [5], this waste fraction could be exploited to a larger extent for the production of higher-value bioproducts in a biorefinery-like scenario [6]. Besides contributing to the urgent need for ensuring materials and energy supply [7], such a scenario would also justify the significant economic investment required to build a robust and efficient source-separated collection system [3, 6]. In this urban biorefinery context, the widely implemented technology of AD for biogas production can still be used as a complementary technology to handle intermediates [8]. Also, due to its robustness, it can be considered a tool to reduce the risk derived from the heterogeneity and variability of the OFMSW as a substrate.

Enzymatic hydrolysis has emerged as a powerful technology during the development of lignocellulosic biorefineries to break down macromolecules into their functional units [9]. So, its application to municipal waste streams has also been gaining interest [10–13]. The main components of the OFMSW are carbohydrates and fibers, representing up to 85% of its composition [5], hence their fractioning can generate a variety of fermentable sugars [14]. From a biorefinery perspective, these sugars act as building blocks for different fermentative processes, especially when collected in a liquid fraction [15]. Several papers have explored the use of sugars obtained from the OFMSW in submerged fermentation, for instance, the production of acetic acid [16], succinic acid [17], bacterial poly(3-hydroxybutyrate) [18], and biofuels [19]. The remaining partially hydrolyzed and non-hydrolyzed fibers could also be used in fermentation processes, even though the literature on the topic is scarce.

Solid-state fermentation (SSF) is described as the aerobic fermentation process that takes place in near absence of free water [20]. It has been gaining research interest as it can use different solid organic wastes as substrate and does not always demand strict sterile conditions [21–24]. For instance, it has been used to produce fungal and bacterial biopesticides [25, 26], aroma compounds [27], bioplastics [28], and biosurfactants [29, 30]. When working with urban organic wastes, complete sterilization is an arduous and energy-intensive task. Therefore, robust microorganisms that can thrive in non-sterile environments would facilitate process implementation in real scenarios. In this sense, *Bacillus thuringiensis* (Bt) species have been grown successfully under not completely sterile conditions in soy residues [31], OFMSW [24], digestate [22], and wastewater [32]. Biopesticides derived from Bt constitute almost 90% of the world's biopesticide market [33], a market growing annually at a rate of 15% [34]. These microorganisms are Gram-positive soil bacteria that produce endotoxin proteins accumulated in parasporal crystals during the sporulation phase, which are selectively toxic for pest insects mainly belonging to the orders Lepidoptera, Diptera and Coleoptera [35]. Specifically, *Bacillus thuringiensis* var. *israelensis* (Bti) has been described as the most environmentally friendly agent for the control of larval mosquitoes [35] Conventionally, Bt has been produced in liquid media through submerged fermentations but its sporulation process can benefit from solid cultivation [33]. Also, SSF opens up doors for more simple solid formulation possibilities. Like many bacteria, Bt thrives at a pH near neutrality [36, 37]. However, pH control and monitorization during the course of SSF processes are difficult due to the heterogeneity of the solid matrix, a limited amount of free water and a lack of suitable online pH measurement methods. Also, the solid substrate itself might have a buffering effect due to its complex chemical composition [12]. Another major challenge of this technology is the heat and mass transfer-related issues that arise during the scale-up. Therefore, monitoring process temperature from early implementation stages is important to prevent heavy efficiency losses at larger scales [38, 39]. Also, an adequate temperature of around 30 °C must be ensured during the growing phase of Bt [36].

In this paper, the use of the solid hydrolysate from enzymatic hydrolysis of OFMSW is evaluated as a substrate for Bt growth via SSF. Two operational strategies have been tested to adjust pH near neutrality and maximize Bt growth and sporulation at 0.5 L: chemical pH modification and the use of cosubstrates with high buffering capacity. Specifically, byproducts of the AD process of urban wastes have

been selected as cosubstrates. Process development to a 1.5 L reactor without temperature control and using non-sterile substrates has also been done to study its suitability for full-scale implementation in a biorefinery-like environment for OFMSW.

Materials and Methods

OFMSW Collection and Hydrolysate Production

The substrate of the enzymatic hydrolysis was the OFMSW collected from a door-to-door collection system selected due to its high quality. It was collected upon arrival at the MSW treatment plant of Mancomunitat La Plana (Malla, Barcelona). The few inert materials (< 1% w w^{-1}), such as plastic, metals, glass or textiles, were removed manually as well as hard shells, bones, hair and excess paper. Then, the OFMSW was shredded mechanically using a home composting shredder (Tecoinsaen SL, Spain) and stored at − 20 °C for a maximum period of three months. Before use, samples were defrosted overnight at 5 °C and sterilized by autoclaving at 121 °C for 30 min. Two different batches of OFMSW (October 2021 and January 2022) were used in this study and their initial characterization can be seen in Table 1.

Enzymatic hydrolysis of homogenized OFMSW was performed using a tailor-made enzymatic cocktail kindly provided by ASA Spezialenzyme GmbH (Wolfenbüttel, Germany) and composed mainly by a blend of cellulases and pectinases but also hemicellulase, ß-glucosidase and α-amylase. Experiments were conducted under sterile conditions in 2 L Erlenmeyer flasks following the provider's recommendations at 50 °C and initial pH of 4.5, modified using 0.05 M sodium citrate buffer, and 0.05 mL of enzymatic cocktail per g of initial dry matter (DM). The initial solid-to-liquid ratio was set to 10% (w v^{-1}). Erlenmeyer flasks were incubated for 24 h at 180 rpm and rapidly centrifuged at 6000 rpm for 15 min at 4 °C. Then, both fractions were separated and the solid fraction was collected and stored at 5 °C until its use in the SSF for a maximum time of 48 h. Characterization of the final hydrolysate can be seen in Table 2.

Table 1 Characterization of the batches of source-separated OFMSW collected in this study and average values from 43 cities in 22 countries reported by Campuzano et al. [5]

Parameter	Batch 1 (10/21)	Batch 2 (01/22)	Literature
MC (%)	77.2 ± 0.5	76.4 ± 1.0	72.8 ± 7.6
DM (%)	22.8 ± 0.5	23.6 ± 1.0	27.2 ± 7.6
OM (%)[a]	88.2 ± 1.1	89.7 ± 0.7	84.6 ± 9.9
RS (%)[a]	17.0 ± 0.7	16.6 ± 0.9	10.5 ± 6
pH	5.6 ± 0.2	5.6 ± 0.1	5.02 ± 0.95
DRI_{24h} (g O_2 kg^{-1} DM h^{-1})	4.9 ± 0.7	3.5 ± 0.5	NA

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

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, *NA* not available

[a]Dry basis

Table 2 Characterization of the solid hydrolysates obtained from each batch of OFMSW

Parameter	Batch 1 (n = 2)	Batch 2 (n = 3)
MC (%)	75.3 ± 1.3	74.7 ± 4.5
DM (%)	24.7 ± 1.3	25.4 ± 4.5
OM (%)[a]	88.7 ± 1.4	91.7 ± 1.7
pH	5.6 ± 0.2	5.3 ± 0.1
RS (%)[a]	14.0 ± 2.9	13.3 ± 1.7
RS liquid fraction (g/L)	35.6 ± 4.2	46.4 ± 4.4

Data presented as mean values of the different hydrolysis rounds for each batch ± standard deviation

MC moisture content, *DM* dry matter, *OM* organic matter, *RS* reducing sugars

[a]Dry basis

Solid-State Fermentation

Microorganism and Inoculum Preparation

All tests were carried out using *Bacillus thuringiensis* var *israelensis* (Bti) strain CECT 5904 obtained from *Colección Española de Cultivos Tipo* (CECT, Valencia, Spain) and preserved at − 80 °C using a seed lot system in cryo-pearls (DeltaLab, Barcelona, Spain). For inoculum preparation, one cryo-pearl was inoculated in 100 mL of sterile Nutrient Broth nº2 (Oxoid CM0067B, England) and incubated at 30 °C and 130 rpm for 20 h when an optical density of 2.5–3.0 was reached. The culture was centrifuged for 10 min at 3500 rpm and 4 °C. First, the obtained pellet was resuspended in 3 mL of the exhausted media and then, diluted 1:10 (v $v.^{1}$) also with supernatant to reach approximately a concentration of 10^8 CFU mL^{-1}. No spores were detected at this point.

Chemical pH Modification of the Solid Hydrolysate

Chemical pH modification was done using calcium carbonate ($CaCO_3$). First, the required amount to reach a pH of 7 was determined by adding increasing amounts (1–12 mL) of a 1 M solution to the solid hydrolysate and thoroughly mixing it manually. A 10% of MC increase was set as the maximum modification possible. The solid was left for 1 h at a cold temperature (5 °C) to settle and then pH was measured according to standard procedures.

Cosubstrates and Mixtures Preparation

Three different organic materials related to urban wastes were evaluated as cosubstrates for the SSF process: (i) digested and dewatered sewage sludge coming from the AD process of a municipal wastewater treatment plant (Sabadell, Spain), (ii) digestate from the AD process of a source selected OFMSW treatment plant (*Consorci per a la Gestió dels Residus del Vallès Oriental*, Granollers, Spain), and (iii) compost from a composting plant of source selected OFMSW (*Planta de compostatge de Sant Cugat*, Barcelona, Spain). Materials were characterized in terms of dry matter, organic matter (OM), pH, conductivity and biodegradability upon arrival, as detailed later, and stored frozen until use for a maximum period of 3 months. Once defrosted overnight at 5 °C, both digestate and digested sewage sludge were subjected to a hygienization pretreatment (1 h at 70 °C) before their use in SSF processes to pasteurize them as specified in the European Regulation Nº 142/2011.

These materials were mixed individually with the solid hydrolysate at two different weight ratios, 25 and 50%, to prepare the final mixtures used in the SSF processes. The same amount of sterile wood chips of particle size between 0.5 and 5 cm (Acalora, Palets Pla d'Urgell, Spain) was added to the mixtures as a bulking agent to provide porosity to the solid matrices.

Experimental SSF Set-Up

SSF experiments were performed at cylindrical polyvinyl chloride packed-bed reactors of two different scales, 0.5 and 1.5 L. Reactors were completely sealed and equipped with an air inlet and outlet port on the bottom and the top respectively. A humidified airflow was provided through a mass flow meter (Mass-Stream D-6311, Bronkhorst, NL) set to constant aeration of 37 mL g^{-1} DM h^{-1} for ensuring aerobic conditions [40]. The oxygen concentration of the exhausted gases was measured after a water trap by an O_2–A_2 oxygen sensor (Alphasense, UK) connected to a custom-built data acquisition system (Arduino® based). The main difference between both scales was that, at 0.5 L, the temperature was controlled and kept constant at 30 °C by placing the reactor in a water bath, whereas at 1.5 L temperature was not controlled but only monitored using button temperature sensors (Maxim Integrated, U.S.) to obtain accurate temperature profiles at different reactor heights (10 and 20 cm).

Initial experiments were performed at 0.5 L evaluating both the effect of the chemical modification of pH and the use of cosubstrates on Bti growth and sporulation. Experiments were performed in duplicate for each condition. The total amount of the final mixture was kept constant for all the conditions and was 90 g of the substrate and 20 g of the bulking agent, corresponding to a ratio of 1:2 v v^{-1}. The different amounts of cosubstrates used for preparing the mixture were calculated based on wet weight (25 and 50%). Materials were mixed manually and inoculated with 3 mL of diluted Bti to reach approximately 10^7 CFUs g^{-1} DM.

Then, the best-performing scenarios were validated in triplicate at 1.5 L and the effect of the mixtures on the temperature was assessed as a preliminary step for scaling up. In this case, 360 g of substrate mixture and 80 g of the bulking agent were mixed and inoculated with 14 mL of diluted Bti inoculum.

Monitoring Parameters

All experiments lasted 72 h, which has been established previously as the maximum spore counting time for Bt [21] and were evaluated in terms of viable cells and spores production. First, a solid-liquid extraction was performed 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 [22]. All plates were incubated at 30 °C for 20 h and viable cells or spores were estimated in terms of colony-forming units (CFUs) and related to the DM of the sample, following the equation:

$$Viable\ cells\ \&\ spores\ concentration\ (CFUs\ per\ g\ DM) = \frac{n^{\circ}\ CFUs \cdot D \cdot Ex}{0.05 \cdot DM}$$

where, nº CFUs is the average of counted CFUs in the Petri dishes, D is the dilution factor of the extract, Ex is the extraction factor (9 mL per g of wet solid), 0.05 is the mL plated and DM is the sample dry matter per g of wet solid.

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:

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

The sporulation yield that expresses the concentration of spores produced per initial viable cell inoculated is calculated using the final spores concentration and the initial cell concentration.

$$Sporulation\ yield\ (spores\ per\ viable\ cell\ inoculated) = \frac{final\ spores}{inital\ cells}$$

With the measured oxygen concentration at the outlet port of the reactor, the specific oxygen uptake rate (sOUR) was

calculated as an indicator of the biological activity according to the following equation [41]:

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

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, 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.

Analytical Methods

Substrates and fermentation samples were characterized in terms of dry matter, moisture content, organic matter and pH according to standard procedures [42]. Reducing sugars (RS) of the solid samples were measured after a solid-liquid extraction with distilled water using the DNS method and expressed per gram of DM [13, 43]. Biodegradability was assessed through two respiration indexes 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 [40, 41]. All measurements were conducted in triplicates.

Results and Discussion

The characterization of the OFMSW samples used in this study was in line with average literature values (Table 1). During enzymatic hydrolysis, the complex carbohydrates that comprise the OFMSW are converted to monomeric sugars released to the liquid fraction. The efficiency of this conversion process depends on many parameters, such as the enzymatic activities selected, the solids load and the time, among others. However, there is always a remaining solid fraction containing partially hydrolyzed and non-hydrolyzed fibers with the potential to be used in further conversion processes. As can be seen in Table 2, the obtained solid hydrolysate is humid and rich in organic matter. Therefore, a potential substrate for SSF processes that require enough water to promote microbial growth [37]. From the RS measurement, it can be seen that a significant amount of easily accessible sugars remains solubilized in the free water content [13]. The pH is rather acidic, which represents a challenge for growing microorganisms that prefer pH near neutrality such as bacteria, and specifically Bti [36, 37]. Therefore, a pH adjustment step is required before growing Bti as was confirmed during an initial experiment in which unmodified solid hydrolysate was used as a substrate (Fig. 1). In fact, not even the expected amount of around

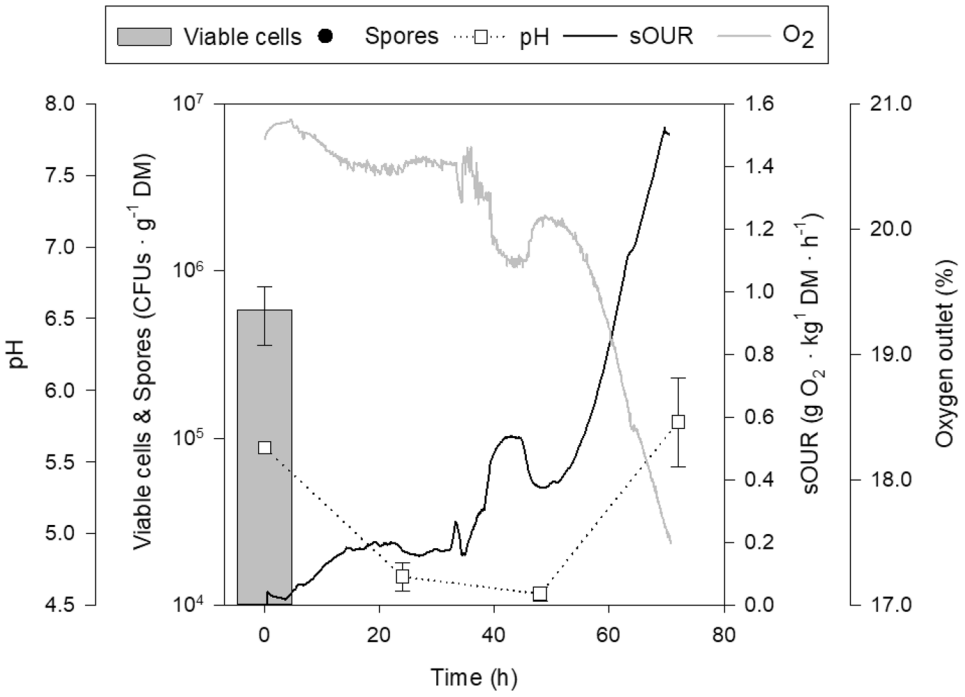

Fig. 1 Process parameters evolution (sOUR, outlet oxygen, viable cells, spores and pH) during the initial evaluation of Bti growth on unmodified solid hydrolysate. Initial spore count was 0

10^7 viable cells per g of DM in the sample of day 0 was reached, indicating that cell death was almost immediately. Even though Bti was not observed after 24 h it can be seen how other opportunist microorganisms started consuming oxygen.

Chemical Modification of the pH

The first attempt to modify the pH of the solid hydrolysate was done using chemicals as it is the most straightforward and commonly used methodology. A substance with a strong buffering capacity ($CaCO_3$) was selected over a strong base, such as NaOH, due to the difficulties in monitoring and modifying the pH during the course of SSF processes [37]. To reach a pH of around 7 it was necessary to add 8.5 mL of 1 M $CaCO_3$ to the 90 g of hydrolysate, which only increased the moisture content of the sample by around 2%. Results from this fermentation can be seen in Fig. 2. As the bar graph shows, Bti was not able to thrive and in only 24 h the viable cells were drastically reduced. This can be explained by the drop in pH below 5.6, reported as inhibitory for Bt species [33], as a consequence of the production of organic acids at the early stages of fermentation [36]. Spores appeared at 24 h, reaching the maximum concentration at 72 h as expected [21, 26]. At his point, viable cells could not be measured because they could not be distinguished from opportunist microorganisms in the Petri dishes, which explains the sharp rise of sOUR after 48 h. Even though spores are produced, it has been reported that spores developed in acidic environments are less viable and robust [44]. Increasing the amount of $CaCO_3$, or other substances involves an increase in the moisture content of the sample, which should not exceed 80% [37]. Besides, it would also increase the production cost and difficult the operation at larger process scales. Therefore, it was decided to test a different strategy for pH modification based on the use of high-buffering capacity cosubstrates.

Selection and Screening of Cosubstrates

The use of cosubstrates to improve process efficiency is a widely researched and used practice in the anaerobic digestion of municipal wastes [45, 46]. For instance, the rapid hydrolysis of food waste leads to an inhibitory pH that can be overcome with the use of sewage sludge or animal manure as a cosubstrate [45]. The use of cosubstrates in SSF has been researched to a lesser extent [29, 47]. Nutrient supplementation is more common but it should be highlighted that these studies are rarely conducted at larger scales [48]. For this study, the cosubstrate needed to provide supplemental alkalinity and, ideally, nutrients. It was decided to evaluate only biomaterials in the framework of municipal waste, as it would ease the implementation of such processes in industrial environments. The selected cosubstrates to be studied were: OFMSW digestate, digested sewage sludge and OFMSW compost. Their characterization alongside an average for the solid hydrolysate (Table 2) is presented in Table 3. All three came from large and well-established municipal waste treatment plants, in fact, sewage characterization reported similar

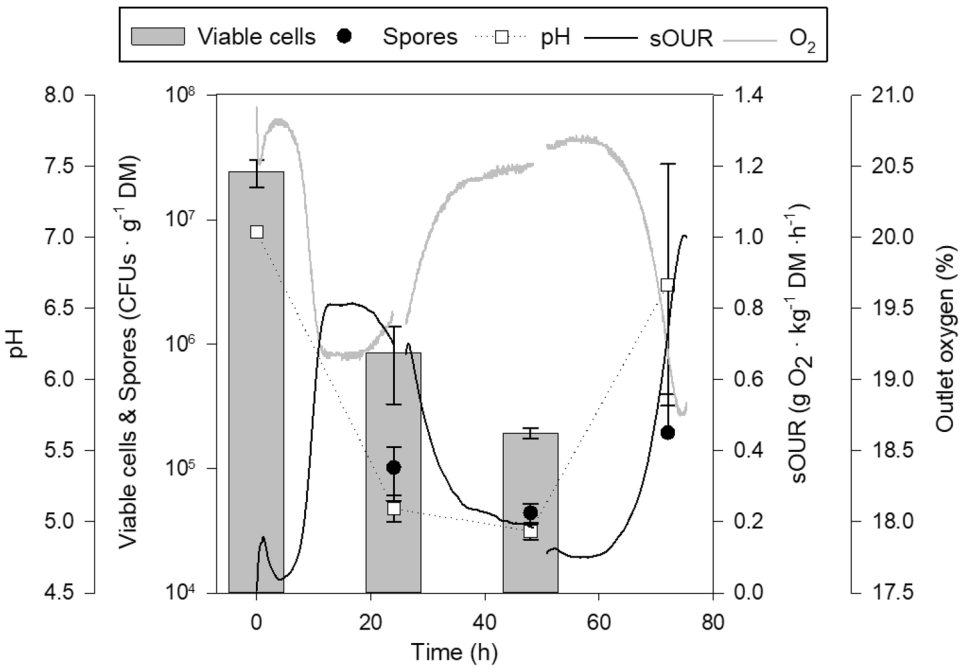

Fig. 2 Process parameters evolution (sOUR, outlet oxygen, viable cells, spores and pH) during the time course of solid hydrolysate with chemically modified pH. Initial spore count was 0

Table 3 Characterization of the hydrolysate and the different cosubstrates used in this study

Parameter	Hydrolysate	Compost	Sewage sludge	Digestate
MC (%)	75.4 ± 2.1^{a}	32.2 ± 0.2	83.5 ± 0.1	77.2 ± 0.4^{a}
DM (%)	24.6 ± 2.1^{a}	67.8 ± 0.6	16.5 ± 0.1	22.8 ± 0.4^{a}
OM (%)*	86.9 ± 1.0	59.8 ± 5.4^{b}	68.8 ± 1.1^{ab}	72.3 ± 6.3^{a}
pH	5.5 ± 0.1	8.1 ± 0.1^{a}	8.3 ± 0.2^{a}	8.5 ± 0.2^{a}
Conductivity (mS/cm)	2.6 ± 0.1^{a}	3.0 ± 0.1	1.1 ± 0.1	2.6 ± 0.1^{a}
DRI_{24h} (g O_2 kg^{-1} DM h^{-1})	1.2 ± 0.1^{a}	0.7 ± 0.0	1.6 ± 0.2^{b}	1.4 ± 0.1^{ab}
AT4 (g O_2 kg^{-1} DM)	39.9 ± 2.4	50.1 ± 3.3	94.8 ± 4.0^{a}	91.1 ± 2.4^{a}

Data presented as mean values ± standard deviation (n = 3). Same letters indicate not significantly different parameters ($p > 0.05$) based on the Tukey test analysis

NA not available, *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

*Dry basis

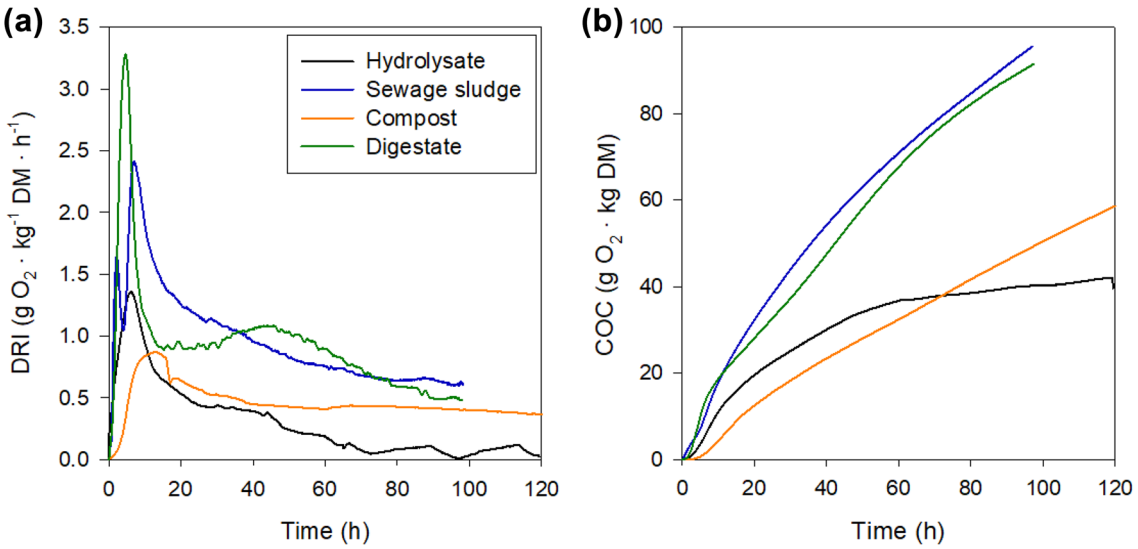

Fig. 3 **a** Dynamic respiration index (DRI) profiles and **b** cumulative oxygen consumption (COC) profiles, for the solid hydrolysate and the cosubstrates evaluated

values to those obtained in a study performed 5 years ago from the same treatment plant [40]. Montejo et al. [49] evaluated 30 compost samples from 10 MSW treatment plants and reported average values of 22 ± 9, 45 ± 8 and 7.6 ± 0.4 for MC, OM and pH respectively. Thus, comparable to the compost evaluated in this study (Table 2).

All three cosubstrates presented a slightly alkaline pH, around three units above the solid hydrolysate. These alkaline materials are well known for their buffering capacity [45, 50]. Digestate was the cosubstrate presenting the highest amount of OM, but there were no significant differences with sewage sludge. The measure of biodegradability is expressed through the DRI index and is an indirect measure of the biodegradable organic matter content [41]. The AT_4 is another useful respiration index that indicates the total oxygen consumed over a four-day period beyond the initial lag phase, which gives a deeper understanding of the sample's biodegradability [40]. For instance, both digestate and sewage sludge did not present significant differences with the hydrolysate in terms of DRI_{24h} but there was a striking difference in the AT_4 values. This might indicate that the hydrolysate contained easily accessible matter but was overall more exhausted. A better insight into the biodegradability potential can be seen in the respiration curves (Fig. 3). The DRI profile of the hydrolysate drops after the first 10 h, while the cosubstrates remain more active during that time leading to higher cumulative oxygen consumptions. Therefore, compost, digestate and sewage sludge represent an increase of 25.6, 128.3 and 137.6% in cumulative oxygen consumption, respectively.

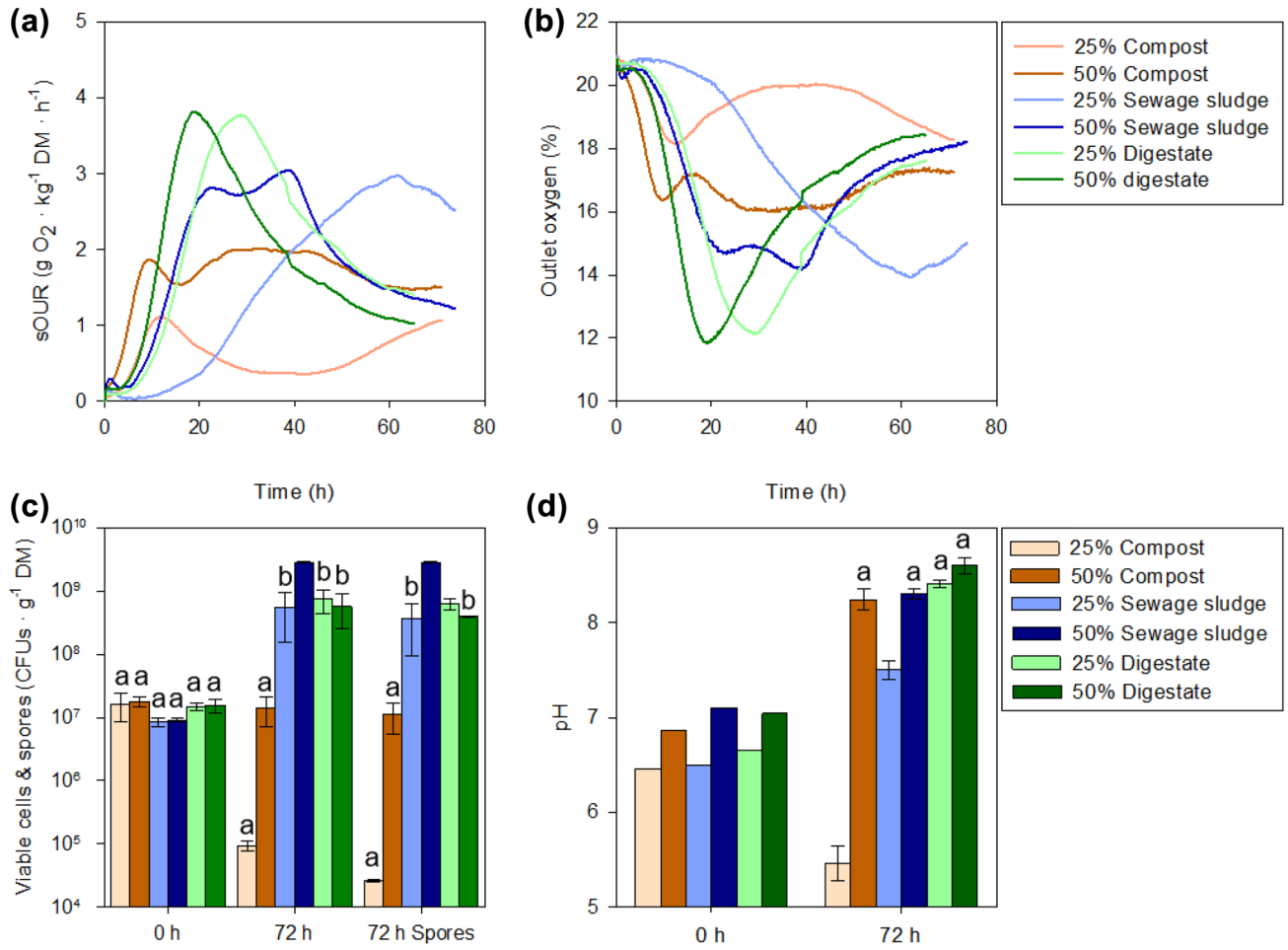

Fig. 4 Process parameters evolution at 0.5 L scale: **a** sOUR profile, **b** oxygen profile, **c** initial and final viable cells and final spore count (initial spore count was 0), and **d** initial and final pH. Same letters indicate no significantly different parameters for each group ($p > 0.05$) based on the Tukey test analysis

Table 4 SSF lag-phase and spores production at 0.5 and 1.5 L for the two different ratios of cosubstrates

Mixtures	Lag phase (h)		Sporulation (%)		Sporulation yield (spores/CFU inoculated)	
	0.5 L	1.5 L	0.5 L	1.5 L	0.5 L	1.5 L
25% Compost	5.0 ± 0.1	NA	28 ± 2	NA	0 ± 0	NA
50% Compost	3.2 ± 0.1	NA	78 ± 2	NA	0.6 ± 0.3	NA
25% Digested sewage sludge	25.1 ± 4.8	33.4 ± 4.9	66 ± 2	78 ± 10	27 ± 15	5 ± 1
50% Digested sewage sludge	10.0 ± 0.5	24.8 ± 0.4	100 ± 1	103 ± 9	196 ± 4	150 ± 13
25% Digestate	12.4 ± 0.9	17.5 ± 2.0	87 ± 19	73 ± 7	42 ± 7	44 ± 10
50% Digestate	8.5 ± 0.5	11.3 ± 0.9	82 ± 27	86 ± 11	27 ± 4	90 ± 7

The lag phase is calculated as the time it takes to reach 25% of the maximum sOUR. Data presented as mean values ± standard deviation (n = 2 at 0.5 L and n = 3 at 1.5 L)

NA not applicable

An initial evaluation of the effect of the selected cosubstrates on the SSF of the solid hydrolysate was performed by assessing them at two different wet weight ratios, 25 and 50%. The initial pH (Fig. 4d) for all the mixtures was above six and therefore, not inhibitory for Bti. The final pH, after 72 h of fermentation remained above six except for the mixture containing 25% of compost, which presented a pH of 5.5. Therefore, this mixture was not alkaline enough to surpass the initial pH drop of the fermentation, which led to a drastic reduction of viable cells at the end of the fermentation (Fig. 4c). In terms of oxygen consumption, the maximum values were presented by both mixtures with digestate and were four times higher than the minimum values, which were obtained for the 25% of compost. None of the fermentations reached oxygen-limiting conditions (Fig. 4b), the lowest oxygen concentration recorded was 12% achieved with a 50% of digestate mixture, as expected from the sOUR (Fig. 4a). From Fig. 4, it can be seen how compost was not an adequate cosubstrate. Even though the mixture with 50% presented a pH similar to that of other cosubstrates mixtures, it did not lead to Bti growth but only to sporulation at a yield of almost 1 spore CFU^{-1} inoculated with a sporulation percentage of 78%. A similar effect has been previously observed when using biowaste digestate as a substrate for growing Bt var *kurstaki* [26]. This may be explained by the low biodegradability of the sample, as this parameter has been shown to positively influence the growth of Bt species in SSF [22]. There were no significant differences in the growth of Bti in the other cosubstrates mixtures (Fig. 4c), except for 50% of sewage sludge which outperformed. This mixture also led to the highest production of spores (Table 4) which also surpassed previous studies that presented a production similar to the other mixtures (around 30 spores per CFU inoculated) [22].

At this point, it should be highlighted that the cosubstrates were not added sterile, as this would have not made sense in terms of process efficiency due to their nature. Instead, they were added after a thermal hygienization step, which is known to reduce the microbial population of digestate and sewage sludge but not completely, as they contain a great variety of microorganisms that arose from their respective anaerobic digestion processes [51, 52]. This implies that, with the use of this kind of cosubstrates, an autochthonous population of microorganisms is also being added to the solid matrix [22]. A direct effect can be seen in the considerable reduction of the lag phase (Table 4) observed for those mixtures with a higher amount of cosubstrates.

Process Verification and Temperature Evaluation

The increment in microbial activity caused by the use of anaerobically digested cosubstrates can in turn cause an increase in temperature as a consequence of higher metabolic heat production [53]. Besides, one of the major challenges when scaling-up SSF processes is the intense heat generation and its inefficient removal, alongside mass transfer issues [20, 48]. Therefore, for the next experiment, we decided to observe the evolution of temperature during the fermentation course on a three-times increased scale (1.5 L) under uncontrolled temperature conditions. To do so, triplicate reactors for each condition were incubated at room temperature (23 °C ± 2) and sensors were distributed inside the solid matrix to monitor changes. For this experiment, compost was discarded as a cosubstrate due to its poor performance at 0.5 L. This experiment was also conducted to study the reproducibility of the process on a different batch of OFMSW and subsequent enzymatic hydrolysis runs.

As can be seen in Fig. 5a, the maximum sOUR achieved remained consistent with those obtained at 0.5 L (Fig. 4a), which makes sense considering that the airflow supplied per gram of DM was maintained between scales. However, the profiles of the mixtures using sewage sludge changed at 1.5 L presenting narrower peaks that may be explained by different microbial profiles as a consequence of uncontrolled temperature. In terms of Bti growth and sporulation (Fig. 5c), 50% sewage sludge still appeared as the best-performing mixture but by a narrower margin and was not significantly different from the mixture with 50% of digestate in terms of final concentration of viable cells. The sporulation achieved was also 100% and the yield dropped to 150 compared with the 0.5 L scale (Table 4). The 25% of sewage sludge mixture also showed lower performance in comparison with the 0.5 L scale. It led to poor growth of Bti, which was significantly lower than the other mixtures using digestate. This can be explained by the relatively low pH achieved at the end of the fermentation (Fig. 5d). Contrarily, the mixtures using digestate increased their performance concerning the 0.5 L scale. Therefore, sewage sludge as a cosubstrate appeared to be more affected by the scale-up than digestate. This may be explained because digestate has higher alkalinity [54] than sewage sludge [55] and thus prevents more effectively the pH drop at the early stages of the fermentation promoting the development of Bti.

Regarding the average temperature profiles within the packed bed reactors (Fig. 4b), the maximum temperatures reached ranged from 34 °C for the mixture with 50% of digestate and 27 °C for 25% of digestate. Minimum temperatures (21 °C) were achieved by the 25% of sewage sludge, which corresponded to the longer lag phase observed (Table 4). Both mixtures with the higher ratio of cosubstrates increased faster their temperature reaching higher values, especially when using digestate. This can be explained because an increase in metabolic activity leads to an increase in temperature, which in turn stimulates microbial activity. This phenomenon can be observed by comparing how

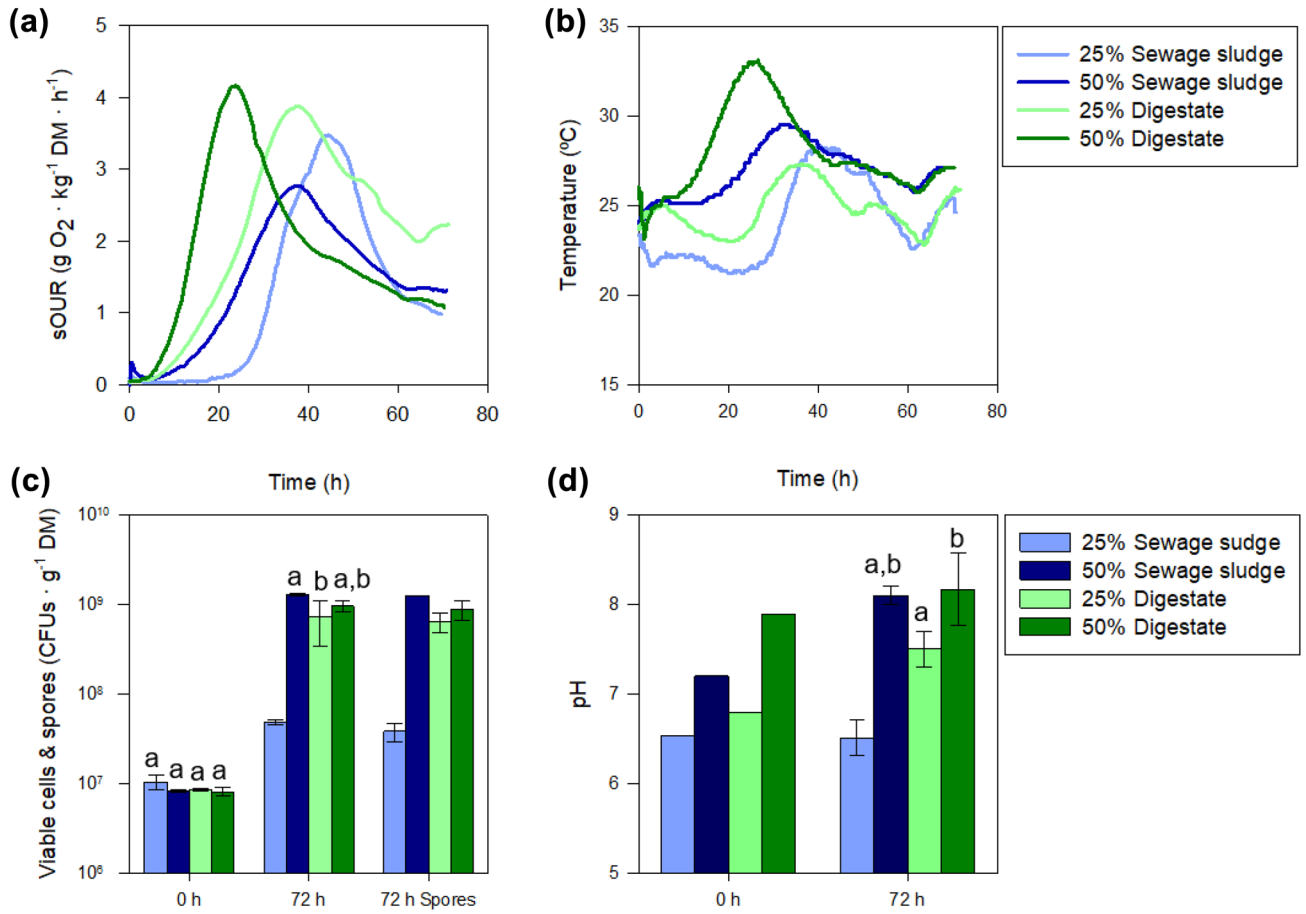

Fig. 5 Process parameters evolution at 1.5 L scale: **a** sOUR profile, **b** average temperature profile, **c** initial and final viable cells and final spore count (initial spore count was 0), and **d** initial and final pH. Same letters indicate no significantly different parameters for each group ($p > 0.05$) based on the Tukey test analysis

the maximum values for sOUR (Fig. 5a) and temperature (Fig. 5b) are obtained at the same time.

Except for 25% of sewage sludge, process robustness using a different batch of OFMSW has been demonstrated at a larger scale, which is of major importance considering the high variability of this type of waste [5]. Though 50% of sewage sludge reported the best results, both growth and sporulation yield were not reproducible and were lower than 0.5 L. It would be essential to evaluate if this downward trend continues as process scale-up does. It should also be given special attention to the use of a 50% digestate mixture for further scale-up because it led to the highest temperatures. It is well known that in SSF processes temperature increases as the scale does due to mass and heat transfer issues, especially for non-sterile substrates [20, 48]. This becomes even more relevant when the room or ambient temperature increase, for instance with seasonality. Therefore, for digestate, the 25% ratio appears as a lower-risk choice as it presented milder changes in temperature and still retained the pH above 7 (Fig. 5d) with a comparable sporulation yield (Table 4). Also, lower ratios of non-sterile cosubstrates imply lesser microbial load into the reactor and thus lesser competitive pressure microbial benefiting the growth of Bti. The final choice might also be influenced by a deeper characterization of the biopesticide activity because even though spore count is considered an indirect estimation method, it does not completely predict the endotoxic potential [56].

Overall, both digestate and sewage sludge, at a 25% and 50% ratio respectively, appeared as suitable cosubstrates for the SSF of an acidic hydrolysate. This gives flexibility to the process and the final choice would depend on the availability of each cosubstrate. From an urban solid waste biorefinery perspective, the use of digestate from the AD process of OFMSW makes more sense as it would lead to better process integration [6]. For instance, the higher quality OFMSW received into the treatment plant can be used for the enzymatic hydrolysis coupled with SSF and submerged fermentation for the liquid fraction while the OFMSW with less quality could be treated through AD. This leads to a multi-platform configuration that not only enhances the recovery

of resources but also provides the biorefinery with more flexibility and resources to adapt to energy requirements and price fluctuation [57, 58]. However, digested sewage sludge is a far more abundant material that in many cases requires treatment before its safe disposal, and therefore novel valorization pathways.

Conclusion

The use of alkaline cosubstrates has been implemented as a successful and reproducible strategy to overcome the pH drop during the SSF of *Bacillus thuringiensis* var. *israelensis* using solid hydrolysate of OFMSW as substrate. Two byproducts of urban waste treatments, digestate and digested sewage sludge, appeared as adequate cosubstrates providing the process with certain implementation flexibility. Spores concentration of 1.1×10^9 spores g^{-1} DM and 6.4×10^8 spores g^{-1} DM were obtained for sewage sludge and digestate respectively, which corresponds to yields of 112 and 48 spores per inoculated Bti cell. This work represents a further step in novel valorization options for the organic fraction of municipal solid waste, in line with a biorefinery scenario for waste management as the future requires. Future studies have to evaluate the biopesticide activity and safety of the final product, as well as its formulation.

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Author Contributions EMP, AS and AA contributed to the study conception and design. Material preparation, data collection and analysis were performed by EMP and NA. The first draft of the manuscript was written by EMP and AS and AA commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Article IV

Filling in the gaps in biowaste biorefineries: the use of solid hydrolysates for the production of biopesticides through solid-state fermentation

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In this final results section, the scale-up of the developed process is performed. A 22 L reactor is used for assessing the scaling-up effects on the process using two different cosubstrates. Furthermore, a deeper evaluation of the final fermented solid is presented together with an overall mass balance for the biorefinery scheme proposed in this thesis.

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Filling in the gaps in biowaste biorefineries: the use of solid hydrolysates for the production of biopesticides trough solid state fermentation

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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 hydrolysate 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.

Keywords

Biorefinery; Biowaste; Biopesticide; Enzymatic hydrolysis; Solid-state fermentation

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 scaling up the process due to heat and mass transfer limitations. At large scale, the accumulation of heat and the 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 less than 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 OFMSW hydrolysates 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 OFMSW hydrolysates may hinder Bt growth and sporulation because this microorganism thrives at pH near neutrality. Moreover, pH tends to acidify at 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 residual solid fraction of OFMSW enzymatic hydrolysis. 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

113 quality with a level of impurities lower than 1%. After collection, bags were opened
114 manually and screened for inert materials (plastic, metal, glass or textile). Also, excess
115 paper, hard shells, hair and bones were removed. Then, around 12 kg of OFMSW were
116 shredded mechanically using a home composting shredder (Tecoinsaen SL, Spain),
117 homogenized and stored at -20ºC for a maximum period of three months.
118 As cosubstrates of the SSF process, two types of digested materials were used:
119 (i) digestate from a source selected OFMSW (DOF) treatment plant (Granollers,
120 Barcelona), which was obtained from a mesophilic wet anaerobic digestion process
121 followed by a solid-liquid separation using a screw press, and (ii) digested sewage
122 sludge (DSS) from a municipal wastewater treatment plant (Sabadell, Barcelona), which
123 was obtained from a mesophilic wet anaerobic digestion followed by a solid-liquid
124 separation using a centrifuge. They were stored at -20ºC for a maximum period of three
125 months. Before use, both materials were defrosted and subjected to a hygienization step
126 to pasteurize them as specified in the European Regulation Nº 142/2011., Materials
127 were kept at 70ºC for 1 h using a previously heated oven and covering them to prevent
128 moisture losses. They were stored in the fridge (5ºC) until their use for less than 24 h.
129 Raw materials were characterized upon arrival at our facilities (**Table 1)**.
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136 **Table 1.** Characterizations of the OFMSW, the digested materials and the enzymatic
137 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

138 *dry basis. OFMSW, organic fraction of municipal solid waste. MC, moisture content.
139 DM, dry matter. OM, organic matter. RS, reducing sugars. DRI_{24h}, dynamic respiration
140 index average in the 24 h of maximum activity. AT_4, cumulative oxygen consumption
141 during the 4 days after the lag phase. NM, not measured. [a]Data presented as mean
142 values ± standard deviation of the sample analysis. [b]Data presented as mean values ±
143 standard deviation of two independent hydrolysis samples.

144 **2.2. Enzymatic hydrolysis**

145 OFMSW samples were defrosted overnight at 5ºC and sterilized by autoclaving
146 at 121ºC for 30 min before use. The enzymatic hydrolysis step was conducted under

147 sterile conditions in 2 L Erlenmeyer flasks using an enzymatic cocktail supplied by
148 ASA Spezialenzyme GmbH (Wolfenbüttel, Germany), which was tailor-made for
149 OFMSW-based materials. Enzyme dosage was 0.05 mL of enzymatic cocktail per g of
150 initial dry matter according to manufacturer's instructions, the solid-to-liquid ratio was
151 set to 10% (w v^{-1}), and the initial pH to 4.5 using 0.05 M sodium citrate buffer. Flasks
152 were incubated at 50ºC and 180 rpm for 24 h. Then, hydrolysates were centrifuged at
153 6000 rpm for 15 min at 4ºC. Samples from both fractions were taken to measure sugar
154 content and the solid fraction was collected and stored at 5ºC until its use in the SSF for
155 a maximum of three days. The mass balance for the enzymatic hydrolysis is presented
156 in Section 3.4.

157 **2.3. Microbial strain and inoculum preparation**

158 *Bacillus thuringiensis* var *israelensis* (Bti) CECT 5904 was obtained from
159 "Colección Española de Cultivos Tipo" (Valencia, Spain) and preserved at -80ºC using
160 a seed lot system in cryo-pearls (DeltaLab, Barcelona). Inoculum preparation was
161 carried out according to the methodology presented by Mejias et al. (2020). Briefly, one
162 cryo-pearl was inoculated in 100 mL of sterile Nutrient Broth nº2 (Oxoid CM0067B)
163 and incubated at 130 rpm and 30ºC for 20 h, until an optical density of 2.5-3.0 was
164 reached. Then, the culture was centrifuged for 10 min at 3500 rpm. The obtained pellet
165 was resuspended in 3 mL of the exhausted media and then, diluted 1:10 (v v^{-1}) to reach
166 approximately a concentration of 10^8 CFU mL^{-1}. No spores were detected at this point.

167 **2.4. Solid-state fermentation process**

168 Two SSF experiments were conducted in a 22L packed-bed bioreactor to
169 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 helical ribbon mixer, as detailed in the supplementary material (Figure S1) and in Martínez et al. (2018). The working volume was approximately 85% of the reactor capacity, corresponding to 4.5 kg of the final substrate mixture. This final mixture consisted of a 50% wet weight ratio of solid enzymatic hydrolysate and cosubstrate (digestate from OFMSW or digested sewage sludge), which was established as the necessary amount to retain an alkaline pH. Both materials were thoroughly mixed with 1 kg of sterile wood chips corresponding to a 1:2 volumetric ratio. The addition of wood chips as bulking agent is essential to provide porosity and ensure proper airflow and oxygen availability. The mixture was inoculated with 25 mL of diluted Bti inoculum per kg. 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} (27 ml h^{-1} g^{-1} of dry matter). The airflow goes first through a humidifier to saturate the air of 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 after mixing (10 min at 12 rpm). Last day, when the reactor was stopped, two additional sampling points at different heights were included to evaluate the material homogeneity.

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,

194 UK) connected to a custom-built data acquisition system (Arduino® based) as detailed
195 elsewhere (Mejias et al., 2017; Ponsá et al., 2010). Specific oxygen uptake ratio (sOUR)
196 was calculated according to:

197 $$sOUR = F \times \left(0.209 - y_{O_2}\right) \times \frac{P \times 32 \times 60 \times 1000^{a}}{R \times T \times DM \times 1000^{b}}$$

198 where, sOUR is the specific oxygen uptake rate (g O_2 kg^{-1} DM h^{-1}); F, airflow rate into
199 the reactor (mL min^{-1}); y_{O2}, oxygen molar fraction in the exhaust air (mol O_2 mol^{-1}); P,
200 the pressure of the system assumed constant at 101325 (Pa); 32.6, oxygen molecular
201 weight (g O_2 mol^{-1} O_2); 60, the conversion factor from minute to hour; 1000^{a},
202 conversion from ml to L; R, ideal gas constant (8310 Pa L K^{-1} mol^{-1}); T, the temperature
203 at which F is measured (K); DM, dry matter of solids in the reactor (g); 1000^{b},
204 conversion factor from g to mg.

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

208 Temperature is another indicator of microbial activity as a consequence of the
209 metabolic heat produced during microbial growth (Arora et al., 2018). This parameter
210 was online monitored in the lower half of the reactor bed employing a temperature
211 probe (Pt-100 sensors, Sensotrans) located in the bioreactor. Also, accurate temperature
212 profiles at different heights of the reactor bed were obtained using temperature sensors
213 (Maxim Integrated, U.S.). Sensors were placed at both the center of the packed bed (at
214 18 cm and 30 cm height) and the edges close to the basket wall (at 12 cm, 24 cm and 36
215 cm height). Room temperature was also monitored.

216 Specific Bti growth was monitored by measuring viable cells and spores. First,
217 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 80ºC for 10 min and then placing them into ice before plating (Mejias et al., 2020). All plates were incubated at 30ºC for 20 h and viable cells or spores were estimated in terms of colony-forming units (CFUs) 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:

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

2.5. Analytical methods

2.5.1. Sugar content

Reducing sugars content of the enzymatic hydrolysis fractions was quantified using the DNS method (Miller, 1959). The liquid fraction was centrifuged (10000 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.

2.5.3. Routine parameters

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

2.5.4. 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 an adhesive paper and dried for further sample metallization with gold.

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 ×20000.

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

263 the final fermented products of both cosubstrates were processed for DNA extraction
264 using the Soil DNA Isolation Plus Kit (Norgen Biotek, Canada). DNA extracts were
265 tested for concentration and quality using a NanoDrop spectrophotometer and used to
266 construct the corresponding genomic libraries by analyzing the variable regions V3-V4
267 of the prokaryotic 16S rRNA gene sequences, which gives 460 bp amplicons in a two-
268 round PCR protocol. First amplification was done with the specific primers with
269 overhang adapters attached that flanks regions of interest, forward
270 (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA
271 G) and reverse
272 (5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT
273 AATCC). Then, using a limited-cycle PCR, sequencing adapters and dual index
274 barcodes, Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA,
275 USA), were added to the amplicon for sequencing pooled together in the MiSeq
276 sequencer with the MiSeq® Reagent Kit v2 (500 cycles) MS-102-2003. Sequencing
277 analysis was carried out at the BaseSpace (Illumina, Inc, USA) with the 16S
278 Metagenomic App that performs taxonomic classification using a taxonomic database.
279 The algorithm used was a high-performance implementation of the Ribosomal Database
280 Project (RDP) Classifier described in Wang et al. (2007).
281 The alpha diversity indices of the different microbial communities were
282 obtained from the EzBioCloud microbiological research platform
283 (www.ezbiocloud.net), while the dendrogram and the Principal Component Analysis
284 were performed using the PAST software for statistical analysis of biological data
285 (PAST 4.05). The phylogenetic tree showing the evolutionary relationship of the
286 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.

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 OFMSW enzymatic hydrolysate in a packed-bed bioreactor of 22 L. As shown in **Fig. 1**., both cosubstrates increased Bti growth by an order of magnitude when compared to inoculated cells. Digested sewage sludge (DSS) (**Fig. 1a**) showed a slower increase in biological activity (measured as sOUR) than in digested OFMSW (DOF) (**Fig. 1b**). They reached maximum activity in 30 h and in 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 indicate 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 hours 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. 1**), 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} 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

311 spore production of 1.6×10^6 spores g^{-1} DM at a 0.5 L scale using an OFMSW solid
312 hydrolysate that had been chemically modified with NaOH to adjust the initial pH
313 (Molina-Peñate et al., 2022b). Therefore, the use of alkaline cosubstrates appears as a
314 more suitable strategy at a larger scale (x44 times) to ensure a pH near neutrality for Bti
315 growth and sporulation, while saving costs of chemical reagents. The use of OFMSW
316 hydrolysate appears beneficial for Bt production compared to using only non-sterile
317 digested OFMSW, which reported lower spores values ($2.8 \cdot \times 10^7$ spores g^{-1} DM) when
318 using Bt var *kurstaki* (Cerda et al., 2019). However, the mixture with non-hydrolyzed
319 biowaste reported slightly higher spore values (4×10^8 spores g^{-1} DM) using a two-step
320 aeration strategy (Mejias et al., 2020).
321 The pH of the solid hydrolysate was 5.3 (**Table 1**) and therefore, inhibitory for
322 Bti (Foda et al., 1985). The use of alkaline cosubstrates increased the pH of the SSF
323 mixtures to 7.5-8 (**Fig. 1**), which is in the optimal range for Bti growth and sporulation.
324 Their high buffering capacity (Karki et al., 2021) prevented the pH from reaching acidic
325 inhibitory values (**Fig. 1**), which are characteristic for OFMSW aerobic treatments
326 (Sundberg et al., 2004). For DSS the pH dropped to 6.3 after 24 h (**Fig. 1a**) in contrast
327 to DOF (**Fig. 1b**), which did not present a pH drop. However, considering the faster
328 development of the DOF fermentation process the pH drop might have occurred during
329 the first 24 h. The use of cosubstrates appeares as an effective alternative to keep pH
330 within safe values for Bti, contrary to the use of chemicals for pH control, which is
331 rather difficult for SSF processes, at large scales (Kumar et al., 2021).
332
333
334

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

Cosubstrate	Lag phase (h)	COC_{96h} (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.

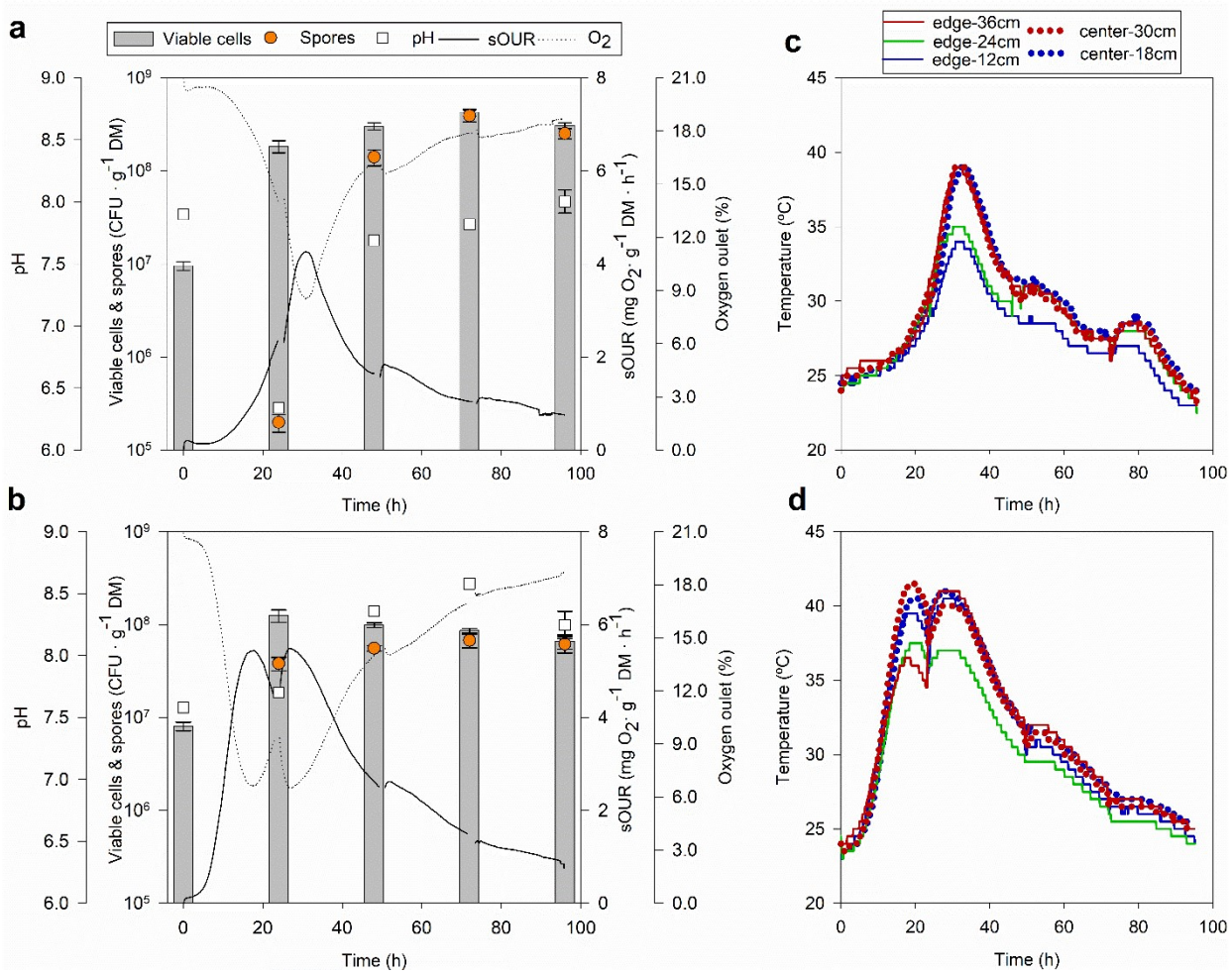

Fig. 1. Monitored parameters during 22 L SSF to produce Bti spores from OFMSW hydrolysate using cosubstrates and thermal behavior inside the respective packed-bed (a, c) digested sewage sludge and (b, d) digested OFMSW.

3.2. 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. 1 c, d**), indicating that the temperature rise was indeed caused by the metabolic heat generation of microbial growth. DOF reached slightly higher temperature values (42ºC) compared to DSS (39ºC) 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. 1 a, b**) despite the highest temperature. Even though this 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 60ºC (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 5ºC lower in the middle point for both scenarios (**Fig. 1d**).

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. (2013).

3.2. Verification of the presence 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. 2** shows SEM images of the pure culture and the fermented products using DSS and DOF. Spores are clearly visible in both SSF (**Fig. 2d** and **Fig. 2e**), 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. 2d**) 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. 2b**. In the fermented products, crystals showed a diameter of 0.7 ± 0.1 (N=50) for SSW (**Fig. 2c**) and 0.9 ± 0.1 (N=50) for SOF (**Fig. 2e**), with a smooth surface. During SEM analysis of the SSF products, vegetative cells (**Fig. 2a**) 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.

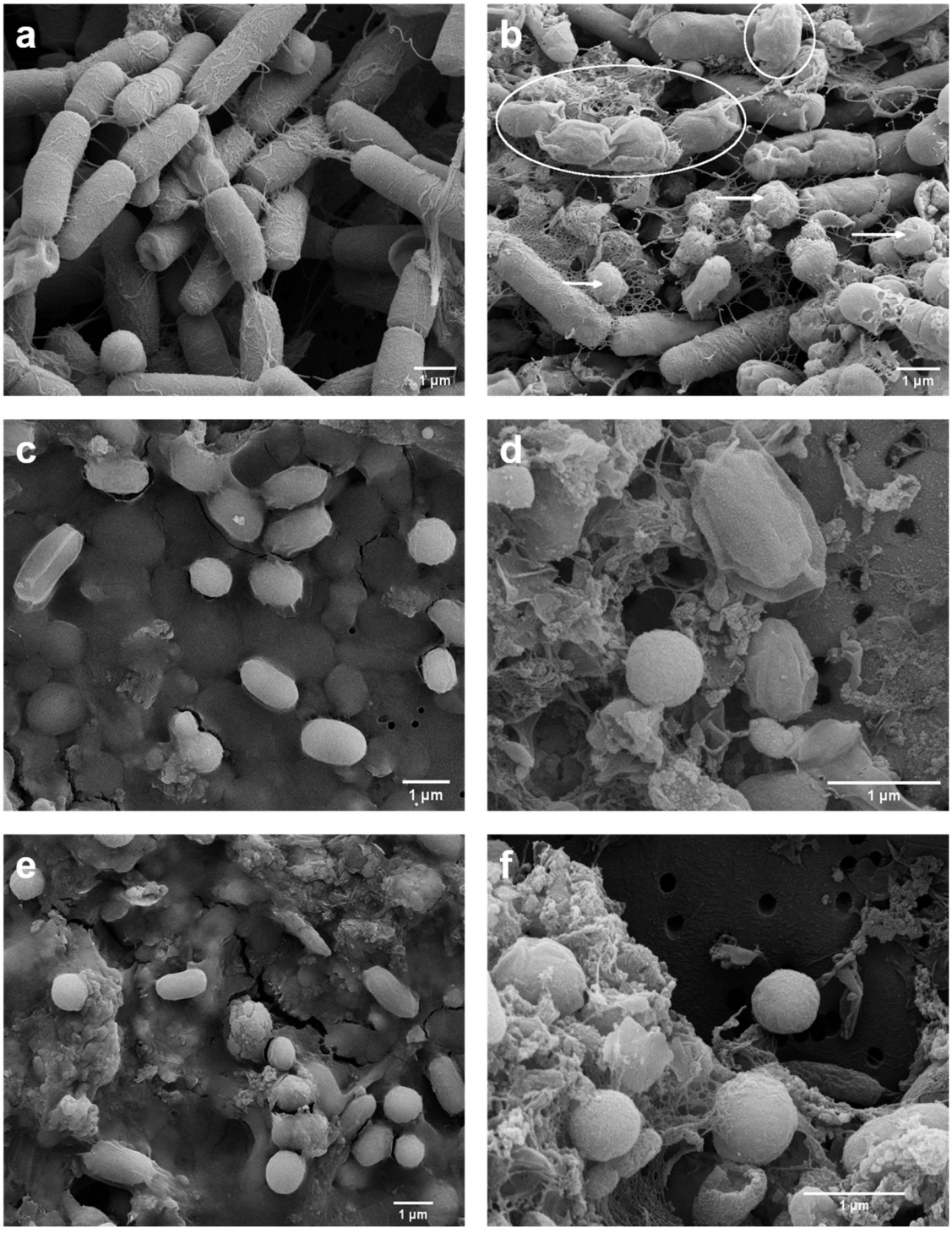

Fig. 2. SEM images of Bti pure culture (a) vegetative cells and (b) spores in circles and spherical crystals pointed with arrows; (c, d) 96 h SSF sample of digested sewage sludge as cosubstrate; (e, f) and 96 h SSF sample of digested OFMSW as a cosubstrate.

3.3. 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. 3a**) 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. 3b**). 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 enzymatic hydrolysate (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 hydrolysate.

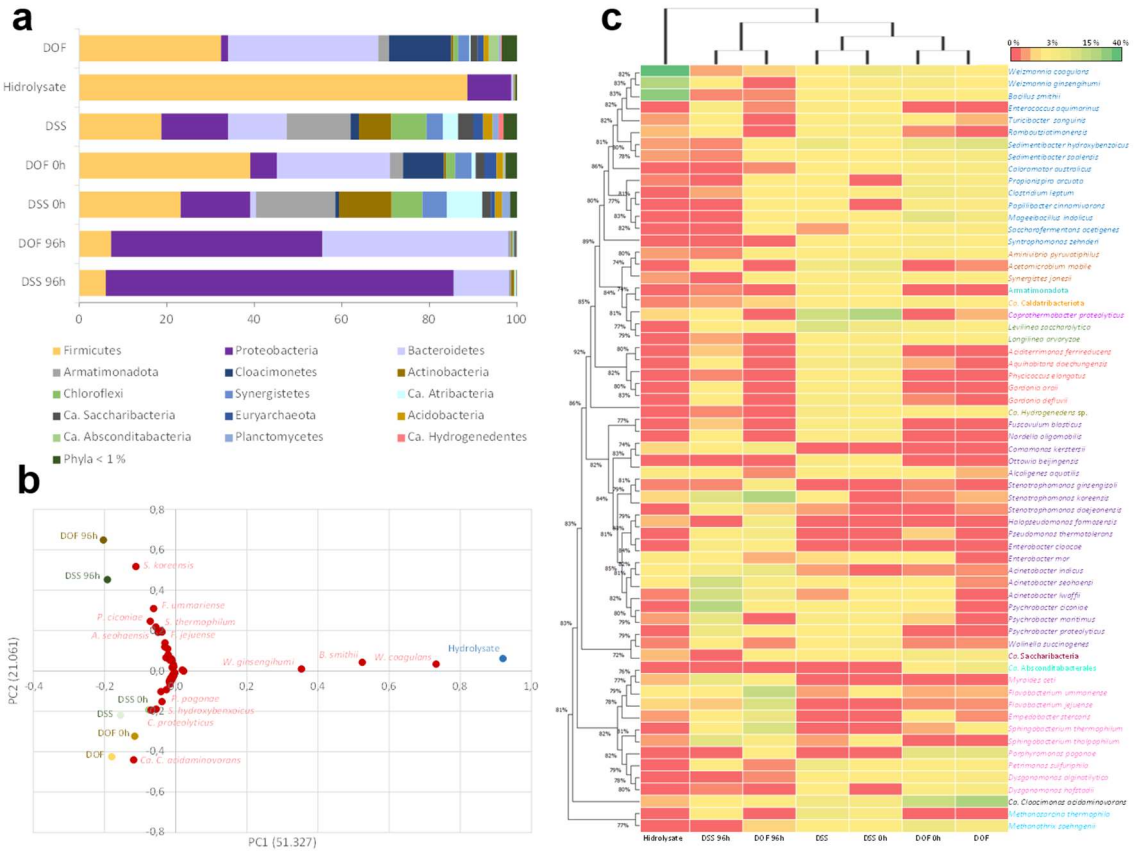

Fig. 3. Prokaryote community structure of the substrates and processes analyzed. a) Distribution by phyla expressed as a function of relative abundances. b) Principal Component Analysis. Biplot graph showing similarity between samples and relationship with dominant species (relative abundance < 1 % in 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.

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 hydrolysate, 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 hydrolysate in relation to its source material seems to be common.

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
Good´s 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.

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. 3b**) as it is clear that both processes, regardless of the starting 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. 3c**). 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 96h, *Psychrobacter* stood out, while *Flavobacterium* did the same at DOF 96h. 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 96h 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 hydrolysate 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 (50ºC, 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.

3.4. 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. 4**. 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.

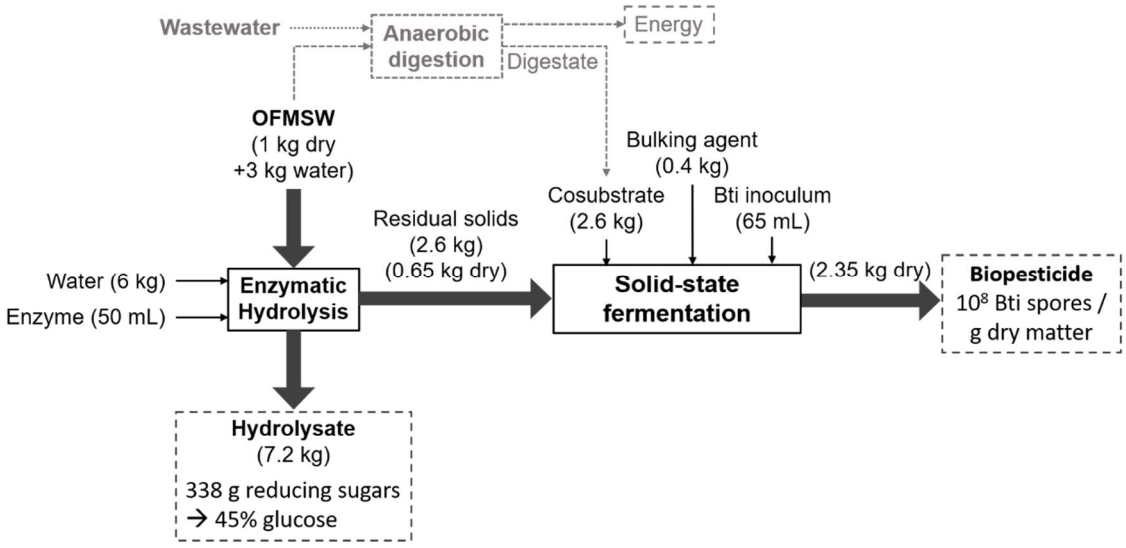

Fig. 4. Scheme and overall mass balance of the proposed OFMSW valorization route.

4. Conclusions

Bacillus thuringiensis var *israelensis* has been successfully grown on residual solids from 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 (<45ºC). 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.

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Supplementary material

Filling in the gaps in biowaste biorefineries: the use of solid hydrolysates for the production of biopesticides through solid-state fermentation

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Figure S1. (a) Experimental set-up of the 22L bioreactor and (b) Reactor's views: empty and loaded with material.

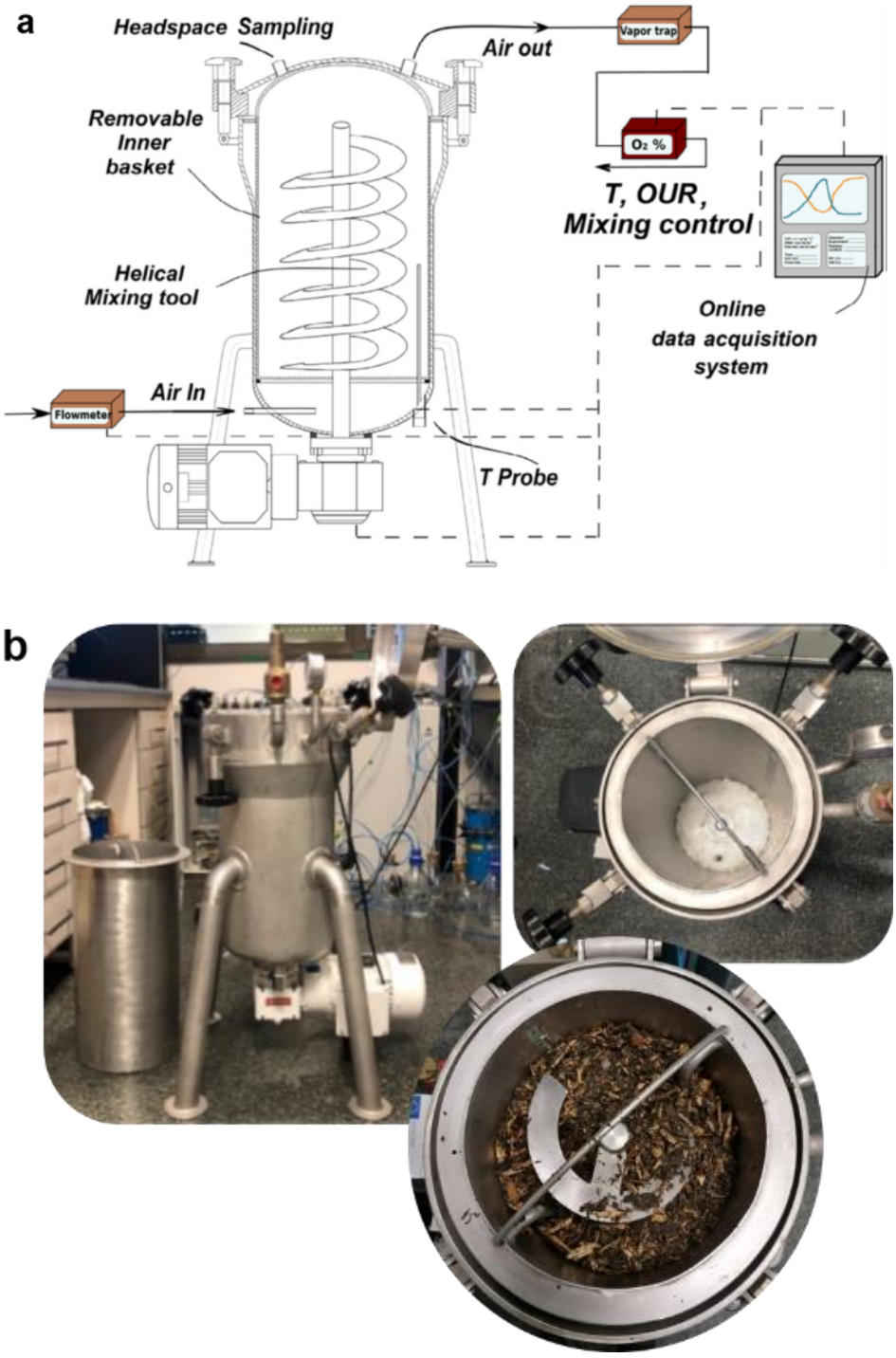

Table S1. SSF process parameters for each cosubstrate.

Cosubstrate	Initial pH	Final pH	Initial DM (%)	Final DM (%)	COC_{96h} (mg O_2 / g DM)
50% Digested sewage sludge	7.9	8.0 ± 0.1	41 ± 4	42.0 ± 0	140.6
50% Digested organic fraction	7.6	8.2 ± 0.1	38 ± 3	41 ± 3	244.8

Chapter 5 **Discussion**

The data obtained in Chapter 4 (Articles I, II, II and IV) are used in this section to elaborate a general perspective of the process. The challenges related to the use of OFMSW in a biorefinery-like scenario are presented and discussed. A preliminary economic evaluation is also included.

The SCALIBUR project was a highly multidisciplinary project that targeted different aspects of the value chain of urban biowaste. The work packages (**Table 5.1**) were divided by thematic areas including collection and characterization activities, social awareness activities, technical activities targeting three types of urban biowaste (OFMSW, HORECA and sewage sludge), communicative activities, and activities to evaluate the economy, quality and safety of the developed processes and their respective products.

Table 5.1 List of SCALIBUR work packages

WP number	WP title
WP1	Project management
WP2	Stakeholder engagement and social innovation actions
WP3	Collection, characterization and homogenization of urban biowaste
WP4	**Biochemical conversion of OFMSW into biodegradable polyesters and biopesticides**
WP5	Insects to valorize organic waste from HORECA
WP6	Bioconversion of sewage sludge and OFMSW through biochemical and bioelectrochemical routes
WP7	SCALIBUR interactive stakeholder platform
WP8	Environmental, techno-economic, social impact and safety assessment
WP9	Communication, dissemination and awareness raising
WP10	Exploitation and replication
WP11	Ethics requirements

WP, work package.

The SCALIBUR's biochemical conversion of the OFMSW (WP4) comprised partners from six different geographical locations (**Figure 5.2**). First, the OFMSW was separately collected in Madrid and homogenized. Then, it was sent to Navarra for the enzymatic hydrolysis process, which was performed with tailor-made enzymes designed and produced in Germany. The enzymatic hydrolysis was optimized and scaled up to a demo scale during the project. Also, the downstream process to concentrate the sugar hydrolysate and reduce viscosity was evaluated. Subsequently, the liquid hydrolysate was processed in Italy for the production of 1,4 butanediol, which was formulated into bio-polyesters and tested for packaging applications in

Valencia. In Navarra, the liquid fraction was also evaluated for the production of Bt-based biopesticides. Lastly, the solid fraction was sent to Barcelona (us) for the production of Bt-based biopesticides through SSF. In light of the difficulties with sample shipments and standby times, it was decided to reproduce the value chain in our facilities to ensure an abundance of solid hydrolysate to develop the SSF process.

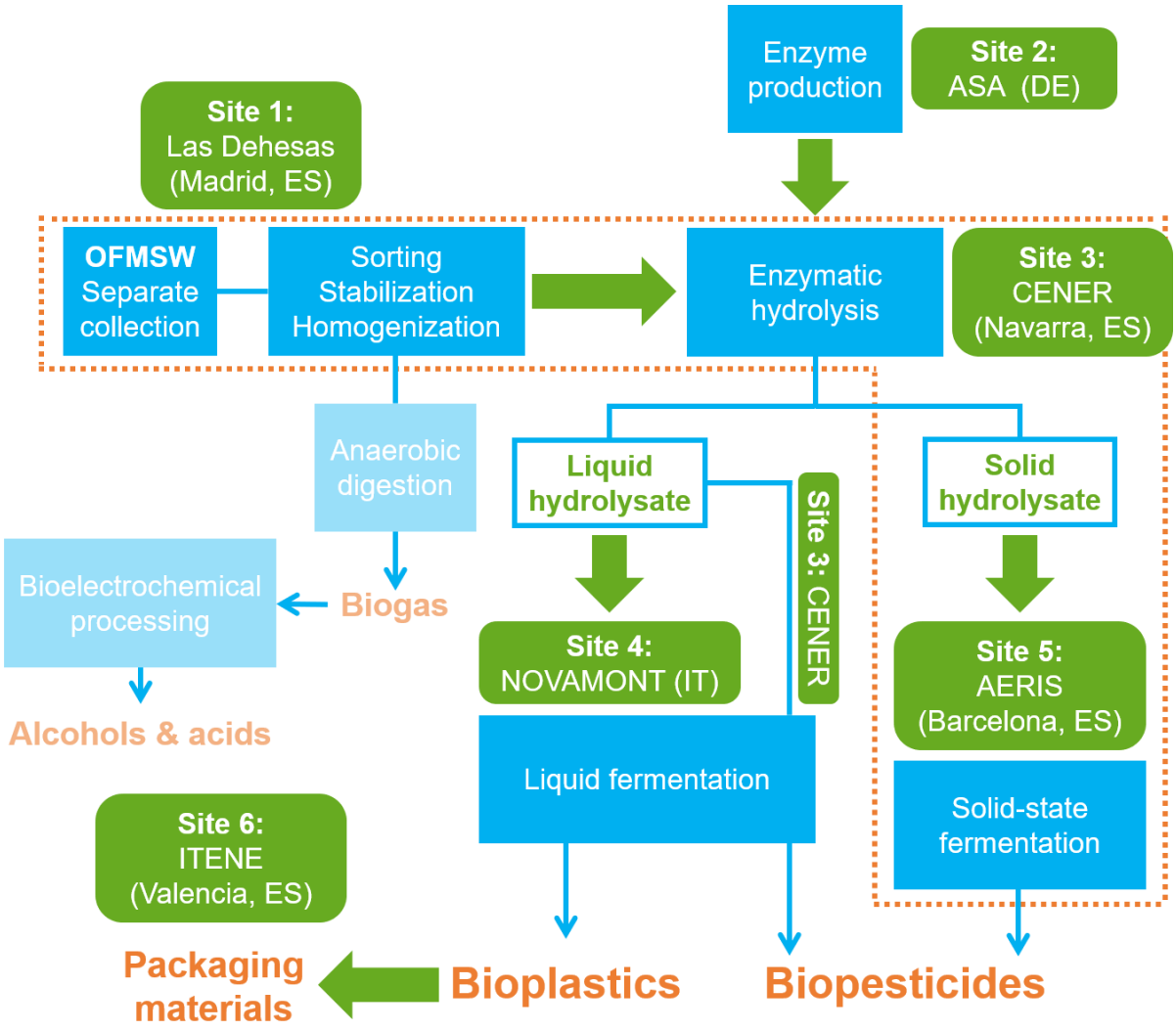

Figure 5.1 Value chain of the SCALIBUR project for the OFMSW fraction. Green arrows indicate material shipment. The dotted lines highlight the parts reproduced during this thesis.

During this thesis, the valorization pathway for OFMSW proposed in the SCALIBUR project has been reproduced and evaluated, focusing on the use of the solid hydrolysate by SSF to produce Bt-based biopesticides. The solid enzymatic hydrolysate produced in the SCALIBUR project was also evaluated for its use as a substrate of SSF. In the coming section, the challenges of each step from an overall perspective are discussed.

5.1 Challenges of using the OFMSW as a substrate for biotechnological processes: heterogeneity and variability

As mentioned in **Chapter 1**, the European compulsion to increase separate collection systems for MSW will lead to an increase in the availability and purity of the OFMSW. However, this fraction still possesses some intrinsic features that can challenge its use in refined biotechnological processes. In **Article I,** it is emphasized that the composition of OFMSW is heterogeneous and variable in contrast to other agricultural and industrial waste. The heterogeneity refers to the chemical diversity of its components, in terms of types of molecules, macromolecules and elementary composition. This complex chemical composition hinders the characterization of the OFMSW but also entails the great potential for developing biotechnological processes. The variability refers to the differences observed between the different OFMSW samples due to several regional, seasonal, technological and socioeconomic factors, which are compiled in **Figure 5.2**. Variability hinders the standardization of biotechnological processes, especially those highly sensitive to substrate variations. In terms of availability, the OFMSW is less affected than agricultural waste because its generation is less dependent on seasonality, except for municipalities with drastic changes in the number of citizens over the year, such as highly touristic places.

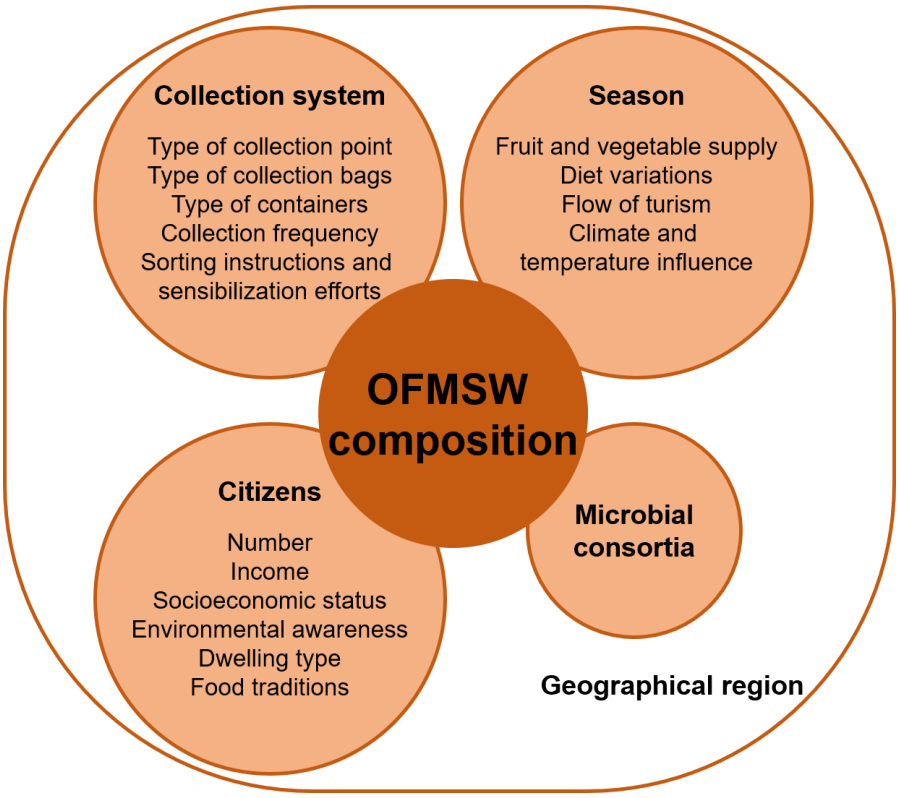

Figure 5.2 Main factors affecting the composition of the OFMSW.

Not all factors have the same significance. For instance, Hansen et al. (2007) statistically analyzed the chemical composition of 40 different samples of source-sorted OFMSW from five Danish cities and concluded that the collection system (bag types and sorting instructions) was the most influential parameter, followed by seasonality for some compositional characteristics, such as sulphur or ash content, whereas dwelling type had no significance. Puyuelo et al. (2013), observed that a compostable bag collection system was effective at improving the compostability of the OFMSW without a significant gaseous emissions increase. Another study on the composition of samples from 43 cities in 22 countries (Campuzano and González-Martínez, 2016) highlighted that the variability is specific for each characteristic. For instance, total phosphorous, sulphur, hemicellulose, free sugars or lignin variated among different samples more than carbon, hydrogen or humidity. Therefore, site-specific characterization and process adaptation to the waste particularities would favor process efficiency and product quality.

In this thesis, a total of eight samples from a door-to-door collection system were taken from the same solid waste treatment facility. Their characterization is presented in **Table 5.2**. Values are within the range of the previous examples and sugars and ashes also appeared as the parameters with the greatest variability, together with the biodegradability indices (DRI and AT_4). This waste has been characterized in terms of impurities or materials content by the Catalan agency of waste (ARC, 2016). For the period from June 2019 to October 2022, the average content was 1.1 ± 0.9% for 141 samples. These low values of impurities can be explained by the type of collection system. In comparison with mechanical separation, the source-separate collection has been shown to significantly improve the OM content of the OFMSW (López et al., 2010). Moreover, the MSW treatment plant of *Mancomunitat de La Plana* deals with OFMSW directly collected at generating households (door-to-door collection system) of small and medium-sized municipalities, which are both factors associated with improved OFMSW quality. For instance, in the composting plant of *Montcada i Reixac* (Ecoparc 2), which deals with OFMSW separately collected from communal collection points of a large municipality (Barcelona), the impurities average content for the same period was 17.1 ± 10.6% for 162 samples. Therefore, the origin of the OFMSW should be taken into account when considering its use in more refined biotechnological processes.

Table 5.2 Average characterization of all the OFMSW samples collected.

Parameter	Average	Range	% Deviation
[a] MC (%)	77 ± 3	73 - 81	4%
[a] DM (%)	23 ± 3	19 - 27	12%
[a] OM (%*)	89 ± 2	84 - 90	2%
[a] ash (%*)	11 ± 2	10-16	19%
[a] pH	5.4 ± 0.2	5.1 – 5.8	4%
[b] RS (%*)	18 ± 4	14 - 24	20%
[b] DRI_{24h} (g O_2 kg^{-1} DM h^{-1})	6 ± 2	4.2 – 8.6	35%
[b] AT_4 (g O_2 kg^{-1} DM)	260 ± 65	179-335	25%

*dry basis. [a] Data presented as mean values of n=8 samples. [b] Data presented as mean values of n=5 samples. 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.

Considering the inherent variability of OFMSW, the success of any biotechnological process for its valorization relies on its ability to tolerate slight substrate variations. Certain process features increase robustness, such as the presence of microbial communities (Stenuit and Agathos, 2015). The most widely implemented technologies for OFMSW recycling, composting and anaerobic digestion, involve heterogeneous microbial populations with diverse dynamics and complex interactions that are constantly challenged by fluctuating environmental conditions (Cerda et al., 2018). Though these technologies can tolerate variations in feedstock, substrate purity and stability still have an impact on the quality of compost and digestate for fertilizer applications, and on biogas potential (Cerda et al., 2018; Mata-Alvarez et al., 2014). In this line, during this work, it has been observed that the quality of the OFMSW impacts the quality of the resulting solid hydrolysate. A total of seven samples of SCALIBUR's solid hydrolysate were received As shown in **Table 5.3**, SCALIBUR's hydrolysate contains a considerably lower amount of OM than the OFMSW's of high quality (**Table 5.2**), which can be explained by the higher percentage of impurities in the feedstock (17-25%) ("SCALIBUR final conference," 2022), especially considering that many inert materials, such as glasses or metals, remain in the solid fraction after the hydrolysis. Lower amount of OM content is related to lower biodegradability (Barrena et al., 2011) and therefore, inferior performance as a

substrate of SSF processes. Furthermore, the final application of the product should be considered when evaluating the risk of low-quality OFMSW. As proposed later, a potential formulation for the final product is as a stabilized solid with biopesticide activity, and therefore, the presence of inorganic impurities could limit its application in soil. As well as, the presence of heavy metals or phytotoxic components (Cerda et al., 2018).

Table 5.3 Average characterization of solid hydrolysates produced from high-quality OFMSW using ASA's enzymatic cocktail and from the SCALIBUR project.

Parameter	High-quality OFMSW hydrolysate [a]	SCALIBUR hydrolysate [b]
MC (%)	75 ± 3	60 ± 4
DM (%)	25 ± 3	40 ± 4
OM (%*)	90 ± 2	67 ± 12
ash (%*)	10 ± 2	33 ± 11
pH	5.2 ± 0.1	6.0 ± 1.3
RS (%*)	1.2 ± 0.1	0.7 ± 0.1 [c]

*dry basis. [a] Data presented as mean values of n=5 samples. [b] Data presented as mean values of n=7 samples. [c] Data presented as mean values of n=2 samples. MC, moisture content. DM, dry matter. OM, organic matter. RS, reducing sugars.

5.2 Enzymatic hydrolysis: relevance of the enzymatic cocktail

As mentioned in **Article I**, the application of enzymatic hydrolysis to organic municipal waste has been studied to a lesser extent than as a pretreatment method of lignocellulosic biomass. The mixed sugars obtained have been used for fermentation into biofuels, mostly bioethanol, and other biomaterials (Choi et al., 2015; Sun and Cheng, 2002). The main factors affecting an enzymatic hydrolysis process are substrate composition and concentration, reaction conditions and enzymatic activity (Sun and Cheng, 2002).

First, the use of OFMSW as substrate involves the discussed challenges related to its composition (**Section 5.1**), specifically in relation to the bromatological properties, i.e. content in carbohydrates, proteins and fats (Campuzano and González-Martínez, 2016). The contents of starch and fibers (cellulose, hemicellulose and pectin) are of great relevance for the sugar extraction potential, as well as lignin, which hinders access to the polysaccharides. In **Article II** (Table 1), the content of cellulose plus hemicellulose was between 14-26% on a dry basis,

whereas lignin content remained below 8% for the two samples analyzed. These values are within the range reported by Campuzano and González-Martínez (2016) and lower than those for agricultural biomasses (Sun and Cheng, 2002). In contrast, OFMSW presents higher values of free sugars, starch and pectin (Hansen et al., 2007). Therefore, this fraction does not contain high amounts of recalcitrant materials facilitating its processing through enzymatic hydrolysis and reducing the need for complex pretreatments.

Reaction conditions were optimized in **Article II** for the commercial cocktail Viscozyme® L. Even though this cocktail is not specific for OFMSW, it includes a wide range of carbohydrases, including arabinase, cellulase, β-glucanase, hemicellulase, xylanase, and pectinase (Rodrigues et al., 2014), which were beneficial for the heterogenic composition of OFMSW. After optimization of the process, around 50 g L^{-1} of reducing sugars were obtained, which was comparable to other processes using OFMSW. For bioethanol production in a biorefinery, a concentration of at least 80 g L^{-1} is targeted for producing enough ethanol for a cost-effective distillation (Cheng et al., 2020). However, these biorefineries commonly employ agricultural waste with higher cellulose contents (up to 80%) than the OFMSW (Zhang et al., 2009). Therefore, detailed cost assessments are required to elucidate if the sugar production potential from OFMSW is enough to ensure process economic viability.

Lastly, concerning the enzymatic activity, the use of cocktails with different enzymatic activities is beneficial due to the synergistic effect (Guo et al., 2018). In this thesis, two different cocktails have been evaluated. The commercial cocktail Viscozyme L (**Article II**), for which the process was optimized as recently discussed, and a tailor-made cocktail for OFMSW developed within the SCALIBUR project by ASA Spezialenzyme GmbH (**Article III** and **Article IV**). ASA's cocktail included exo-cellulase, exo-hemicellulase, endo-cellulase, β-glucosidase, pectinase and α-amylase. The sugar profiles obtained with each cocktail after 24 h of hydrolysis were analyzed by HPLC (**Figure 5.3**). As can be seen in **Figure 5.3a** and **Figure 5.3b**, profiles were very similar for both cocktails, with glucose as the main sugar as expected (**Figure 5.3c**). This can be explained because both cocktails contained similar enzymatic activities, even though the specific proportions of each activity are unknown. The main advantage of Viscozyme L is the lower operating temperature, which involves lower energy expenses (**Article II**). Considering the variability of the OFMSW between regions, a universal tailor-made cocktail is not achievable, and therefore efforts should be put into versatile cocktails that could be then used under optimal reaction conditions.

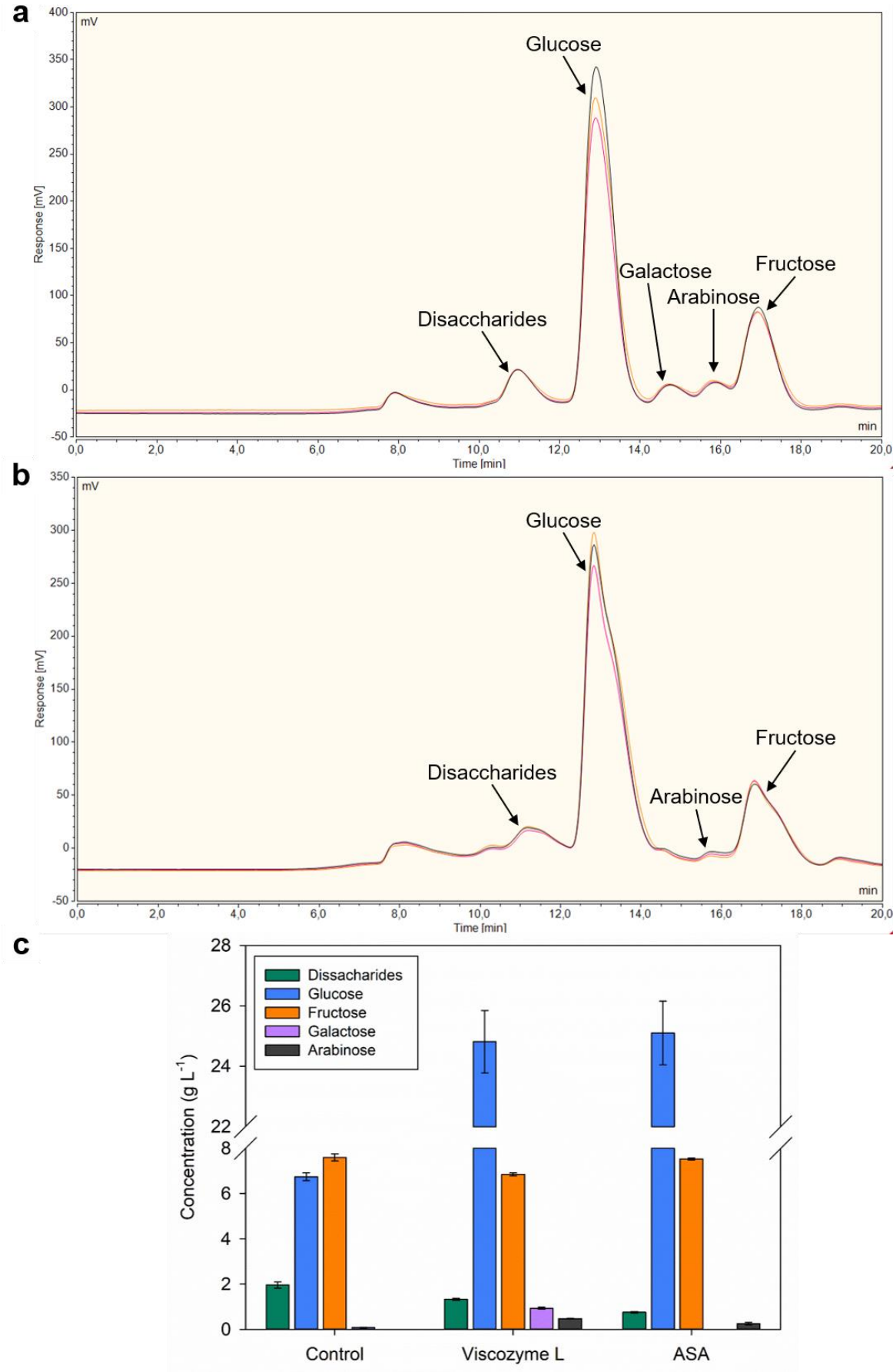

Figure 5.3 HPLC chromatograms for the liquid hydrolysates of (a) Viscozyme L and (b) ASA's cocktail, and (c) estimated concentrations for the monosaccharides identified.

The use of enzymatic hydrolysis to extract sugars reduces the impact of OFMSW's variability on the fermentation process. This compartmentalization enables the implementation of more sensitive processes. For instance, those using pure cultures for the production of specific high-value bioproducts.

5.3 Solid-state fermentation: overcoming the exhausted hydrolysate acidic nature

The production of Bt through SSF using organic waste has been previously evaluated in the GICOM research group. Even though enzymatic hydrolysates have never been used as a substrate, Bt spores have been produced successfully on soy residues, OFMSW and digestate from the AD process of OFMSW (Ballardo, 2016; Mejias, 2020; Rodríguez, 2019). Digestate and enzymatic hydrolysates have certain resemblances as both come from OFMSW that has been already treated, although the extension of both treatments is quite different. Both materials present low biodegradability (**Article III**), which has been defined as a significant parameter for Bt growth in SSF (Mejias et al., 2020). However, digestate is a material with high buffering capacity and, generally, alkaline whereas solid hydrolysate is acidic due to the conditions at which the enzymatic hydrolysis occurs. As mentioned in **Chapter 1**, the substrate is one of the most influential factors in an SSF process. The characteristics of the substrate determine the need for bulking agents, moisture adjustment, nutrient supplementation or pH control.

For the solid hydrolysate, the addition of bulking agent was indispensable due to the low porosity and high moisture content. Porosity was modified from 38% to around 75% (corresponding to a bulk density of 0.7 and 0.35 kg L^{-1}) by the addition of two volumetric parts of bulking agent (18% in weight). This value was previously optimized by Mejias (2020). High porosity values have been reported to yield higher enzyme production due to increased oxygen availability and reduced shrinkage in packed-bed bioreactors (Perez et al., 2021).

It is important to highlight that the process does not take place under completely sterile conditions, and therefore, the presence of other microorganisms can be expected. Especially, when using cosubstrates with an indigenous microbial community, such as digestates from AD processes. Given that we are working with MSW, this represents an advantage for process implementation in real scenarios. However, it does pose some risk since these microorganisms can compete with Bt and overcome it under unfavorable conditions. During this work, it was observed that if Bt was not growing within the first 48h, other microorganisms would colonize the substrate. Remarkably, in most of the unsuccessful fermentation, fungal contaminations

could be seen macroscopically after 72h. During the microbial analysis of samples at a 22 L scale (**Article IV**), only five species of the dominant population had a biosafety level of two instead of one. These were *Enterobacter cloacae*, *Acinetobacter seohaensi*, *Acinetobacter lwoffii*, *Sphingobacterium thermophilum* and *Sphingobacterium thalpophilum*. They were only present in the initial samples and non-hygienized samples but not in the final products. Also, regulated pathogens (*Escherichia coli* and *Salmonella*) were not found in the final samples (relative abundance > 0.5%).

5.3.1 The pH challenge

The incompatibility between the acidic pH of the enzymatic hydrolysate and the optimal pH values near neutrality for Bt growth and sporulation has been the greatest challenge encountered in this work. This has been intensified by the difficulties in modifying and controlling the pH of solid substrates during SSF, as explained in **Chapter 1**. One outcome of the preliminary assessment (**Article II)** was that the poorer performance of Bt on the Viscozyme hydrolysate from harsher conditions was due to the lower pH. The pH tolerance of the Bt strain employed was evaluated in liquid media (**Figure 3.6b**) and complete growth inhibition was observed for initial pH lower than 5. At first, it was attempted to modify the pH of the solid hydrolysate with different chemicals. In **Article II** a strong base (sodium hydroxide, NaOH) was used for Viscozyme hydrolysate, whereas in **Article III** a carbonate buffer (calcium carbonate, $CaCO_3$) was employed for ASA hydrolysate. Despite that NaOH allowed for Bt growth on Viscozyme hydrolysate, both scenarios led to a spore production lower than the initial cells inoculated. Also, the use of chemicals for pH control involves several disadvantages. On the one hand, low volumes with high strengths are required to prevent drastic changes in the moisture content of the solid substrate. However, achieving proper homogenization by mixing small quantities with the bulk of the solid bed is difficult. On the other hand, the use of chemicals increases process complexity and operating expenses, and thus hinders process scale-up. The buffering capacity of some solid substrates and the use of urea instead of ammonium salts, when an additional nitrogen source is required, have been the most widespread pH control strategies in SSF systems (Lonsane et al., 1992).

Article III was focused on developing a reproducible strategy to maintain pH within adequate values for Bt growth. To change the pH and buffering capacity of the substrate, we decided to evaluate the use of cosubstrates. In this sense, digested materials from AD processes appeared as a viable and scalable option (**Article III** and **Article IV**). Digestate is an abundant biomaterial due to the proliferation of AD plants in the last decade, with almost 1900 biogas

plants in 2019 in Europe (EBA, 2021). This tendency is expected to continue with the EU's REPowerEU plan to reduce fossil fuel dependency (EC, 2022), which targets the production of 35 billion cubic meters of biomethane per year by 2030. Currently, digestates are used as an organic fertilizer, but the latest amendment to the EU fertilizer regulation tightens the quality requirements for specific raw materials and excludes as input materials sewage sludge and mixed MSW (ECN, 2022). Therefore, valorization pathways for digested materials are also a research area with increasing interest (Cerda et al., 2019).

To illustrate the effect of pH on the SSF performance, the initial pH has been plotted against the lag phase and the final pH against the final viable cell count for all the fermentations at 0.5 L presented in **Chapter 4** (**Figure 5.4**). In **Figure 5.4a** it can be seen that the lag phase is inversely related to the initial pH. Typical values for the lag phase of Bt growth in SSF are expected around 12 h (Mejias, 2020). The batches with the lowest pH values (2 and 4) correspond to the unmodified hydrolysates of Viscozyme under extreme conditions (**Article II**) and of ASA (**Article III**), both unsuccessful fermentations. The adequate pH must be maintained throughout the fermentation, and even though middle points were not always available, **Figure 5.4b** shows a linear correlation between the pH and the viable cell count after 72 h. Those batches that reached higher pH at 72 h showed higher viable cell count. The tendency of low pH and poor fermentation performance is clear, even though it should be highlighted that the correlations have been made using data from experiments with remarkably different conditions.

Another approach to the pH challenge could have been the use of microorganisms adapted to acidic pHs, such as the fungi *Trichoderma harzianum*, which has been previously studied as a microbial biopesticide producer in SSF systems (Sala et al., 2019). In this case, the target pest would have been different. Either way, process development from a laboratory scale is necessary to adapt the operational parameters and strategy for the specific combination substrate-microorganism and enhance the success of SSF scale-up.

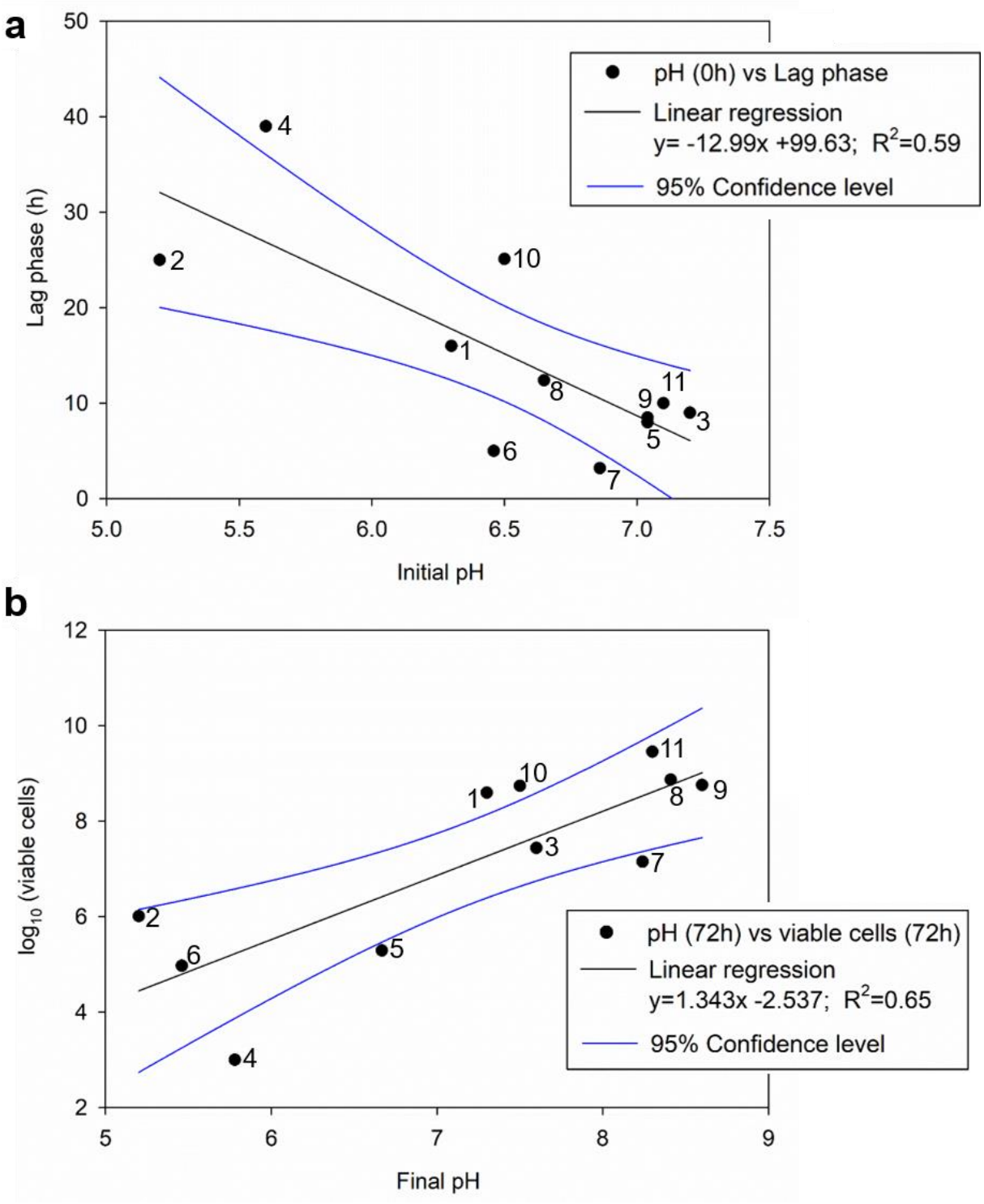

Figure 5.4 Correlation between (a) initial pH and lag phase duration and (b) final pH and viable cell count, of all the tests presented at 0.5L scale.1) Viscozyme, mild conditions; 2) Viscozyme, extreme conditions; 3) Viscozyme, extreme conditions with modified pH; 4) ASA; 5) ASA with modified pH; 6) ASA +25% compost; 7) ASA +50% compost; 8) ASA +25% digested OFMSW; 9) ASA +50% digested OFMSW; 10) ASA +25% digested sewage sludge; 11) ASA +50% digested sewage sludge.

5.3.2 Scale-up efficiency

One of the goals of the SCALIBUR project was to demonstrate the scalability of the novel valorization technologies proposed in the project. In this thesis (**Chapter 4**), the SSF process to valorize the residual solid hydrolysate has been developed at laboratory scale (**Article III**) and scaled up to 22 L for two different cosubstrates (**Article IV**), digested sewage sludge and digested OFMSW at a 50% wet weight content. It is important to highlight that few published studies evaluate the scalability of SSF processes, which remains the main challenge of this technology (Oiza et al., 2022). SSF experiments are mostly performed at a laboratory scale and in some cases using fewer than 10 g of the solid substrate. At such scales, most of the SSF heat and mass transfer phenomena are disregarded and therefore, productivity performance is not representative of what might happen at larger scales. This gains particular relevance when working with heterogeneous substrates such as OFMSW. In this sense, the quantity of solids used in sampling procedures is also relevant, since it has to be representative of the solid matrix.

In **Table 5.4**, the performance parameters for each scale are compared for the two cosubstrates. Regarding oxygen consumption, it can be seen that for both scenarios the maximum sOUR remained stable at the laboratory scale but not at the 22 L scale. Considering that the air supply per g of DM (27 mL h^{-1} g^{-1} DM) and the initial moisture content (around 64-67%) was maintained, it appeared that the scale-up led to increased microbial activity, which might be related to different temperature dynamics inside the bioreactor. Another effect of the scale was a decrease in spore concentration, which was reduced around an order of magnitude for both scenarios and led to a consequent reduction of the spore yield. Therefore, the higher microbial activity does not appear to be related to Bt but to other microorganisms present in the digested materials. However, in the 22 L reactor, the production was not as homogeneous, which reduced the representativeness of single samples (**Article IV**). It should be evaluated whether the tendency of spore decrease is maintained in further scale-ups or is stabilized as observed by Zhang et al. (2013) in a sterile system. The most stable parameter, without significant differences between scales, is the sporulation %. Therefore, sporulation metabolism of Bti did not appear to be affected by the scale effect, which is promising for future scale-up as long as the growth is also maintained. Despite the efficiency losses at 22 L, the results obtained for spore concentration and yields are in line with those reported for Bt in SSF (**Table 1.2**). However, more experimentation at this pre-pilot scale would be necessary to properly adapt the process and standardize it for other substrates with similar characteristics.

Table 5.4 Performance parameters of the SSF processes performed at three different scales and comparison with the pilot test (100 L) using SCALIBUR's hydrolysate.

Mixtures	High-quality OFMSW hydrolysate with 50% Digested sewage sludge			High-quality OFMSW hydrolysate with 50% Digested OFMSW			SCALIBUR hydrolysate with 50% Digested sewage sludge
	0.5 L	1.5 L	22 L	0.5 L	1.5 L	22 L	100 L
Lag phase (h)	10.0 ±5	24.8 ±2	18	8.5 ±6[a]	11.3 ±8[a]	8[a]	50
sOUR max ($g\ O_2\ kg^{-1}\ DM\ h^{-1}$)	3 ±3[a]	2.9 ±3[a]	4.3	3.8 ±5[a]	4.1 ±2[a]	5.5	2.3
Spore concentration ($CFU\ g^{-1}\ DM$)	3×10^9 ±2	1×10^9 ±20	3×10^8	4×10^8 ±1	9×10^8 ±3	7×10^7	1×10^7
Sporulation (%)	100 ±1[a]	103 ±9[a]	92[a]	82 ±33[a]	86 ±13[a]	79[a]	NM
Sporulation yield (spores/CFU inoculated)	196 ±2	150 ±9	44	27 ±15[a]	90 ±8	8[a]	4

The lag phase is calculated as the time it takes to reach 25% of the maximum sOUR. Spore concentration, % of sporulation and yield are given for 72 h of processing. NM, not measured. Data presented as mean values ± % of standard deviation (n=2 at 0.5 L and n=3 at 1.5 L). [a] not significantly different parameter between scales for each cosubstrate ($p>0.05$) based on the Tukey test analysis.

To validate the strategy developed at a larger scale, the hydrolysate material of the SCALIBUR project was used (obtaining the amounts of hydrolysate from the high-quality OFMSW needed for a 100 L reactor was highly labor-intensive for the equipment available, i.e. hydrolysis vessels and centrifuge). Digested sewage sludge was selected as cosubstrate because it presented better results in the 22 L scale (**Table 5.4**). A 100 L bioreactor with the same configuration as the 22-L reactor was employed (Rodríguez et al., 2019). It was loaded with a total amount of 21 kg of solids, composed of 8 kg of solid hydrolysate, 8 kg of hygienized digested sewage sludge and 5 kg of wood chips (1:2.5 volumetric ratio). The mixture was inoculated with 0.5 L of diluted Bti inoculum and the airflow was set to 3.8 L min^{-1} to maintain approximately the same air supply as in previous scales (27 mL h^{-1} g^{-1} DM). Sampling was avoided until the oxygen consumption began to prevent process disturbances. A sample was taken at 72 h of processing from the upper part and three samples at different heights of the packed bed were taken when the reactor was dismantled at 120 h. Results are presented in **Figure 5.5**.

Before discussing the results, it should be highlighted that no successful fermentation (spore production higher than inoculated cells) at the laboratory scale was achieved in this thesis for SCALIBUR's hydrolysate. On the one hand, this hydrolysate material presented low quality, i.e. low organic matter content and high improper material content (**Table 5.3**), due to the characteristics of the OFMSW collection system of Madrid but also because of the longer standby times during storage and transportation. On the other hand, properties were highly variable due to upstream process variations, which hampered the SSF process development. For instance, the pH would vary from 4 to 7 in each batch depending on the hydrolysate processing.

In **Figure 5.5a** a considerably long lag phase can be observed, which was a preliminary indicator of poor Bt's performance. The maximum sOUR (**Table 5.4**) was lower than those observed for the high-quality hydrolysate. After the sOUR peaked, oxygen consumption remained constant for 40 h, indicating an active microbial community inside the reactor. At the beginning of the fermentation, the pH was 6, which was considerably lower than the initial pH at 22 L (7.9) for the high-quality hydrolysate, indicating a stronger acidity in SCALIBUR's hydrolysate. After 72 h, the pH remained at 6 and increased to almost 7 at the end of the fermentation. However, lower pH might have occurred at the early stages of the fermentation, when no samples were taken. Temperature (**Figure 5.5b**) reached 45ºC in the middle-low part of the reactor and 35 ºC in the upper part (the 60 cm sensor was not in contact with the solid

bed). So, maximum temperatures remained below critical temperatures for Bt according to Ballardo et al. (2016).

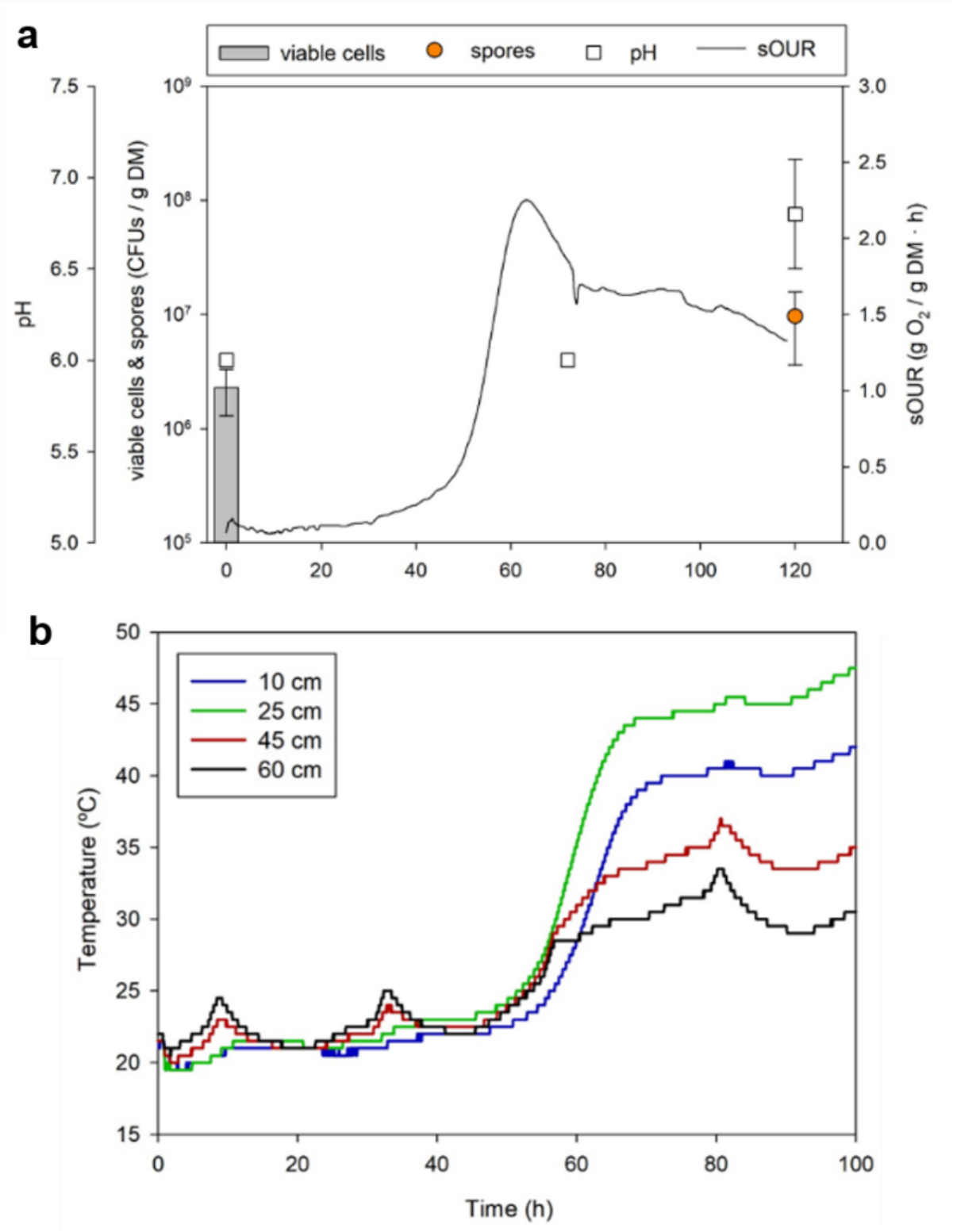

Figure 5.5 (a) Monitored parameters during 100 L SSF using SCALIBUR's hydrolysate and (b) the thermal behavior inside the packed bed at different heights from the bottom.

Regarding the growth of Bt and the efficiency of the process, it should be highlighted that it was difficult to follow because of the abundance of opportunist microorganisms, especially fungi, which covered the solid matrix in only 72 h of processing (**Figure 5.6**). Viable Bt cells could not be accurately distinguished on the Petri dishes, so only spores were monitored. Final spore production was on average 1×10^{7} spores g^{-1} DM, corresponding to a spore rate of 4 spores per initial viable cell. In light of the origin and complex value chain of SCALIBUR's hydrolysate, a decrease of one order of magnitude compared to the high-quality hydrolysate at 22 L is an acceptable result, given that the same decrease in efficiency was observed from the laboratory to the 22 L scale. However, excessive fungal growth might interfere with Bt biopesticide activity.

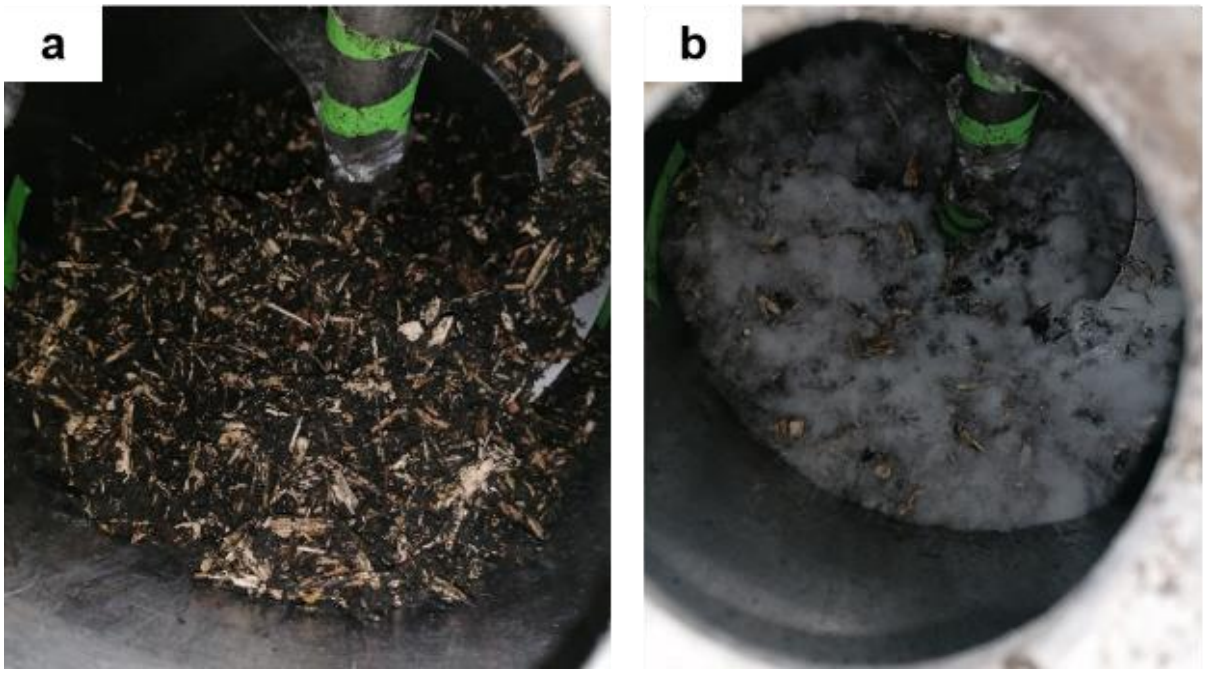

Figure 5.6 View inside the 100 L batch at (a) 0 h and (b) 72 h.

The study of Mejias (2020) evaluated the SSF process for Bt spore production using a similar substrate (digested OFMSW with raw OFMSW) on a larger scale (300 L). As in this work, the competitive advantage of Bt over other autochthonous or opportunist microorganisms was lost at the pilot scale. After six batches at such a representative scale under different operation conditions, Bt growth was not achieved and the spore concentration decreased two orders of magnitude in comparison with the previous scale (22 L). The maximum spore yield reported was 0.35 for Bt var *kurstaki* under limiting oxygen conditions at the early fermentation

stages. This example, alongside the work performed in this thesis, exemplifies the difficulties of scaling-up SSF processes, even when they are relatively simple.

Therefore, future research efforts should be focused on understanding the dynamic changes of Bt at a large scale, alongside that of the microbial community present in the substrate. A better comprehension of the scale-up effect on Bt and on the solid bed would enable standardization of the process for similar substrates.

5.4 Final product recovery

The downstream processing was out of the experimental scope of this thesis so, in this section, some considerations are given based on previous experiences in the GICOM group and from the literature.

First, it is noticeable the lack of research in this area, with few published documents about Bt-based biopesticide production on SSF systems and lesser addressing the downstream and formulation steps (Oiza et al., 2022). More studies can be found for the formulation of Bt-based biopesticides produced in SmF systems. The downstream process depends on the final formulation targeted, which can be divided between dry solid products, such as dust or granules, and liquid suspensions (Brar et al., 2006). The main objectives of formulation development are to ensure product stability during storage, to maintain product activity after field application, and to ease the handling and application of the product (Brar et al., 2006). The formulation should also consider the method of application, whether it is aerial, foliar or terrestrial. As a biological agent, Bt-based biopesticides are susceptible to temperature and UV radiation (De Oliveira et al., 2021). Therefore different strategies have been proposed to offer protection from environmental factors, such as the addition of adjuvants (Brar et al., 2006) or, more recently, encapsulation strategies (De Oliveira et al., 2021).

Most research using SSF for the production of Bt-based pesticides or other bioproducts, whether from municipal waste or different solid substrates, propose the extraction of the bioproduct (toxins, proteins and spores for Bt) from the fermented solid (de Carvalho Barros Cortes et al., 2022; El-Bendary et al., 2016). However, Catalán et al. (2019) showed in a life cycle analysis that in such a scenario for the extraction of cellulases, the downstream part was the main contributor to the environmental impact of the process due to the high energy associated with drying processes. So, if the bioproduct was to be extracted, minimal downstream processing should be performed to avoid reducing the environmental benefits of using waste materials as a substrate. El-Bendary et al. (2017) outlined a method for the

downstream processing of Bt toxins using tap-water extraction, air-drying, and a formulation with talcum powder that retained biopesticide activity for eight months. Instead of extracting the bioproduct, Ballardo et al. (2020) presented an interesting approach, which consisted on a solid formulation of the biopesticide product. Once the sporulation of Bt was achieved, the fermented product was stabilized into a compost-like enriched with Bt. This type of formulation could be used for soil applications, for instance, against root-knot nematodes (Abbasi et al., 2019).

Considering that the type of substrates used in this work are commonly employed in composting processes and that process simplicity has been prioritized at every step, a stabilized fermented solid with biopesticide activity would be an adequate formulation for our process. However, more research is needed to determine biopesticide activity and product stability in time. In this sense, Rodríguez (2019) evaluated the preservation and storage of Btk biopesticide (solid and liquid formulations) produced via SSF from digestate. After 30 days at different temperatures (5ºC and 25ºC), solid formulations preserved above 98% of initial spores for both temperatures whereas liquid formulations were more sensitive to temperature and extractive volume preserving around 60-100% of initial spores. Therefore, even though biopesticide activity should also be evaluated, Bt spores produced via SSF can maintain stability within the fermented solid. Furthermore, to be applied as a fertilizer, it should be ensured that the product is stable in terms of biodegradability and safe in terms of pathogens and heavy metal content. The specific evaluation should be performed to ensure that values are within regulatory limits for solid organic fertilizers according to EU regulation (2019/1009).

5.5 Evaluation of the proposed OFMSW biorefinery

The biorefinery-like management scenario for OFMSW proposed in the SCALIBUR project (**Figure 5.1**) and evaluated during this thesis, aims at obtaining several high-value bioproducts using enzymatic hydrolysis followed by liquid and solid fermentative processes. In **Article IV** (**Figure 4**) an overall mass balance for 1 kg of dry OFMSW is presented, but for the present discussion, the balance has been recalculated for 1 kg on a wet basis (average values) to ease comparison with other methodologies (**Figure 5.7**). Even though the mass balance presented is not exhaustive, it gives an initial overview of the potential of the biorefinery and, together with the data from the SCALIBUR project, enables an evaluation of the system.

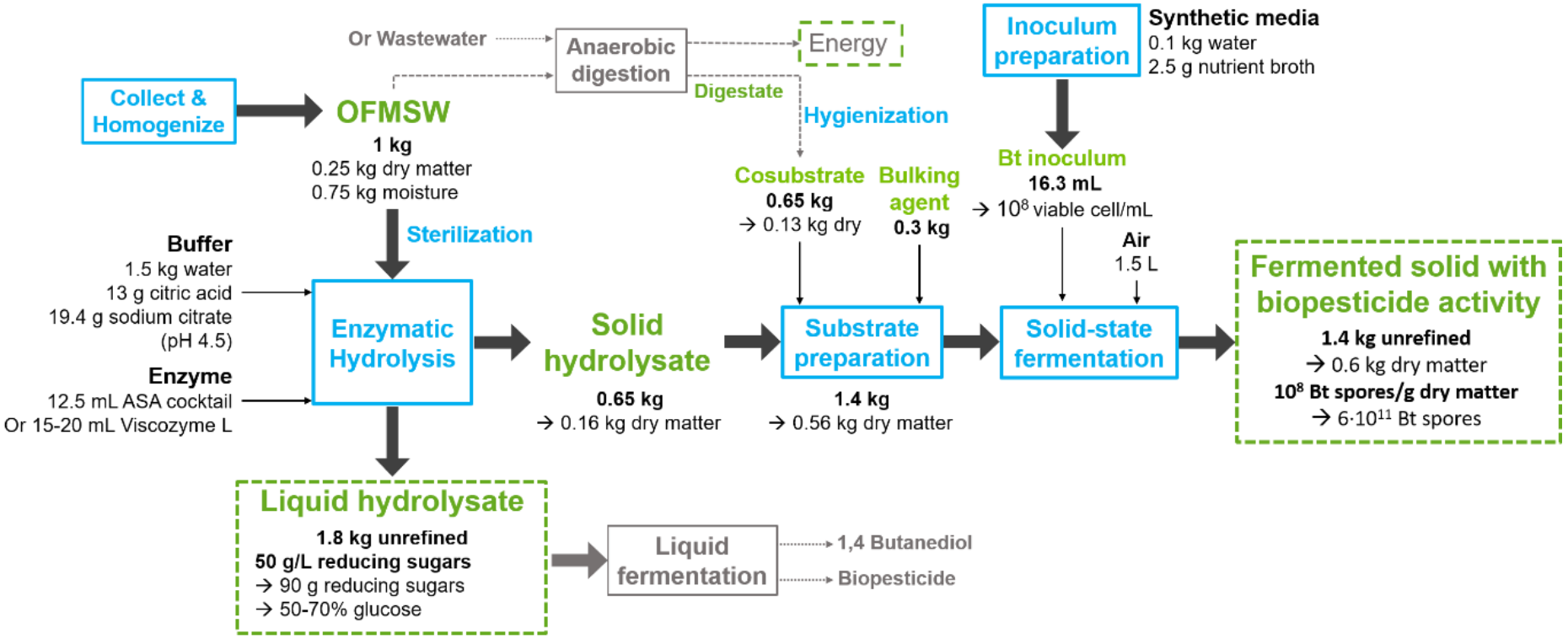

Figure 5.7 Overview of the proposed biorefinery for treating 1 kg of OFMSW. Grey areas are outside of the experimental scope of this thesis.

From 1 kg of OFMSW, 90 g of reducing sugars (or 360 per kg DM) can be obtained considering the highest production achieved in this thesis (**Article II**). Around 45-63 g are glucose (or 180-252 g per kg dry). Other recent studies using different types of waste in biorefinery scenarios have reported 335 g of glucose per kg of dry sugarcane bagasse (Dai et al., 2021), 310 g of glucose per kg dry of wheat straw (Liu et al., 2021) or 220 g of glucose per kg dry of cotton stalks (Christopher et al., 2017). In contrast to these examples, no pretreatment (other than autoclaving) has been used in this work, which involves economic advantages for the OFMSW biorefinery despite the lower glucose titers. Ebrahimian and Karimi, (2020), also used OFMSW to obtain fermentable sugars and produced 380 g of glucose after an acid pretreatment followed by enzymatic hydrolysis. It represents a 1.5-fold increase in glucose content compared with our highest value, but they consumed 2.5 to 4-fold more enzyme (211 mL) on top of an acid pretreatment.

Parallelly, 1.4 kg of fermented solid with a concentration of around 10^8 Bt spores per g DM is also produced. This value has been obtained from the highest scale available (22 L, **Article IV**) and assuming that no matter is lost during the SSF process or the recovery of the fermented product. It should be noted that in a real scenario, the bulking agent would be removed, and ideally reused as in a composting process. The results regarding the loss of competitive advantage of Bt at larger scales suggest that an autoclaving or hygienization step for this recovered bulking agent is recommended in order to prevent it from becoming a support for the accumulation of unwanted microorganisms.

5.5.1 Preliminary economic assessment

The main limitation of the industrial application of enzymes is their market price, which can contribute up to 25-30% to operational costs in lignocellulosic biorefineries (Guo et al., 2018). In this study, Viscozyme L was purchased at the price of 265 € per 250 mL (Merck KGaA, November 2020), which makes a cost of 883 € kg^{-1} of cocktail (density of 1.2 kg L^{-1}) (Rodrigues et al., 2014). This price is exorbitant as it is acquired as a fine chemical. For an industrial application, the economy of scale and a bulk formulation would reduce the price. The largest amount that has been found available for purchase is 30 kg per 874€ (Fischer Scientific, n.d.), i.e. 29 € kg^{-1}, a more reasonable amount and in line with Liu et al. (2016), who evaluated the cost of cellulases for industrial-scale ethanol production that ranged from 1.25 to 23.3 € kg^{-1} protein.

The optimum dosage (**Article II**) found during the optimization was between 0.06-0.08 L kg^{-1} of DM, depending on the reaction temperature, i.e. 15-20 mL of Viscozyme L for 1 kg of OFMSW (**Figure 5.7**). Therefore, an enzyme cost of roughly 0.54-0.72 € kg^{-1} of OFMSW is obtained, i.e. 540-720 € t^{-1}. For the same enzymatic cocktail, Zhang et al. (2016) estimated a price of 5 € kg^{-1} based on the hypothesis that developments in enzyme production will reduce the cost. In such a scenario the cost would be reduced to 90-120 € t^{-1}. The calculated price hardly competes with the current management alternatives, composting and anaerobic digestion technologies. Abad et al. (2019) presented a treatment cost of 100 € t^{-1} of OFMSW for an MSW treatment plant in Catalonia that processes 50,000 t of source-selected OFMSW per year producing 4,275,000 Nm^3 of biogas and 5000 t of compost. This was an exhaustive calculation including staff, wastewater treatment, cleaning or analysis cost among others. Therefore, even in the reduced-price scenario, only the cost of enzymes surpasses the entire value chain for actual treatment plants.

However, it should be highlighted that the products obtained in actual treatment plants (biogas and compost) have low economic value. The revenues that Abad et al. (2019) presented were 0.26 € t^{-1} of OFMSW from the sale of compost and 18.64 € t^{-1} from the sale of electricity, which aided in reducing the final production cost to 83.27 € t^{-1} of OFMSW. Nevertheless, the situation for electricity has drastically changed in the past year due to the energetic crisis in Europe. In Spain, the wholesale electricity price has increased from 0.062 € MWh^{-1} in January 2019 to 0.116 € MWh^{-1} in November 2022, peaking at 0.283 € MWh^{-1} on March 22 (Ember, 2016). This corresponds to a 1.9-fold and 4.6-fold increase respectively (a 7-fold increase for the European average price). In these circumstances, the production of electricity from OFMSW through AD becomes more profitable and an attractive scenario for ensuring future energy supply needs, as reflected in the previously mentioned REPowerEU plan (EC, 2022).

Then, for the proposed biorefinery to be economically viable, the benefits from the byproducts must justify the cost of enzymes. During the SCALIBUR project, the liquid fraction rich in sugars was explored as media for producing 1,4 butanediol and Bt-based biopesticides. For the former, the market price is $2.5 per kg (2.38€) (Satam et al., 2019), thus at least 0.3 kg has to be produced per kg of OFMSW to only cover the cost of Viscozyme (0.72 € kg^{-1}). Burgard et al. (2016) reached a yield of 0.35 g BDO per g of glucose with a genetically modified and optimized *E. coli* strain at a commercial scale. Assuming the same yield and that all of the obtained glucose after the enzymatic hydrolysis (63 g) can be used in the fermentation process, around 22 g of 1,4 BDO would be produced from our liquid hydrolysate. Therefore, not enough

to cover the cost of Viscozyme per kg of OFMSW. For the latter, the market price is $10 per kg (9.5€) (Kumar et al., 2019), thus 0.075 kg with effective biopesticide activity has to be produced per kg of OFMSW to cover the cost of Viscozyme. While the cost of enzymes would be allocated among all the bioproducts produced in the biorefinery (**Figure 5.7**), including the biopesticide produced by SSF and potentially the energy generated by AD, it clearly constitutes an economic restriction.

An alternative to explore for reducing the cost of enzymes is to integrate their production within the biorefinery system (Johnson, 2016). One of the partners of the SCALIBUR project (ASA Spezialenzyme GmbH) was responsible for the development of tailor-made and economic enzymatic cocktails for OFMSW. They declared a production cost of 12.5 € kg^{-1} cocktail ("SCALIBUR final conference," 2022), which is half the price of the recently estimated for Viscozyme L (29 € kg^{-1}). Considering that from ASA's cocktail we needed 0.05 L kg^{-1} of DM, that makes a cost per kg of treated OFMSW of 0.19 €. Therefore, almost a 4-fold decrease with regards to Viscozyme cost.

In this scenario, the use of SSF for the production of enzymes gains particular relevance as it is the most common application of this technology, especially by exploiting the enzyme battery of fungal species, which are the preferred microorganisms for SSF (El-Bakry et al., 2015; Sala et al., 2019). As seen in **Table 5.5**, different types of enzymes can be produced by SSF using waste as substrate. The inocula employed are mainly belonging to the fungal genus of *Aspergillus* or autochthonous microbiota of waste materials. The enzyme production on-site using waste materials involves several economic advantages (Reis et al., 2023). For instance, it simplifies the formulation because it eliminates the need for long-term storage and transportation. Also, it allows for the formulation to be adapted to the immediate application reducing downstream purification steps required for more sophisticated industries (food or pharmaceutical) (Reis et al., 2023).

Table 5.5 Examples of published works on SSF for enzyme production.

Reference	Enzyme type	Substrate	Inoculum
Llimós et al., 2022	Cellulases and xylanases	Brewer's spent grain	*Aspergillus niger*
Mejias et al., 2018	Cellulases and xylanases	Digested OFMSW	Native and *Trichoderma reesei*
Marín et al., 2019	Cellulases	Orange peel, apple pomace and rice fiber	Compost
Marín et al., 2018	Proteases	Soy fiber & cow hair	Native
Biz et al., 2016	Pectinases	Citrus waste	*Aspergillus oryzae*
Sahnoun et al., 2015	Amylases	Agro-industrial waste	*Aspergillus oryzae*
Veerabhadrappa et al., 2014	Lipases and proteases	Biodiesel byproduct	*Aspergillus versicolor*
Santis-Navarro et al., 2011	Lipases	Winterization residue	Wastewater sludge

5.5.2 Alternative biorefinery configuration

In this thesis, enzymatic hydrolysis has been explored as a methodology for extracting sugars from OFMSW (**Figure 5.7**), but it can also be applied as a pretreatment method before the AD to facilitate the hydrolysis of complex components, which is the first and rate-limiting step of the process (Mata-Alvarez et al., 2014). Martínez-Valdez et al. (2017) studied the implementation of an aerated phase prior to the AD to produce enzymes using the inherent microbiota of OFMSW (SSF-like). They reported a 20% net increase in methane production when it was co-digested with raw OFMSW. Therefore, the loss of organic matter during the SSF was compensated by the production of hydrolytic enzymes. Considering that biorefineries must be energetically self-sufficient and that the future perspectives for OFMSW management in Europe point towards an increase in AD plants for energy production, which is a more robust and well-established technology than SSF, another scenario for the OFMSW biorefinery is proposed in **Figure 5.8**.

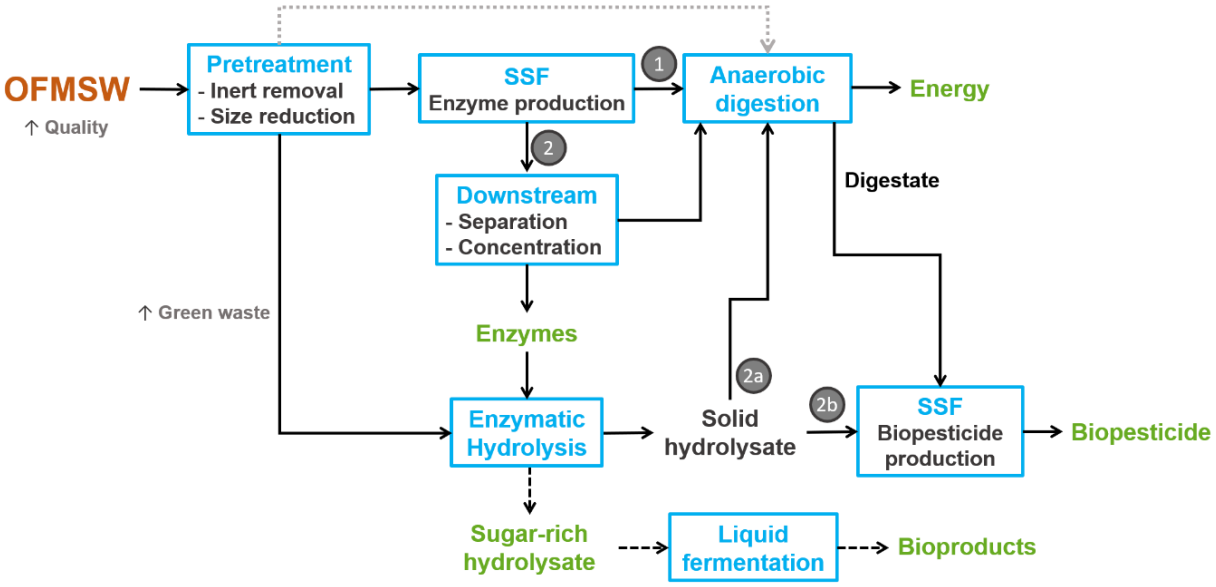

Figure 5.8 Diagram of the proposed biorefinery with on-site enzyme production. Circled numbers represent configuration alternatives. Discontinuous black lines represent liquid fractions. Grey line represents the current scenario.

In this case, AD technology takes the main role and energy production is prioritized. Enzymes produced through SSF can be applied either as a pretreatment to enhance methane content, i.e. energy potential (scenario 1), or when there is surplus energy, they can be recovered to perform the enzymatic hydrolysis for sugar extraction (scenario 2). Ideally, the collection circuits with higher lignocellulosic content (higher green waste content), which is more recalcitrant and difficult to digest in the AD process, would be redirected to the enzymatic hydrolysis. In this scenario, SSF can also be applied to valorize the solid fraction after the enzymatic hydrolysis and/or the digestate after the AD process into biopesticides. A deeper economic evaluation is needed to define the best configuration for an OFMSW biorefinery. For instance, a sensitivity analysis could assist in determining whether it is more profitable to process the solid hydrolysate via AD or SSF.

Overall, it can be said that SSF can be applied in MSW treatment plants as a complementary tool to the consolidated technologies to provide flexibility and higher-value bioproducts, rather than as a substitute. Nonetheless, to ensure the viability of the process, the quality of the collected OFMSW must be guaranteed in the first instance. To do so, an efficient logistic system in terms of collection, standby times or storage, need to be implemented.

Chapter 6 **Conclusions and future work**

In this chapter, the main conclusions of the articles from Chapter 4 are organized into topics together with those derived from the discussion of Chapter 5. Finally, future work is proposed.

Enzymatic hydrolysis of OFMSW

- Enzymatic hydrolysis was applied to obtain sugars from OFMSW using a commercial cocktail (Viscozyme L) at a laboratory scale. The process was optimized for temperature, pH and enzyme dosage, achieving a maximum of 50 g L^{-1} of reducing sugars in the liquid fraction and almost a 2-fold increase in total reducing sugars available.
- Viscozyme L performed properly under mild conditions (25ºC, pH 4.5 and 0.06 mL of cocktail per g DM), which is beneficial from an environmental and economic perspective.
- A comparison with a tailor-made cocktail from the SCALIBUR project, which included similar enzymatic activities at different rates, did not reveal significant differences in terms of sugar production.

SSF for the valorization of the exhausted solid fraction

- *Bacillus thuringiensis* var *israelensis* was grown successfully on the exhausted solid fraction after the enzymatic hydrolysis of OFMSW. It was negatively affected by acidic pH at the beginning or in the first hours of the fermentation course.
- A reproducible SSF operational strategy to overcome the challenge of pH incompatibility between the substrate and the microorganism was developed at a laboratory scale by using alkaline cosubstrates. Digested materials appeared more effective to keep pH within the neutral range than chemicals.
- The effect of the use of cosubstrates on process temperature was evaluated at a higher scale (1.5 L), achieving temperatures below 35ºC and a spore concentration of around 10^8 spores g^{-1} DM. The mixtures using 50% cosubstrates were more robust and reproducible, even though they also implied higher temperatures.
- The operational strategy to control pH was scaled up successfully to a 22 L reactor with a capacity for 4 kg of solid mixture. A considerable reduction in yield, in terms of spores production, was observed but results were similar or higher to those from the literature. The produced crystals and spores of Bt were identified by SEM imaging and the microbial population of the final product was analyzed.
- The process was also tested in a hydrolysate from low-quality OFMSW but reproducibility was not achieved. An attempt to scale up the process to a 100 L packed-bed bioreactor with a capacity of 21 kg of the solid mixture showed problems related to microbial contamination and heat accumulation.

General conclusions about the proposed biorefinery scheme

- An alternative scenario for the management and valorization of OFMSW was evaluated. Two bioproducts were obtained, a sugar-rich fraction with the potential for fermentative processes and a fermented solid with biopesticide activity. This option is complementary to existing and consolidated technologies.
- The quality of the OFMSW appeared as a limiting factor for the performance of the enzymatic hydrolysis, and especially, the successive SSF process due to the cumulative effect of inert materials in the solid residual fraction. Anyhow, the OFMSW biorefinery needs to tolerate variations in composition, inherent of this waste fraction.
- Enzymes were the cost-limiting factor and alternatives to reduce operational costs should be technically explored, especially those related to the production of hydrolytic enzymes from waste.

Future work

- A better understanding of the scale effect on the dynamics of Bt and the autochthonous microbial population of the digested substrates is required at 22 L to deeper understand the process and develop standardized strategies for similar substrates. In this line, more proportions of cosubstrates can be evaluated, to refine their amount and deeper evaluate their impact on the process performance.
- Other microorganisms more tolerant to acidic pH could be explored for the SSF process. However, it should be kept in mind that as an exhausted material, the use of cosubstrates may still be required.
- The final product formulation as a fertilizer with biopesticide activity should be developed and evaluated in terms of toxicity and storage to certify the produced biopesticide for market applications.
- Exhaustive mass balance and economic and environmental evaluations are necessary to get an accurate sustainability analysis, which can be used as a decision-making tool.
- The integration of enzyme production within the biorefinery using SSF technology should be technically and economically evaluated as an option to overcome the market price of enzymes.
- Lastly, the alternative uses of liquid hydrolysates should also be explored to gain an integral perspective on the developed biorefinery.

Chapter 7 **References**

Finally, all the references used in Chapters 1, 3 and 5 of this document are collected hereafter. The references of Chapter 4 are listed at the end of each section, corresponding to each published article.

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