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Report on the possibilities of digestate (and other wastes) as raw materials for SSF processes to obtain certain bioproducts

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

DECISIVE

A DECENTRALISED MANAGEMENT SCHEME FOR INNOVATIVE VALORISATION OF URBAN BIOWASTE

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A Decentralised Management Scheme for Innovative Valorisation of Urban Biowaste

D4.5 - Report on the possibilities of digestate (and other wastes) as raw materials for SSF processes to obtain certain bioproducts.

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ABSTRACT

This documents aims at providing a complete overview of the possibilities of valorisation of digestate and other wastes into bioproducts using the solid state fermentation technology. This report details the productivities of the process and the different optimization strategies for the improvement of the productive process.

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Table of contents

Glossary

DG, DE	stands for "Granollers Digestate", "Ecoparc Digestate"
AD	stands for “Anaerobic Digestion”
AP	stands for "Apple pomace"
BA	stands for “Bulking Agent”
BMP	stands for "Biochemical Methane Potential"
Bt	stands for "*Bacillus thuringiensis*"
Biowaste	Biodegradable garden and park waste, food and kitchen waste from households, restaurants, caterers and retail premises, and comparable waste from food processing plants
C/N	stands for "Carbon to Nitrogen ratio"
CC, SC	stands for "cell count" and "spore count”, respectively.
CFU	stands for "Colonia Forming Units"
COC	stands for "Cumulative Oxygen Consumption"
DGO	stands for "Granollers digestate and olive oil"
DGG	stands for "Granollers digestate and glucose"
DGGA	stands for "Granollers digestate, glucose and oleic acid"
DM	stands for “Dry Matter”, also called “Total Solid -TS”
FTIR	stands for "Fourier transform infrared spectroscopy"
FPU	stands for "Filter Paper activity Unit"
FW	stands for "Food Waste"
OFMSW	stands for "Organic Fraction of Municipal Solid Wastes"
OM	stands for “Organic Matter”, also called “Volatile Solid - VS”
SB	stands for “Sequential Batch”
SL	stands for "sophorolipids"
sOUR	stands for “specific Oxygen Uptake Rate”
SSF	stands for “Solid State Fermentation”
WC	stands for "Water content"

1. Introduction

In order to decrease the cities' environmental impacts and to contribute to a better resilience of urban areas addressing energy or food supply crisis, waste management systems have to be rethought to drive prevention and local valorisation of waste through new circular systems. In this context, the objective of the DECISIVE project is to demonstrate the ability to decrease the generation of urban waste (from households and assimilated) and increase recycling and recovery by focusing efforts on decentralised management and valorisation of the organic fraction of waste (i.e. biowaste), in a short cycle. To achieve this objective, the DECISIVE project will develop and demonstrate eco-innovative solutions that include, among other tools, the use of Solid State Fermentation (SSF) technology.

In general terms, SSF presents important advantages over conventional submerged fermentation such as reduced energy requirements, high productivity and less inhibitory effects for enzyme production (Kuhad *et al.*, 2016). However, its application at the industrial scale appears to be hindered by technology issues, such as reactor design, heat transfer issues or sterilization costs (Mitchell *et al*., 2006).

Deliverable 4.5 from WP 4.2.1 aims at reporting the possibilities of digestate (and other wastes) as raw materials for SSF processes to obtain certain bioproducts. The first step for this goal is the characterization of the digestates and the optimization of SSF conditions for the production of high added-value bioproducts. The information gathered in this document is a statement of the main concerns on the SSF process which may evolve and be completed with the work performed in other work packages (especially WP4.1, 3.2 and 7) that could highlight possible exceptions to these general specifications. According to the Grant Agreement (Annex 1, Document of Action), specifications include both the design and operation concept of the SSF system (e.g. size and type of bioreactor, type and quantity of biowaste to be valorised)and also a list of regulations, safety and sanitary constraints, social features and economic data. To ensure a clear reading and understanding of those specifications, D4.5 is divided into five parts:

Chapter 2 proposes a state of the art of SSF developments worldwide and their achievements.

Chapter 3describes the routine analysis and the experimental setup that will be used in SSF processes proposed in the DECISIVE project. This chapter is sorted into three categories: biowaste handling, SSF setup and routine analysis performed during the SSF.

Chapter 4 focuses on the first results obtained in the targeted bioproducts generated using the digestate as the substrate. Based on these results, there is a selection of the most suitable bioproducts and the development of strategies to optimize the productive processes. To make reading easier, SSF process setup and specific methodologies are explained for each bioproduct in this chapter.

Chapter 5 is the section of conclusions. This section summarizes the most remarkable results and further actions to improve the productivities of the studied processes.

2. State of the art

SSF is an emerging technology for the bioconversion of organic solids into value-added products. There is a great interest in the study of these processes that is reflected on the high rate of production of related scientific literature. For example, some studies explore the use of the SSF to produce enzymes and other compounds of pharmaceutical and industrial interest (El Bakry *et al*., 2015). Some of these studies aim at producing enzymes such as proteases (Abraham *et al*., 2013), lipases (Santis-Navarro *et al*. 2011), amylases (Cerda *et al*., 2016), cellulases (Cerda *et al*., 2017), peroxidases (Vassilev *et al*., 2009) and xylanases (Dhillon *et al*., 2012). Many of these studies investigated the optimization of process parameters:water activity, addition of nutrients, and optimization of culture medium (Mahanta *et al*., 2008; Dhillon *et al*., 2012). However, most of the research carried out in SSF is performed using few grams of substrate. In addition, most of the above mentioned studies have been performed under initial sterile conditions, using specific microorganisms and systems with controlled temperature. Taking into account these conditions, the scaling up of the process appears as an extremely difficult issue, considering the widely known constraints of working with large amounts of solid substrates and the costs associated to the conventional operational conditions.

There are few recent studies on the overcoming of some of the disadvantages and limitations regarding the SSF scaling-up processes. An extensive analysis on the design and operation of bioreactors in SSF has been published by Thomas *et al*. (2013). Enzyme production using these designs has been successfully carried out by several researchers (Brijwani *et al*., 2011; Dhillon *et al*., 2011; Hansen *et al*., 2015). Although these approaches are a good alternative, there is still a requirement for temperature control and heat removal. For an efficient SSF scale-up it is necessary to have a deep knowledge of the process engineering: mass transfer phenomena and energy flow models, etc., and a wide experience on methodologies used to analyse physical properties affecting these phenomena (Ruggieri *et al*., 2009a; Ruggieri *et al*., 2009b).

In terms of SSF, the limits can be solved with a strong background in composting science as held by UAB. Currently, and based on that knowledge, UAB is developing and facing an easily scalable SSF process. SSF can be applied to urban wastes to obtain valuable products. The potential transformation of the targeted wastes into value-added products is of high interest for all the communities, providing a solution for the management of these materials and an economic benefit by the use or commercialization of the products obtained. A small scale multi-feedstock biorefinery will allow for the management of wastes in-situ, avoiding economic and environmental costs related to waste transportation, and providing new business models for the circular economy, also very necessary in many areas. As a consequence, SSF is a promising technology to be considered as a completely innovative solution for biowaste in the EU: no patent describing bioproducts production from SSF of urban biowaste has been identified in different sources such as the European Patent Office (EPO).

In particular, ecological (environment-protective) advantages of SSF reflect the fact that the processes of high value molecule production are conducted in the absence of a free aqueous phase. This results in minimum water consumption and thus, a low production of effluent water by the process, use of antifoam agents is not required, and the possibility of conducting SSF processes in certain applications under semi-sterile conditions. Due to SSF process conditions, the growth of contaminating bacteria and fungi is minimized. This means that, in optimal cases, energy and sterilization equipment will be not necessary.

3. Materials and Methods

This chapter lists general methods that must be taken into account for the development of a SSF process. First, the targeted biowaste typology is presented followed by the most relevant concerns on material conditioning and storage. The experimental set-up used for the development of the targeted bioproducts is also explained. However, specific methods and procedures to produce each bioproduct are specified in Chapter 4 for clarity purposes.

3.1 Biowaste typology

3.1.1 Conditioning

The DECISIVE project aims to produce a value added-product from biowaste. In a first stage digestate and the organic fraction of municipal solid wastes (OFMSW) were assessed. These wastes were used in the same conditions as delivered; however, these materials can be highly heterogeneous and may contain large amounts of impurities. Therefore, in a later stage, a conditioning stage was included as a pre-treatment to the SSF process as described below.

If required, i.e. high presence of impurities, the digestate was sieved using a 0.5 mm net, in order to remove the impurities. Additionally, and as discussed in Deliverable 4.1(Degueurce et al., 2017), a sanitation process is mandatory according to the current legislation hence it was included as a part of the conditioning of the materials. This sanitation consists in holding the residue for 1 h into a previously heated oven at70ºC. Then, the residue is cooled down to room temperature.

After sanitation, the digestate is conditioned in order to meet suitable conditions for microbial growth in terms of porosity and moisture. The porosity and moisture content must be around 30% and 60%, respectively for the proper development of the process (Ruggieri et al., 2009). Figure 1 shows digestate sanitized and conditioned with the addition of different bulking agents.

FIGURE 1. SANITIZED AND CONDITIONED DIGESTATE PREVIOUS TO ITS USE IN SSF PROCESS USING SPONTEX (LEFT) AND WOOD CHIPS (RIGHT) AS THE BULKING AGENT.

3.1.2 Characterization

The potential to produce biopesticide, hydrolytic enzymes and sophorolipids was assessed using different raw materials. In addition to the composition of the wastes, the main factors considered to select the most suitable materials were the proximity to the UAB facilities and the availability of the substrates. In this sense, two different digestates of different sources were assessed: one from Ecoparc and another from Granollers treatment plant. The digestate obtained from Ecoparc (DE) (Montcada i Reixac, Spain) is currently being obtained from the processing of

OFMSW using a mechanical biological treatment plant. This process consisted in a mesophilic dry anaerobic digestion followed by a centrifugation stage. The Granollers digestate (DG) (Granollers, Spain) is currently being obtained from with source selected OFMSW sited. This process consisted in a mesophilic wet anaerobic digestion followed by a solid/liquid separation stage carried out using a screw press.

The average characteristics of the OFMSW and digestate from Ecoparc and Granollers used in the different SSF experiments are listed in Table 1.

As the biowaste has an intrinsic heterogeneity, the UAB team has developed a database to record the characterization of the substrates as arrived to UAB facility. This information will provide useful information to assess the impact of the wastes variability in the productive processes carried out during the development of the DECISIVE project. An example of this database is presented in Annex I.

TABLE 1. AVERAGE CHARACTERISATION OF THE OFMSW AND DIGESTATES FROM ECOPARC AND GRANOLLERS USED IN THE FERMENTATION PROCESSES.

Source of input	Ecoparc1	Granollers2	OFMSW
Parameter	Average (± s.d)		
Moisture (%, wb)	83.35±0.92	75.60±5.11	59.28±2.06
Dry Matter (%, wb)	16.65±0.92	24.40±5.11	40.72±2.06
Organic Matter (%, db)	59.20±6.22	63.00±1.62	67.40±1.92
pH (1:5)	8.43±0.01	8.31±0.07	5.58±0.1
Carbon (%, db)	28.14±0.19	34.14±2.38	42.3±1.0
Nitrogen (%, db)	3.45±0.06	4.32±0.33	2.5±2.2
Hydrogen (%, db)	2.28±0.25	2.89±0.25	-
Sulphur (%, db)	0.49±0.01	0.33±0.14	-
C/N ratio	12.34±1.30	11.85±0.70	16.92±0.91
Hemicellulose (%, db)	13.29±3.11	10.06±0.90	-
Cellulose (%, db)	11.16±2.61	10.42±1.25	-
Lignin (%, db)	12.48±1.73	17.54±1.96	-
Fats (% db)	-	5.05±0.04	-

(1)number of samples equal to 2. (2)number of samples equal to 8. wb wet basis, db dry basis

3.1.3 Storage

The materials were stored in a cold chamber (−4 C) until use for a maximum of two days.

3.2 Experimental set-up

The SSF experiments were carried out at different scales, using 0.45, 4.5 and 10L reactors.

3.2.1 SSF reactors

This setup consists in 12 lines, each one able to carry out 3 independent fermentations using specific reactors. These reactors are cylindrical polyvinylchloride packed-bed reactors (13x7cm) each one provided with one mass airflow-meter and the exhaust gases connected to CO_2 and O_2 sensors. A specific software was developed by GICOM research group (UAB team) to allow the on-line monitoring and the continuous storage of the experimental data. A schematic diagram of the setup is presented in Figure 2.

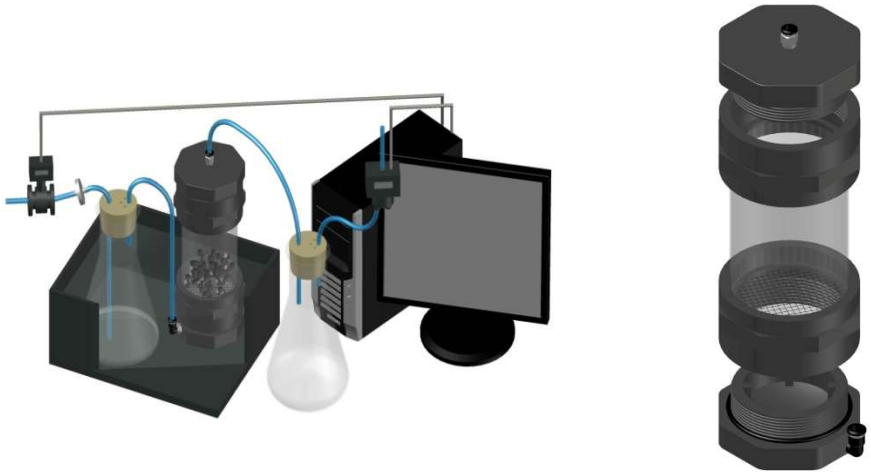

FIGURE 2. SCHEMATIC DIAGRAM OF 0.45 L REACTOR'S SETUP USED FOR LAB-SCALE SOLID-STATE FERMENTATION.

The reactors are submerged in a thermostatic bath in order to keep the temperature stable in a fixed value in a range of 20-90ºC. This temperature depends on the process carried out and the optimum temperature of the specific microorganisms.

3.2.1.1 Reactors of 4.5 and 10 L

Solid-state fermentation can be performed in either 4.5 L or 10 Lair-tight packed-bed reactors, thermally isolated to work under near adiabatic conditions. Experimental setup is presented in Figure 3. Air was continuously supplied to the reactors by means of a compressor joined to a mass airflow controller (Bronkhorst, Spain). Airflow was adjusted either automatically or manually depending on the process and the scale in order to maintain oxygen content in the reactor in levels above 5% and avoid anaerobic conditions. In this context, air flow was supplied through a pipeline from the bottom of the reactor where, by means of a plastic diffuser, the air circulated through the solid bed of the reactor. The exit of the exhausted gas is located on the top of the reactor, and led to an oxygen analyser. The oxygen content was measured using an oxygen sensor in a range of 0-20.9%.

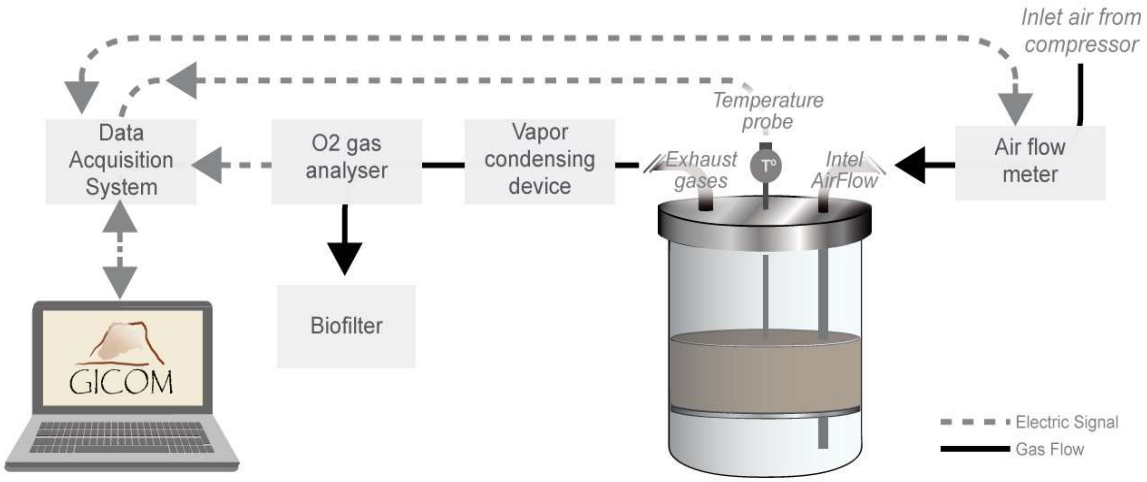

FIGURE 3. EXPERIMENTAL SETUP OF THE BENCH SCALE SOLID-STATE FERMENTATION SYSTEM. SOURCE: CERDA *et al.* (2016).

Between the reactor and the oxygen sensor there is a vapour-condensing device to avoid humidity to reach the sensor and consequently damage it. All outlet pipelines were connected to a biofilter.

3.2.2 SSF monitoring

Airflow, temperature and oxygen content were continuously monitored during all fermentations. Using the on-line measurement of the oxygen content, the specific oxygen uptake rate (sOUR) was calculated on-line for continuous monitoring in order to provide more accurate information on biological activity. The calculations were carried out according to Ponsá (2010). Monitoring was performed by a self-made acquisition and control system based on Arduino® and self-made software.

3.3 Routine analysis

Routine analysis of pH, total (TS) and volatile solids (VS) were performed according to standard methods (The US Composting Council, 2001). Cellulose, hemicellulose and lignin content was determined according to Van Soest *et al*. (1991) using the Ankom200 FiberAnalyzer incubator (Ankom Technology, Macedon, NY), adding amylase and sodium sulphite solutions.

4. Results for different products

4.1 Biopesticides

B. thuringiensis (Bt) is a spore former, facultative anaerobic gram-positive bacterium present in soil, water and plant surfaces. It is a producer of a paraesporal crystal protein also called d-endotoxin. The toxin has a great potential to cause mortality to insects belonging to different orders such as Diptera, Coleoptera and Lepidoptera, pests that destroy more than 40% of the world's food, forage, and fiber production. Conversely, these toxins are innocuous for plants, animals and human beings. The biopesticides used in biological control of plagues are an environmentally safe alternative to synthetic pesticides. They have been used worldwide for many years for food crops and forestry pests (Chandler *et al*., 2011). The production of Bt based-biopesticides has been studied mainly by submerged fermentation and applied at industrial scale, with few studies in solid-state fermentation. In these cases, different wastes have been used as substrates, such as soy residues, wastewater treatment sludge, kitchen waste, wheat bran, among others (Devi *et al.,* 2005; Zhang *et al*., 2013; Zhuang *et al*., 2011). So far, all the SSF studies have been performed under sterile conditions and mesophilic temperatures (Smitha *et al*., 2013).

Taking advantage of the ability of Bt to produce spores in adverse conditions, the aim of this part of the study is to assess the potential of digestate as a substrate for the development of a soil amendment with biopesticide effect. In this sense, the challenge is to make this microorganism thrive in a complex solid matrix under non sterile conditions at a representative scale.

4.1.1 Experimental procedure

4.1.1.1 Screening for suitable conditions for biopesticide production

SSF tests were performed using sterile and non-sterile DG as substrate in order to assess the effect of the presence of autochthonous microbiota in the DG and the ability of *Bacillus thuringiensis* (Bt) to survive in a competitive media. In the experiments carried out using sterile substrate the digestate was subjected to a sterilization process (121ºC, 31min) previous to inoculation.

In 0.45 L reactors tests (described in Section 3.2.1.1), a mixture of DG as the substrate and wood chips as the bulking agent (BA) was used in a 5% (w/w) ratio. The total weight of the inoculated solid matrix was 120 g of mixture per fermentation. Two sets of experiments were performed, one without inoculation (control) at sterile and non-sterile conditions and another inoculated at the beginning of the process at sterile and non-sterile conditions. The microorganism used was *Bacillus thuringiensis var.kurstaky* (*Bt*) and it was added in a 10% (w/w) ratio to the DG-BA mixture with a concentration of10^7 CFU mL^{-1}. These fermentations were carried out in triplicates at 30ºC using a constant airflow of 20 mL min^{-1} during 96 h.

Samples were collected at 0,12,24,48 and 96h of the fermentation time to ascertain the evolution of viable Bt cells. Cell (CC) and spore (SC) counts were performed according to the methodology described below in Section 4.1.2. All the counts were done in triplicates and were expressed as colony forming units per mL (CFU mL^{-1}).

4.1.1.2 Scale up to bench scale SSF for biopesticide production

After the assessment carried out at laboratory scale, the process was scaled-up using 4.5 and 10 L reactors (described in Section 3.2.1) using, in all cases, a non-sterile substrate. A description of the experiments performed is presented in Table 2.

TABLE 2. SUMMARY OF THE EXPERIMENTAL CONDITIONS FOR BIOPESTICIDE PRODUCTION BY SSF AT A BENCH SCALE.

	4.5L Reactors	10L Reactors
Substrate	Non-sterile DG	Non-sterile DG
Bulking agent (w/w)	1:1	1:1
Inoculum (w/w)*	8%, with a concentration of 105CFU mL-1	5%, with a concentration of 107CFU mL-1
Total weight (g)	1,295	5,009
Operational conditions	Manually controlled airflow ranging between: 400-800 mL min-1	Manually controlled airflow ranging between: 400-800 mL min-1
Replicates	Duplicates	Duplicates

*the amount of inoculum is different due to reactors used for growth preparation were not the same in every experiments. Nowadays we have standardized the inoculum preparation procedure to the conditions presented in the 10L reactor.

In both cases, prior to the scale up experiments, an initial batch was performed without inoculation, in order to monitor the temperature profile obtained by the substrate that will determine the proper growth of Bt.

Sampling was performed at 24, 48, 72h and at the end of the fermentation. Routine analysis, CC and SC were measured according to the methodology described below in Section 4.1.2.

4.1.2 Specific analytical methods

B. thuringiensis var. Kurstaky NRRL HD-73 (CECT 4497) (Bt) was purchased from the "Colección Española de Cultivos Tipo" (CECT), located in Valencia, Spain. The strain was kept stored frozen at -80 ºC in cryo-vials with 10% (v/v) glycerol until use.

Bt was incubated in Petri dishes on supplier's recommended agar media Nutrient Agar (OXOID®) and for liquid cultures Nutrient Broth No. 2 (OXOID®) as described by Ballardo *et al*. (2016). During the preparation of the inoculum the growth of *Bt* was monitored by means of optical microscopy and optical density. Optical microscopy was used in order to prove the absence of contamination in the inoculum and the optical density was used to measure the growth of microorganisms.

The determination of total cell (CC) and spore (SC) quantification was carried out according to the specification reported by Ballardo *et al*. (2016a). All the counts were done in triplicates and were expressed as colony forming units per mL (CFU mL^{-1}).

4.1.3 Results for biopesticide production

4.1.3.1 Screening for suitable conditions for biopesticide production

Results of the SSF carried out using DG as the substrate at sterile and non-sterile conditions are presented in Figure 4.

Figure 4a shows sOUR profiles for the control and Bt-inoculated fermentations (R1, R2 and R3) during 96h of SSF performed with sterile DG. Due to the sterilization stage, it was expected that sOUR remained in values near 0. However, Figure 4a shows that the control presented a sOUR near 1 mg O_2 g^{-1}. DM, which was lower than the observed in the Bt-inoculated fermentations but not as low as expected. It is possible that the sterilization process on large amounts of solids is a complex process due to heat transfer onto the solid matrix. Then, if sterilization was not properly performed some microorganisms were able to survive, which explains the sOUR vale in the control fermentation.

A peak of sOUR was observed at 35h of fermentation with a value of 0.82 mg O_2 g^{-1} DM h^{-1}, decreasing until negligible values at the end of the process. Related to sOUR profiles for Bt inoculated reactors, a similar trend than the control was observed. Also, the differences among the triplicates were minimal and therefore the results are reliable and reproducible. Maximum sOUR was obtained at 20h of operation with an average value of 1.98±0.08mg O_2 g^{-1} DM h^{-1}. Bt inoculation generated a rapid start-up of the process, generating 2-fold increase on the biological activity reflected as sOUR.

Figure 4a also shows the viable CC throughout Bt-inoculated fermentations. The triplicates started with the same value of initial viable CC of 1.31 10^7 ± 0.1 CFU g^{-1}DM. Viable CC in all replicates showed an initial increase of the biomass in the first 24h. After that period, there are no significant differences between the CC in the next 48h. It is after that period the CC increased, reaching a peak of CC 1.15±0.12 (10^9) CFU g^{-1}DM. These results are very positive, because they reflect the ability of Bt to not only survive in a complex solid matrix, but to thrive and increase in nearly10^2 units the initial viable CC. Other studies using other substrates working at the same conditions observed a final CC of 6.6 $10.^{10}$CFU g^{-1} DM (Devi *et al*., 2005) and 6.2 10^{11} CFU g^{-1} DM (Ballardo *et al*., 2016a), which is higher that the obtained in the present study using DG as the substrate. However, it has to be considered that the substrates used by Devi *et al*. (2005) and Ballardo *et al*. (2016a) were wheat bran and soy fiber, respectively, which show less heterogeneity and were (in the case of Devi *et al*., 2005) provided with external carbon and nitrogen sources. Ballardo *et al*. (2016b) also worked with the OFMSW, in order to partially assess a highly heterogeneous material. In this case, the authors observed a viable CC of $1.9 \cdot 10^9$ CFU g^{-1} DM, which is in range of the obtained in the present work.

In addition to the mentioned experiments carried out at sterile conditions, another series of experiments were carried out under non-sterile conditions. The results obtained are presented in Figure 4b. Control and Bt-inoculated SSF reactors (R1,R2 and R3)presented very similar sOUR profiles, with slight differences associated to the heterogeneity of the material. In all cases the biological activity was higher than in the SSF performed under sterile conditions, which is likely related to the higher presence of microbial populations in the reactors. Maximum sOUR was found at 11h of operation in all cases, with values of 2.79 mg O_2 g^{-1} DM h^{-1} for the control and an average of 3.23±0.83 g O_2 g^{-1} DM h^{-1} for the Bt-inoculated triplicates. Although the triplicates trend is very similar, there are differences between them which can be partially attributed to the mixing and sampling during the process. The non-sterility of the fermentation implied a higher number of microorganisms able to colonize the solid matrix, which generated an overall increase on biological activity.

Regarding the viable CC, the triplicates of the Bt-inoculated fermentations started with a viable CC of 9.7±0.5 (10^9) CFU g^{-1}DM. In all cases, the CC decreased during the first 24h. After that moment, the replicates started to present slight differences that led to the achievement of different CC values at different moments but in any case, surpassing the initial viable CC. For R1, maximum viable CC was found at 58h of operation. For R2 and R3, maximum viable CC was observed at 72h of fermentation with an average value of 1.52±0.24 (10^9) CFU g^{-1} DM. R1 showed the lower maximum of sOUR with very different trends, which led to a lower CC. These differences might be attributed to the complexity of the digestate and its heterogeneity.

Previous research performed at the same conditions using soy fiber and the OFMSW as the substrate, presented a higher viable CC of 3.8 10^{11} CFU g^{-1} DM (Ballardo *et al*., 2016b). The same authors confirmed that the final CC depended on the initial inoculum added to the solid matrix. In this sense, they stated that when Bt was added in a 7%, 9% and 12% to the substrate (OFMSW) the viable CC obtained were 9.9 10^5 CFU g^{-1} DM, 1.1 10^7 CFU g^{-1} DM and 2.5 10^7 CFU g^{-1} DM, respectively (Ballardo *et al*.,2016b). All of these values are lower than the observed in the present work.

More interestingly, from the present study it can be stated that Bt growth using DG, as substrate, has been demonstrated possible both under sterile and non-sterile conditions at controlled temperature. This is of great importance for the further scale up of the process at a bench and pilot scale that is forecasted in the DECISIVE project and also, it is particularly important to decide whether sanitation step should be processed before or after the AD step.

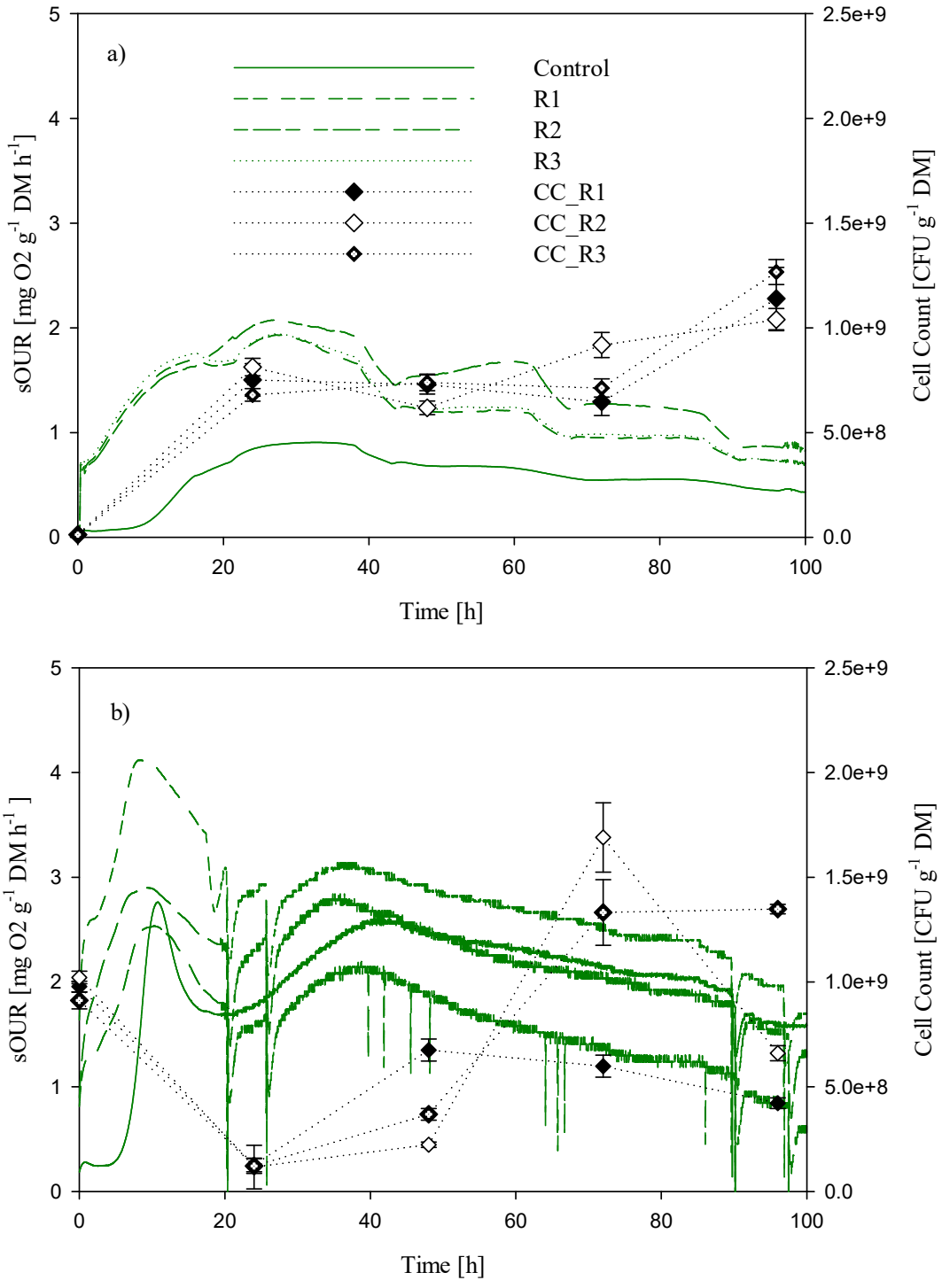

FIGURE 4. SOUR AND VIABLE CELL COUNT PROFILES OBTAINED IN 0.45 L SSF REACTORS UNDER A) STERILE AND B) NON-STERILE CONDITIONS USING DG AS SUBSTRATE.

4.1.3.2 Scale-up to bench scale SSF for biopesticide production

Experiments at bench scale were undertaken under non-sterile conditions and not controlled temperature. A control experiment with DG without Bt inoculation was performed. The main objective of this preliminary experiment was to assess the temperature evolution of DG during the operation on an isolated packed-bed reactor. The obtained

temperature profile is presented in Figure 5a. The maximum temperature reached was 41.4°C, in the mesophilic range, that can be suitable for the growth of Bt.

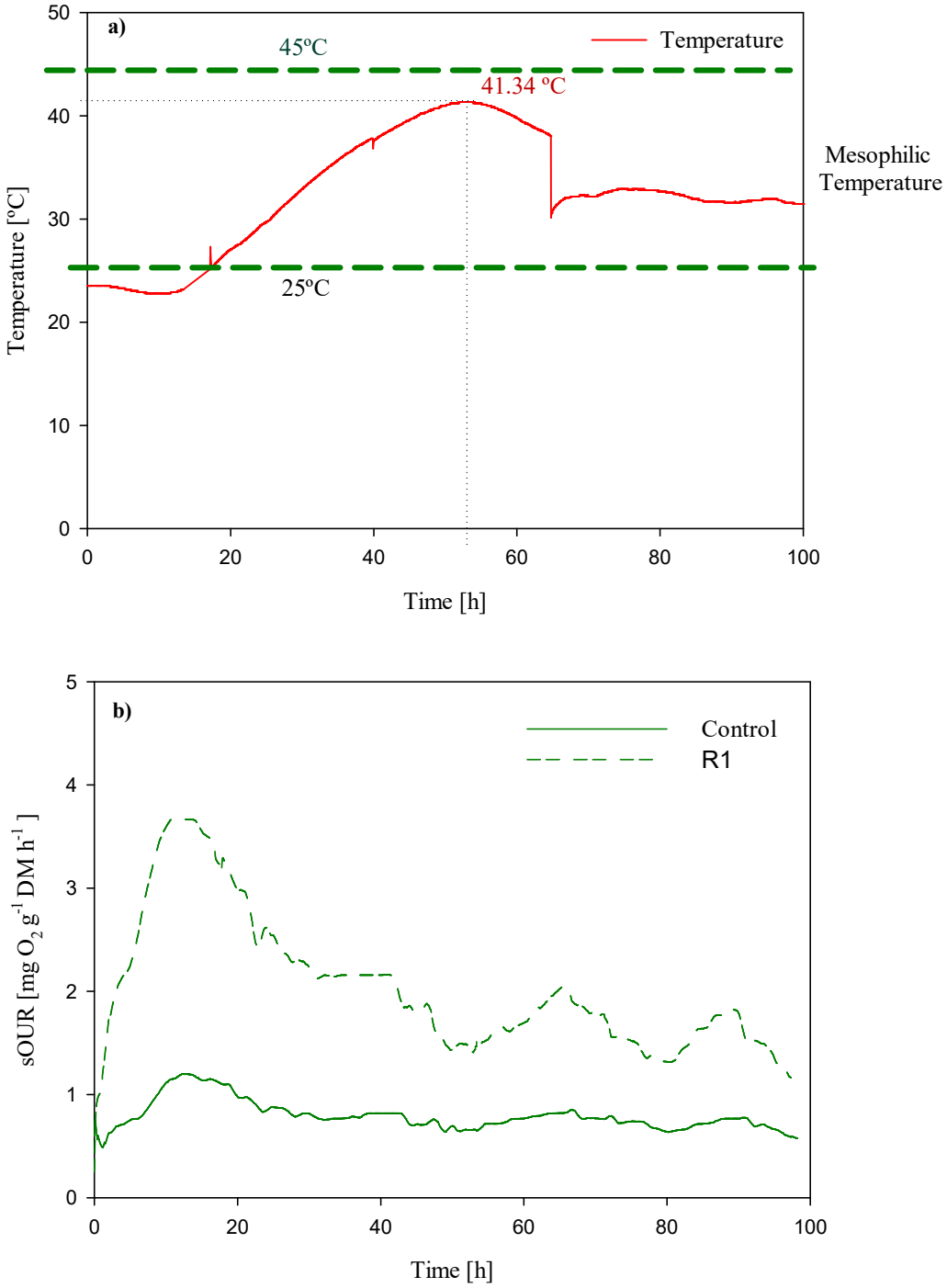

FIGURE 5. OPERATIONAL PROFILES OF SSF CARRIED OUT USING DG AS SUBSTRATE IN A 4.5L REACTOR. A) TEMPERATURE PROFILE OF A CONTROL SSF AND B) SOUR PROFILES OF CONTROL AND BT-INOCULATED REACTOR (R1).

Figure 5b presents the sOUR profile of the control and the Bt-inoculated (R1) fermentations. There is a similar sOUR profile in both fermentations, with the consequent increase on the biological activity when Bt was inoculated. In this sense, the experiments carried out in 4.5L reactors showed the same trend than the obtained at lab scale (0.45 L reactors). Both fermentations reached a maximum sOUR at 12h of operation, with a maximum value of 1.2 and

3.67 mg $O_2 g^{-1}$DM h^{-1} for the Control and Bt-inoculated SSF respectively. After that period, the biological activity decreased to values below 1 mg $O_2 g^{-1}$DM h^{-1} in both cases.

Figure 6 presents the complete operational profile (sOUR, temperature, CC and SC) of the reactor inoculated with Bt.

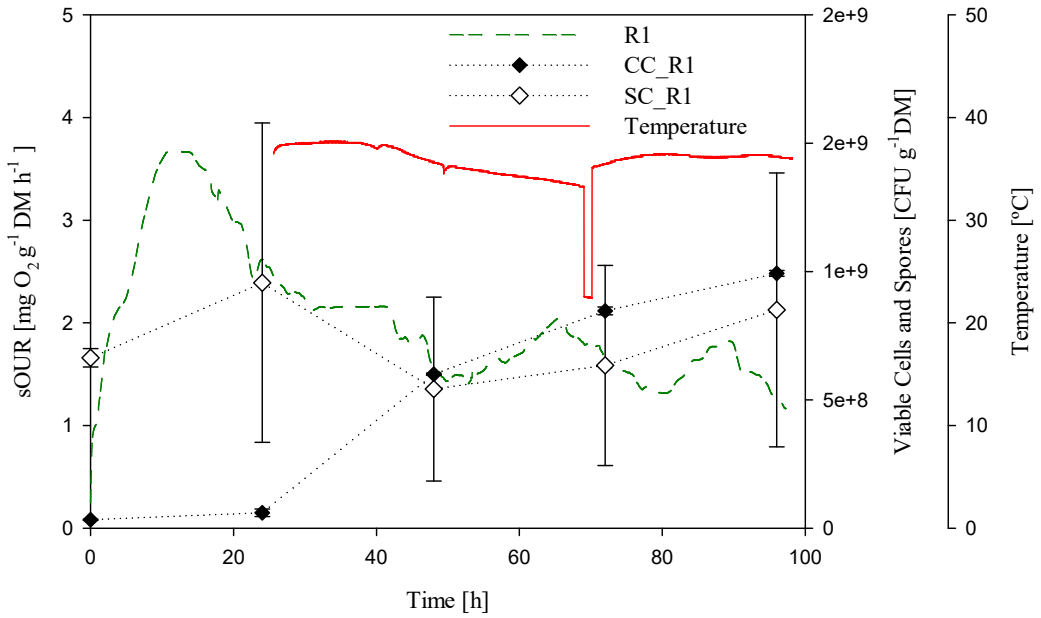

FIGURE 6 OPERATIONAL PROFILES OF SSF CARRIED OUT USING DG AS SUBSTRATE BT-INOCULATED IN A 4.5L REACTOR.

There is a gap in the temperature profile in the first 24h of fermentation, as showed in Figure 6. This was due to a malfunction on the aeration and data acquisition system. Regardless, once the issue was fixed the system was able to recover and the temperature remained in the mesophilic range as expected, reaching a maximum of nearly 40ºC. This temperature range provided proper growth conditions for Bt, therefore the survival and colonization of Bt onto the solid matrix was expected. It is important to mention that at 72h of operation, during the sampling, there is a drop in the temperature. This is associated to the fact that the temperature probe was at room temperature while the sampling was performed. However, it is not a reflection of the biological process carried out.

The initial viable CC of this reactor was 3.28±0.27 (10^7) CFU g^{-1} DM and from the beginning of the fermentation started to consistently increase with a maximum CC found at the end of the fermentation (9.93± 0.11 (10^8) CFU g^{-1} DM after 96h of fermentation). These results are very promising, due to the proper colonization of Bt in a very simple and easily scalable process. Moreover, these results are very positive when compared with the reported literature. Ballardo *et al*. (2016b) obtained a viable CC of $2.7 \cdot 10^5$ CFU g^{-1} DM working at the same conditions but with a larger initial CC (3.8 10^{11} CFU g^{-1} DM.) and using the OFMSW as the substrate.

Results related to spore count showed a sustained value of SC during the process with no significant differences and an average value of 8.24±1.48(10^8) CFU g^{-1} DM.

In light of the results obtained at 4.5L a further scale-up was assessed, to 10-L reactors. As in 4.5L reactors, a previous experiment without considering inoculation was performed in order to assess the temperature evolution at this scale (Figure 7a). In this case, the temperature reached thermophilic conditions with a peak of 55.8°C in nearly 18h. The higher temperature reached at this scale can be attributed to the higher work scale and the consequent heat generation but also to the characteristics of the substrates. Digestate used for 4.5 L and 10 L fermentations came from different batches and therefore the initial organic matter content showed slight differences. Organic matter content of the digestate used for these fermentations was 63.7±1.5 and 79.0±1.0% (db) for 4.5 L and 10 L fermentations, respectively. The higher organic matter content could have generated an increased temperature obtained in these fermentations due to the more available substrate. The higher temperature reached on the 10 L

fermentations directly affects the performance of the reactor and the proliferation of the different microbial populations present in the solid matrix, and thus, they will probably affect Bt growth.

Figure 7b presents sOUR profile of the control and two replicates of Bt-inoculated reactors (R1 and R2). In this cae the control showed a different trend that the Bt-inoculated reactors. The control fermentation showed a clear trend with a single sOUR peak of 4.5 mg O_2 g^{-1} DM h^{-1} at 5 h of operation, while Bt-inoculated reactors showed two sOUR peaks. These peaks were observed after 4 and 24 h of fermentation with values of 6.22 and 3.84 mg O_2 g^{-1} DM h^{-1}in the first peak for R1 and R2 respectively. In this sense, it is likely that the temperature could have affected the development of the microbiome of the reactors and hence changing the biological activity parameters. Additionally, total COC obtained in these processes was 190 mg O_2 g^{-1} DM for the Control and an average of 331±40mg O_2 g^{-1} DM for the Bt-inoculated fermentations.

4.2 Proteases

Proteases are hydrolases that catalyze the cleavage of peptide bonds in proteins. Proteases are a highly complex group of enzymes that differ in their substrate specificity and catalytic mechanism. Therefore, proteases are the most important industrial enzymes that account for about 60% of the world market of industrial enzymes (El-Bakry *et al*., 2015).The importance of these enzymes is reflected in their tremendous applications in both physiological and commercial fields, for example, in detergent formulations, textile, food, and pharmaceutical industries (Queiroga *et al*., 2012). Therefore, this is an attractive bioproduct to target in the framework of the DECISIVE project.

4.2.1 Experimental procedure

The fermentations were carried out using two different digestates: DG and DE. The ratio of digestate and Spontex (as the BA) changed depending on the characteristics of the substrate, mainly in terms of moisture content (Table 1). Then, the experiments carried out with DE were set using a DE:BA ratio of 90:10(w/w) while the experiments using DG used a DG:BA ratio of 95:5 (w/w). In both cases, SSF tests were carried out using a total weight of 120g.

These fermentations were carried out in triplicates using 0.45 L reactors (described in Section 3.2.1.1) at 37ºC using a fixed airflow of 20 mL min^{-1} during 96 h. Sampling was performed when maximum sOUR was achieved and from that moment, each 24h until the end of the fermentation. During the process, sOUR and protease activity monitoring was performed. In addition, several physical-chemical parameters were also measured: pH, dry matter, organic matter (Section 3.3).

4.2.2 Specific analytical methods

4.2.2.1 Enzyme extraction

Fermented solid material was mixed thoroughly with 50 mMHCl-Tris (tris(hydroxymethyl)aminomethane) buffer, pH 8.10, in a ratio 1:5 (w:v) for 45 min and the extract was separated by centrifugation at 10000 rpm for 10 min at 4ºC and further filtration through 0.45 µm (Abraham et al., 2013). The filtered supernatant was used as crude enzyme extract.

4.2.2.2 Enzyme activity assay

Alkaline protease activity was determined using a method previously described by Alef and Nannipieri (1995). Briefly, 1 mL aliquot of the enzyme extract was added to 5 mL of 2% casein solution and incubated at 50ºC and 100 rpm for 2 h. One unit of alkaline protease activity was defined as 1 µg of tyrosine released under the assay conditions.

4.2.3 Results of protease production

The profile of sOUR and protease production at a laboratory scale (0.45 L reactors) using DE and DG is presented in Figure 9. In Figure 9a the operational profiles of the triplicates of the fermentations using DE as the substrate are presented. It is noticeable that the triplicates had a proper reproducibility in terms of the biological activity reaching a maximum value of 4.6±0.1 mg O_2 g^{-1} DM h^{-1} at 35h of operation.

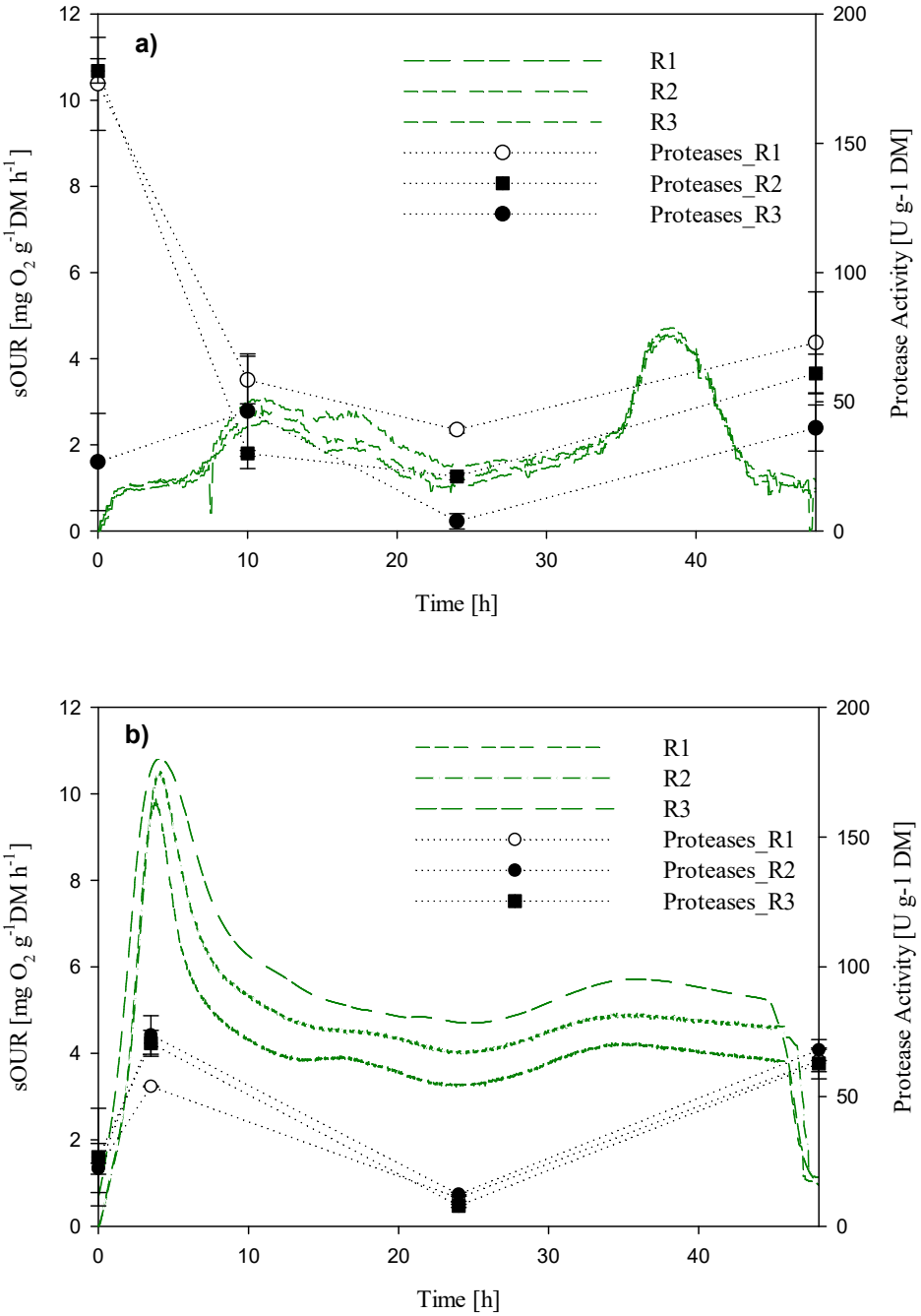

FIGURE 9.OPERATIONAL PROFILES OF SSF CARRIED OUT IN TRIPLICATES USING A) DE AND B) DG AS SUBSTRATES IN A 0.45 L REACTOR. PROFILES OF SOUR AND PROTEASE ACTIVITY ARE PRESENTED.

Results obtained using DG as the substrate for protease production are presented in Figure 9b. Triplicates of the fermentation were monitored and, as observed in Figure 9b, the same profile was obtained. Maximum sOUR was obtained at 3h of fermentation with a maximum average value of 10.4±0.5mg O_2 g^{-1} DM h^{-1}. This peak was stable for few hours and then dropped until stable values around 5 mg O_2 g^{-1} DM h^{-1}.

Considering these results, it is clearly observed that the substrates presented different stability at the beginning of the fermentation. DG presented high biological activity which is likely associated with a high pruning residues content of the sample, which could have increase the initial sOUR. DE, on the other hand, presented a moderate biodegradability with two differentiated peaks during the SSF process.

As for protease activity production, both substrates presented high initial enzymatic activity with values of 166±22 and 36.9±0.2 U g^{-1}DM for DE and DG respectively.

In the SSF using DE as substrate there was a clear decreasing trend during the process, i.e, no enzyme production using this experimental configuration at the presented operational conditions. At the end of the process, protease activity was reduced to values around 44.06±11.10U g^{-1} DM.

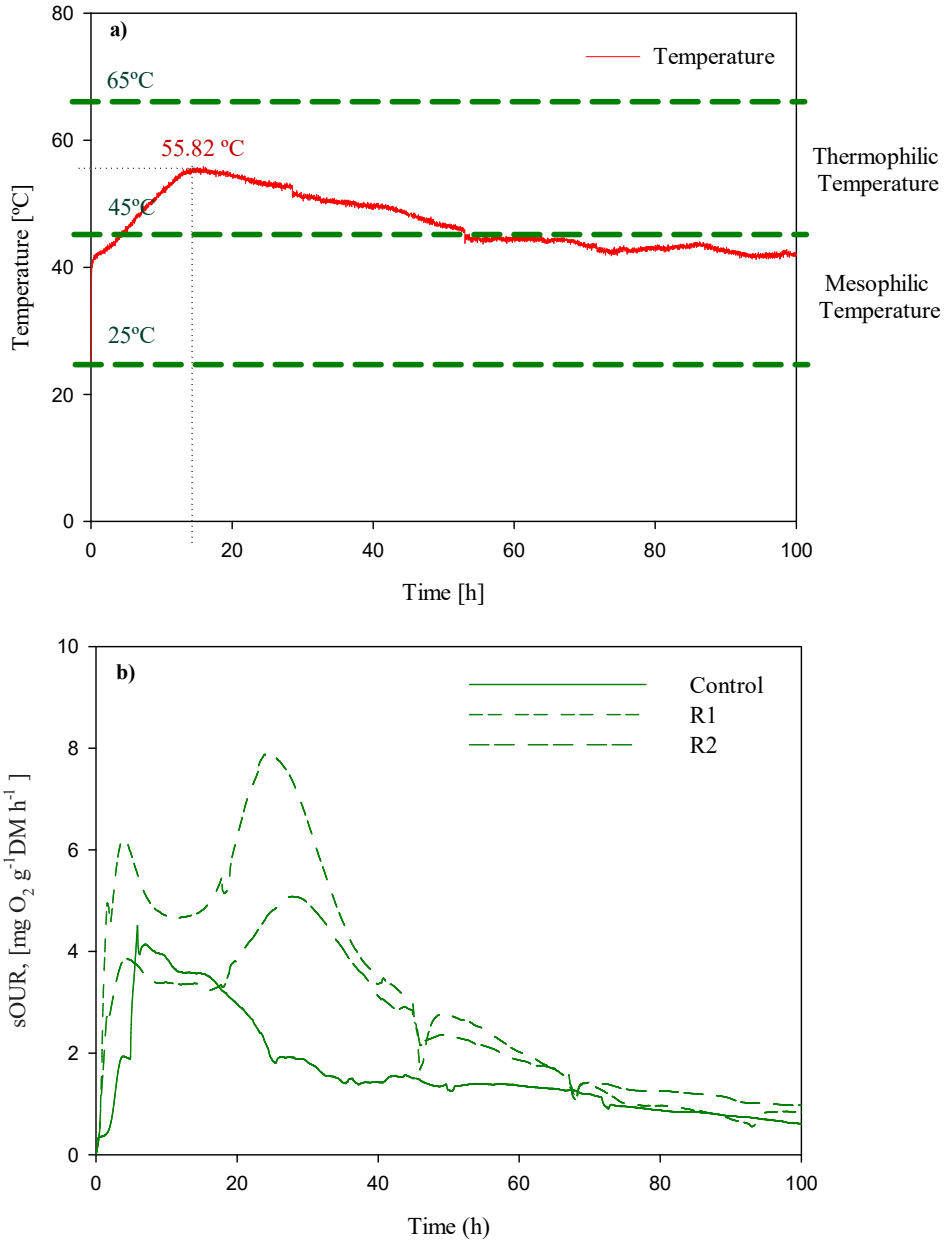

FIGURE 7. OPERATIONAL PROFILES OF SSF CARRIED OUT USING DG AS SUBSTRATE IN 10L REACTORS: A) TEMPERATURE PROFILE OF A CONTROL SSF AND B) SOUR PROFILES OF CONTROL AND BT-INOCULATED REACTORS (R1 AN R2).

Protease production by SSF using DG as the substrate presented a different behavior. Two discrete enzymatic peaks were detected, one at the beginning and another at the end of the fermentation. Both peaks reported a protease production of nearly 65 U g^{-1} DM. The obtained values of proteases production are in the lower range of those

reported in literature using different substrates. Abraham *et al*. (2013) use the same SSF configuration for protease production using soy fiber, coffee husk and hair waste with values of 310.33±9.17, 89.01±0.59, and 141.75±17.6 U g^{-1} DM, respectively.

It was observed that in the fermentations using both DG and DE protease production was low when compared with reported literature. In this case, digestate from different sources was found not suitable as a substrate for protease production. In this sense, protease production is not an attractive product for further study in the context of the DECISIVE project. In Figure 8 the operational profiles of the Bt-inoculated reactors (R1 and R2) are presented.

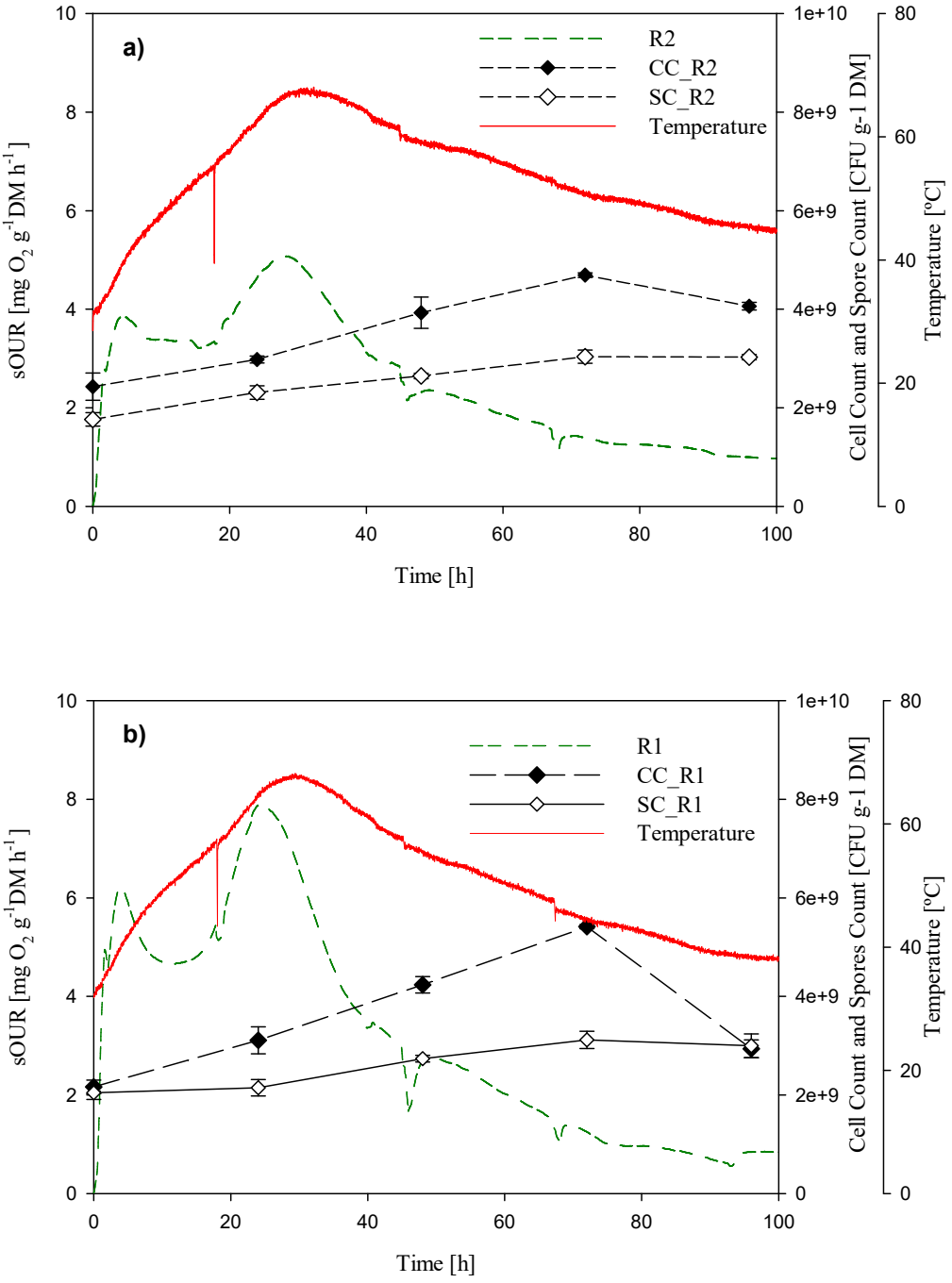

FIGURE 8 OPERATIONAL PROFILES OF SSF CARRIED OUT USING DG AS SUBSTRATE INOCULATED WITH BT IN 10-L REACTORS (DUPLICATE).A) REACTOR R1 AND B) REACTOR R2.

The average initial viable CC and SC for both Bt-inoculated reactors (R1 and R2) were 2.16±0.14(10^9) CFU g^{-1}DM and 2.05±0.14(10^9)CFU g^{-1} DM respectively. In both replicates the viable CC and SC presented a similar profile, with a sustained increase until 72h of operation, achieving average values of 5.42±0.04(10^9) CFU g^{-1} DM and 3.12±0.1(10^9) CFU g^{-1} DM respectively. Previous experiments reported by Ballardo *et al*. (2016a) using soy fiber as the substrate showed a decrease in Bt when compared with the initial CC of the solid mixture. Initial CC value was 9.5 10^7 CFU g^{-1} DM and after 20 days of fermentation it dropped to1.8 10^7 CFU g^{-1} DM, representing a decrease in nearly 80% of CC .

In light of the results obtained at lab and bench scale using sterile and non-sterile conditions it is possible to state that there is the possibility of managing biodegradable wastes, specifically digestate, by their transformation into a waste-derived soil amendment with enhanced biopesticide effect using an interesting technique such as SSF.

4.3 Cellulases

Cellulases are a group of three enzymes working in series in the transformation of cellulose into fermentable sugars. These enzymes are commercially produced by submerged fermentation mainly from pure substrates (McMillan *et al*., 2011). Cellulases have many applications in different productive sectors, one of the most relevant being the energy production. Bioethanol is one of the most important sources of clean energy available and cellulase production accounts up to 60% of the total costs associated with its production. Despite the fact that the use of commercial cellulases potentially provides high sugar yields, its use is far of being a cost-effective alternative. The enzymatic preparations must be purchased continuously and its production process is complex and energy intensive (Lever, 2005). Submerged fermentation normally includes sterilization systems, control and monitoring of different parameters such as temperature, pH and dissolved oxygen, which implies the use of high amounts of energy in the process (Tolan and Foody, 1999). Thus, cellulase production should be optimized to avoid the high cost of commercial enzymes to perform more sustainable and cheaper productive and processes.

4.3.1 Experimental procedure

4.3.1.1 Screening for suitable substrates for cellulase production

A mixture of substrate and BA was used in a 95:5 ratio (w/w), where the substrate was digestate and the bulking agent was multi-purpose cloth (Spontex®) pieces (1cm x 2cm) giving a total weight of 120g per fermentation.

The fermentations were carried out in triplicates at 37ºC at lab scale (Section 3.2.1.1) with a constant airflow of 20 mL min^{-1}. During fermentation, sOUR and cellulase activity were monitored. Sampling was performed at the beginning of the fermentation, at the moment of maximum sOUR and from that moment, each 24h.

4.3.1.2 Sequential batch operation for cellulase production

Sequential batch (SB) operation is a good strategy for microbial population selection, where microbial communities are forced to adapt to the environment and the provided operational conditions. This strategy could in some level sustain or even enhance cellulase production (Cerda *et al*., 2017).

Two strategies were developed, the first one (SB1) consisted on a non-inoculated reactor in order to promote the autochthonous biological diversity. The second strategy (SB2) included the initial incorporation of compost as inoculum with the objective of providing a diverse microbiota as reported by Cerda *et al*. (2017). In both strategies, the solid retention time was set at 3.5 d.

SB1 initial batch contained 95% (w/w) of digestate as the substrate and 5%(w/w) of Spontex as the bulking agent. SB2 consisted in the same mixture but with the addition of compost in a 10%(w/w) ratio as the inoculum. The content of the solid mixture and operational conditions were the same as described in Section 4.3.1.1.

Once the first retention time is achieved, 90% of the content of the reactor is replaced by fresh substrate as reported by Cerda *et al*. (2017). A total of 8 batches were performed. During the process, sOUR and cellulase activity were monitored.

4.3.2 Specific analytical methods

4.3.2.1 Enzyme extraction

Cellulases were extracted by adding 150 mL of citrate buffer (0.05 M, pH 4.8) to 10 g of fermented solids in a 250 mL Erlenmeyer flask and mixing thoroughly on a magnetic stirrer for 30 min at room temperature. The mixture was separated by centrifugation at 10000 rpm for 10 min, followed by filtration with a 0.45 µm filter. The remaining supernatant was used for cellulases activity determination (Dhillon *et al*., 2012).

4.3.2.2 Enzyme activity assay

Cellulase activity was measured according to the IUPAC filter paper assay (Ghose, 1987). The reducing sugars were determined by the dinitrosalicylic (DNS) colorimetric method (Miller, 1959). One filter paper unit (FPU) was expressed as equivalent to the enzyme that releases 1 µmol of reducing sugars under the assay conditions. The cellulase production has been expressed with respect to the dry matter content, i.e., FPU g^{-1}DM.

4.3.3 Results of cellulase production

4.3.3.1 Screening for suitable substrates for cellulase production

The complete profile of the sOUR and cellulase production using the two different substrates at a laboratory scale is presented in Figure10. Maximum sOUR from DG was found at 3.5h of fermentation with value of 10.3 g O_2 kg^{-1} DM h^{-1}while maximum sOUR from DE was obtained at 37.6h with a value of 4.6 g O_2 kg^{-1} DM h^{-1}. It is likely that the differences between maximum sOUR values are related to the content of readily metabolizable material or even to the characteristics of the substrate. This batch of DG was obtained from a digester which was fed with OFMSW with a high pruning content which generated differences in the performance of the fermentations.

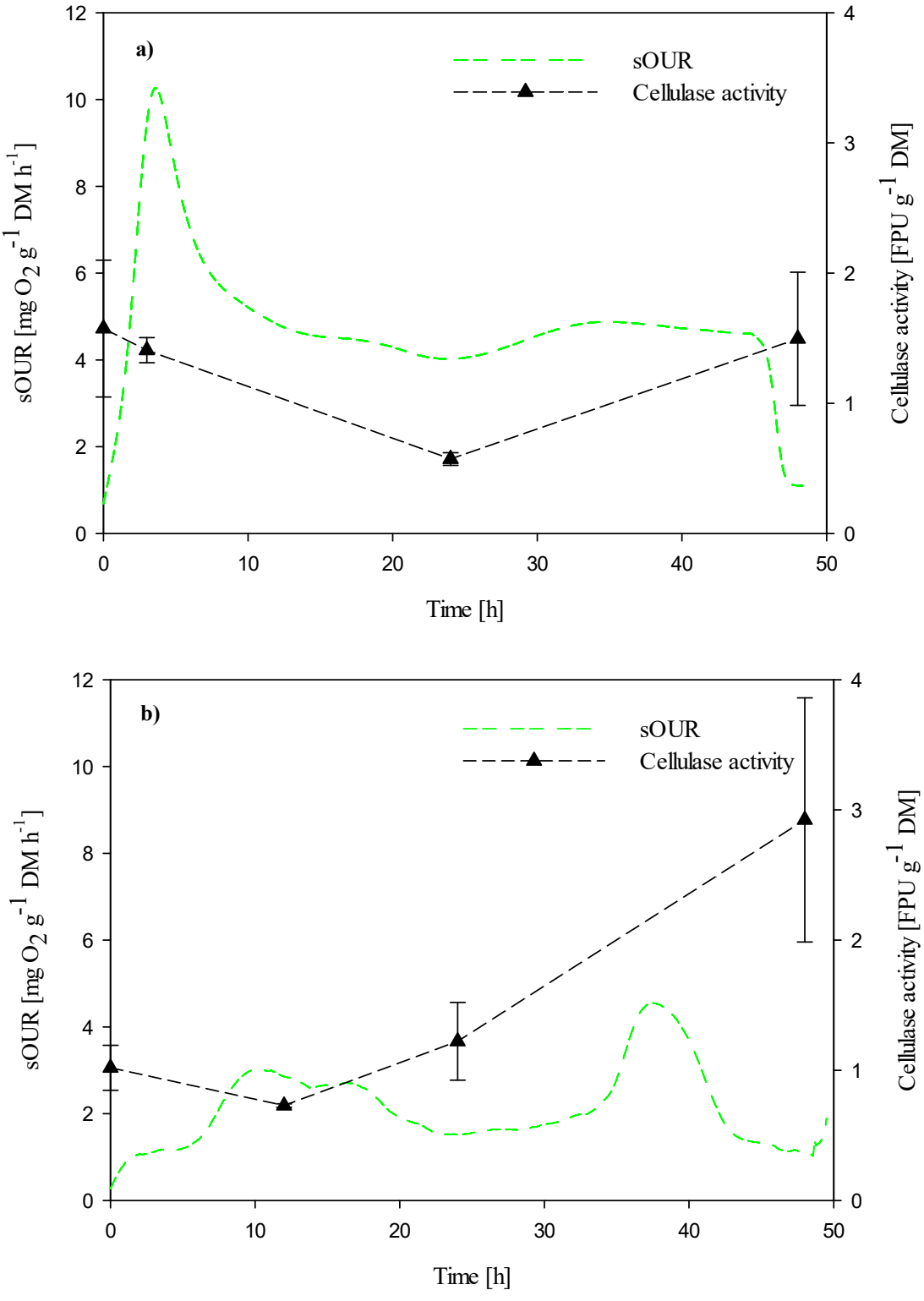

FIGURE 10. OPERATIONAL PROFILES OF SSF USING A) DG AND B) DE AS SUBSTRATES IN 0.45 L REACTOR. AVERAGE SOUR AND CELLULASE ACTIVITY ARE PRESENTED (S.D<5%).

In terms of cellulase activity, both of the proposed substrates provided a cellulase production ranging between 1.5-3.0 FPU g^{-1}DM, which is in the lower range reported at lab scale at similar conditions (Cerda *et al*., 2017) and even lower when compared with SSF using sterile substrates or adding a single strain (Dhillon *et al*., 2012).

In Figure 10 it can be observed that there is no clear relation between maximum biological activity (sOUR) and cellulase activity. This is not in accordance to the reported by Cerda *et al*. (2017), which found that the moment of maximum cellulase and sOUR activities consistently matched. Despite these findings, these authors also observed that there was not a direct correlation between cellulase production and sOUR (or any other relevant parameter), indicating a highly complex process with different microorganisms and/or metabolites working together in a synergetic or antagonistic manner (Eichorst *et al*., 2013; Cerda *et al*., 2017).

In the fermentations using DG and DE, there is a high initial cellulase activity present in the substrates which is probably associated with the previous anaerobic digestion process. In general, there is an initial decline in cellulase production, followed by a recovery, which could indicate a shift in the microbial population or even in the substrate structure. During the performance of the SSF the substrate structure is altered, i.e, the generation of more accessible and simple hydrolysates. These hydrolysates can be of any origin (proteins, lipids or carbohydrates) and therefore do not imply an improvement in the cellulase production.

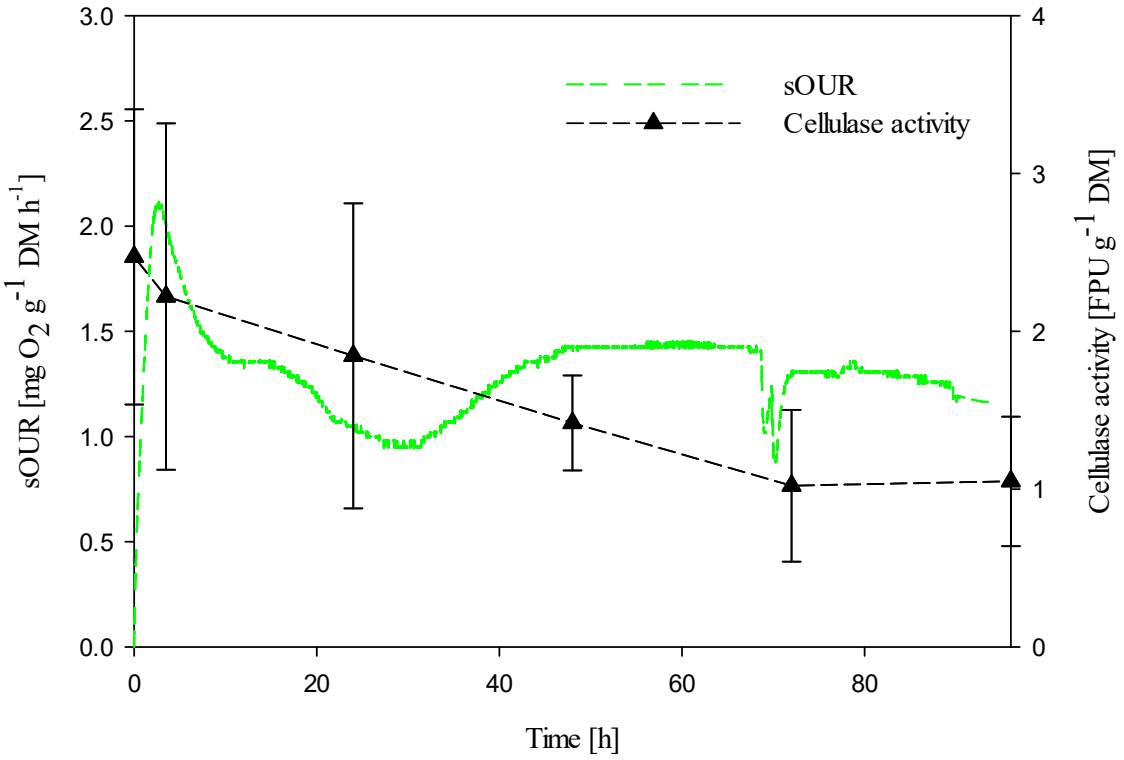

FIGURE 11. AVERAGE SOUR AND CELLULASE ACTIVITY PROFILES USING DG AS A SUBSTRATE IN A SSF CARRIED OUT IN 0.45-L REACTORS (S.D<5%).

Maximum cellulase activities were found at 48h of operation with values of 2.9 and 1.5 FPU g^{-1} DM using DE and DG respectively. Despite the higher cellulase production, DE was discarded because of the presence of a high amount of impurities hence hindering the proper handling of the material.

In order to validate the preliminary results obtained with DG, a new fermentation was set in order to obtain a full profile of cellulase production using a different DG. The substrate was obtained from the source (Granollers Treatment Plant) at a different time, therefore the impact of the initial characteristics of the material was also assessed. Results regarding these issues are presented in Figure 11.

The results observed in Figure 11 showed that the sOUR trend is similar to the observed in previous experiments (Figure 10a), however with values considerably lower. A significant decrease of maximum sOUR can be observed, achieving the value of 2.1 g O_2 g^{-1} DM h^{-1} at 2.9h of operation. This difference in the magnitude of maximum sOUR

using two different DG can be attributed to the presence of pruning remains in the first digestate tested and hence contributing to higher respiration activity.

As for cellulase activity, the production trend was different to the observed in the previous experiments (Figure 10a). In this fermentation, no peak of cellulase activity was found which can be associated to the lack of nutrients or specific substrate to promote the enzyme production. Maximum cellulase activity was found in the initial time of the fermentation (2.5 FPU g^{-1} DM), i.e. enzyme activity from the substrate, and it decreases over time until 1 FPU g^{-1} DM.

Considering that one of the main objectives of the DECISIVE project is to develop an easily scalable SSF using highly variable substrates, a standardization has to be performed. In this sense and based on the work reported by Cerda *et al*. (2017), an adequate alternative to achieve this goal is to use specialized microbiota adapted to degrade this substrate. An interesting alternative to obtain a specialized inoculum is to operate a series of sequential batches with a continuous addition of fresh substrate. Operating in this configuration can provide the generation of an adapted microbiota and even more, an enhancement in the enzyme production (Cerda *et al*., 2016).

4.3.3.2 Sequential batch operation for cellulase production

In relation to SB1 strategy, which was carried out without inoculation, a low cellulase activity was achieved. Results observed after 5 cycles showed that cellulase activity consistently decreased along the process, reaching values lower than 1 FPU g^{-1} DM (data not shown). For this reason, this strategy was discarded and SB2 was implemented. The results are presented in Figure 12.

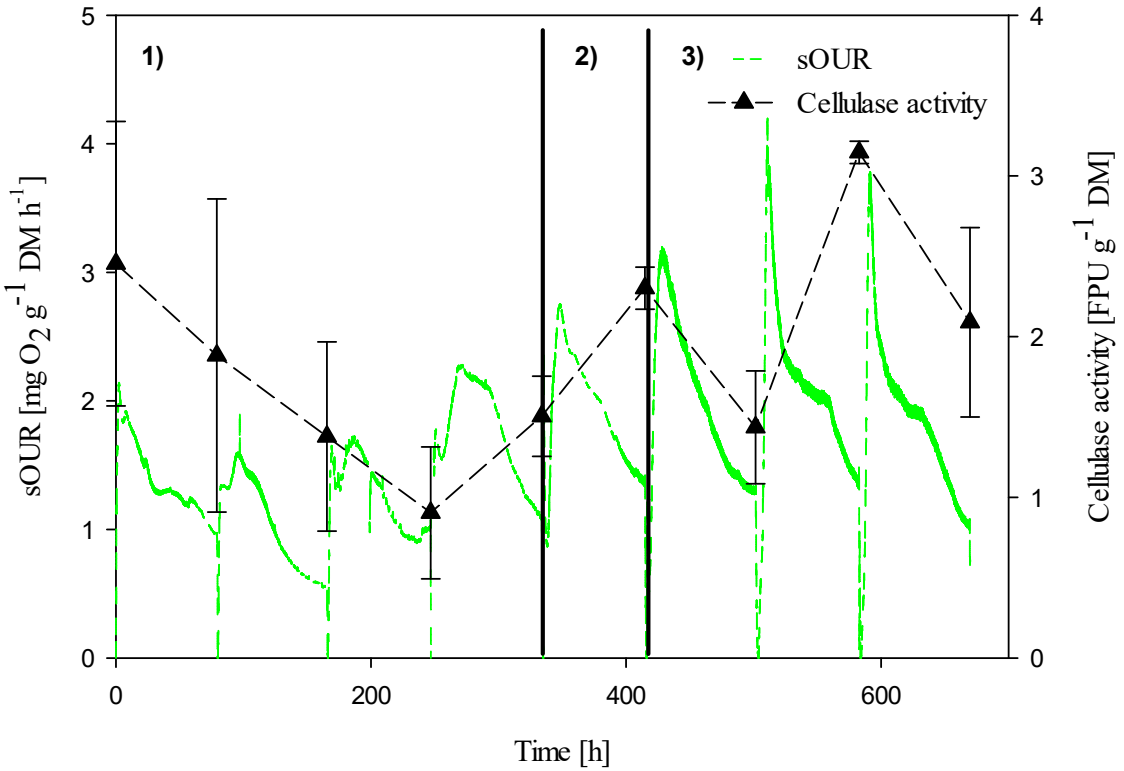

FIGURE 12. SOUR AND CELLULASE ACTIVITY PROFILES OBTAINED FROM A SEQUENTIAL BATCH OPERATION CARRIED OUT IN 0.45L REACTORS USING DG AS SUBSTRATE.

During the process DG was used as the substrate. However, the digestate was collected from the source at different moments. Due to the variability in DG composition three stages can be observed during the sequential batch operation. In stage 1, there is a clear trend, where maximum sOUR is nearly constant during the first three batches with an average value of sOURmax of 1.8±0.2 g O_2 kg^{-1} DM h^{-1}. After that moment, each sOUR profile showed a continuous increase until the end of the fermentation. In stage 2, fresh DG (arrived at UAB facilities the

same day) was fed and major differences between the batches were observed. There was a notorious increase of sOUR max achieving a maximum of 2.7 g O_2 kg^{-1} DM h^{-1}. Finally, in stage 3 of the process fresh and sanitized DG was fed to the fermented solids. This feeding generated a major increase of the biological activity, reaching a maximum sOUR of 4 g O_2 kg^{-1} DM h^{-1}.These results reflected a nearly 2-fold increase in the sOUR measurements observed at the beginning of the fermentation. Considering these results, it can be stated that the sequential batch operation was a successful strategy to adapt the microorganisms to degrade the digestate with a consistent increase in the biological activity. It can also be noticed that DG sanitation did not negatively affect the fermentation but, on the contrary, this pre-treatment resulted in benefits for the fermentation that can be associated with a reduction on microbial diversity or a possible ammonia stripping, although no pH differences were detected.

Cellulase production profile presented in Figure 12 shows a high initial cellulase activity of 2.4 FPU g^{-1} DM which consistently dropped during stage 1, reaching the lowest production at the end of the third batch with a value of 0.9 FPU g^{-1} DM. Once the sOUR started to recover, cellulase production also increased to 1.5 FPU g^{-1} DM at the end of the fourth batch. In this sense, it is likely that the microorganisms present at that moment were associated with the metabolism of cellulose degradation.

During stages 2 and 3 a further increase of cellulase activity was obtained. Maximum cellulase activity was found in seventh batch reaching a value of 3.15 FPU g^{-1} DM. The enzymatic production decreased in the last sampling, which can be attributed to different factors, such as the compaction of the solid bed, depletion of substrate, the generation of toxic/inhibitory compounds or even to the non-productive binding of cellulase into lignin hydrolysates (Van Dyk and Pletschke, 2012; Kuhad *et al*., 2016).

Another aspect to take into consideration is the value of sOUR during sampling, which could have affected cellulase extraction and quantification. In all stages, sampling was performed when the biological activities were at their lowest values (<1gO_2 kg^{-1} DM h^{-1}) and therefore less active. This is an attractive subject to optimize because it can be hypothesized that if sampling would have been performed when the biomass was active the cellulase production could have been higher.

The implementation of SB2 strategy was a success due to cellulase production increased in nearly 60%, however the production was still in the lower range of the reported literature (1-144 FPU g^{-1} DM) (El-Bakry *et al*., 2015). Other strategies are being studied, which contemplates the addition of lignocellulosic materials as the BA or the use of specific cellulolytic strains to enhance cellulase production.

4.4 Sophorolipids

Biosurfactants, surface-active molecules produced by a variety of microorganisms, are an alternative to conventional surfactants because they are readily biodegradable, display low toxicity and can be produced from renewable feedstocks, or even wastes, by fermentation (Mukherjee *et al*., 2016). Biosurfactants can be applied in many fields, such as environmental management, improvement of oil quality, synthesis of new polymers and bioplastics, or in the pharmaceutical industry (Singh *et al.,* 2007). Sophorolipids (SLs) are a group of extracellular biosurfactants produced at relatively high yields by several non-pathogenic yeast species, with *Starmerella bombicola* being the most studied. SLs are glycolipids comprised of a sophorose moiety coupled with a hydroxylated fatty acid. They are produced as a mixture of different molecules with two major points of variation: acetylation in the sophorose, and lactonization. Additionally, SLs have good anti-microbial, anti-inflammatory, anti-HIV and even anticancer effects, which allow these molecules to be used in the pharmaceutical sector (Rashad *et al*., 2014).

4.4.1 Experimental procedure

The digestate is a material that contains low amounts of soluble sugars (0.18%) and fats (5%), which are the main substrates required for SLs productions. Then, considering the requirements of fats and sugars, a set of four experiments were performed to determine the potential use of digestate as a substrate for sophorolipids (SLs) production using *Starmerella bombicola* ATCC22214 as inoculum. A full description of these experiments is detailed in Table 3.

TABLE 3. SUMMARY OF THE EXPERIMENTAL CONDITIONS FOR SOPHOROLIPIDS PRODUCTION BY SSF.

Operational conditions	Initial composition for SSF	
Experiment 1: Fat source effect		
Airflow: 20 mL min^{-1} Temperature: 30°C Time: 5 days Sterile digestate(121ºC, 20 min)	Bulking agent: Spontex (5%) Substrate: Digestate Granollers Inoculum: *S.Bombicola* Abbreviation: DG	Bulking agent: Spontex (5%) Substrate: Digestate Granollers Co-substrate: Olive Oil (10%) Inoculum: *S. Bombicola* Abbreviation: DGO
Experiment 2: Sugar source effect		
Airflow: 20 mL min^{-1} Temperature: 30°C Time: 5 days Sterile digestate (121ºC, 20 min)	Bulking agent: Spontex (5%). Substrate: Digestate Granollers. Co-substrate: Glucose (1%). Inoculum: *S. Bombicola.* Abbreviation: DGG	Bulking agent: Spontex (5%). Substrate: Digestate Granollers. Co-substrate: Oleic acid (10%) and Glucose (1%). Inoculum: *S.Bombicola.* Abbreviation: DGGA
Experiment 3: Sanitation effect		
Airflow: 20 mL min^{-1} Temperature: 30°C Time: 5 days Sanitized digestate (70ºC, 1h)	Bulking agent: Spontex (5%) Substrate: Sanitized Digestate Granollers Co-substrate: Glucose (1%) Inoculum: *S. Bombicola* Abbreviation: DGG	Bulking agent: Spontex (5%) Substrate: Sanitized Digestate Granollers Co-substrate: Glucose (1%) and Oleic Acid (10%) Inoculum: *S. Bombicola* Abbreviation: DGGA
Experiment 4: Food waste and recycled oil as substrates		
Airflow: 20 mL min^{-1} Temperature: 30°C Time:7 days Sanitized digestate (70ºC, 1h)	Bulking agent: Spontex (5%) Substrate: Apple Pomace (AP) Co-substrate: Recycled cooking oil (10%) Inoculum: *S. Bombicola*	Bulking agent: Spontex (5%) Substrate: Food waste (FW) Co-substrate: Recycled cooking oil (10%) Inoculum: *S. Bombicola*

Also, in order to obtain soluble sugars and to suppress the external sugar source addition, an acid pre-treatment was assessed, This pre-treatment was carried out using different concentrations of sulphuric acid (1.5%, 2%, 3% and 5%) at 121°C and then the soluble sugars were measured.

Fermentations were carried out in triplicates at lab scale using the experimental setup described in Section 3.2.1.1. A total of 100 g of mixture were assessed under controlled conditions of airflow and temperature. Routine analysis and specific measurements described in Section 3.3 were performed during the fermentations.

4.4.2 Specific analytical methods

4.4.2.1 Quantification

SLs were extracted from the fermentation mixture according to Jimenez-Peñalver *et al*. (2016). SL yield is defined as grams of sophorolipid per g of total dry mass of fermentation. In the text, SL yield is also reported as grams of sophorolipid per 100 g of substrate mixture for comparison with yields reported in the literature.

Water-soluble sugar content was measured in the supernatant using the anthrone method (Scott and Melvin, 1953).

4.4.2.2 Characterization

The structural identity of the SLs produced was first confirmed by Fourier Transform Infrared Spectroscopy (FTIR) (Tensor 27, Bruker). The instrument had an attenuated total reflection accessory (ATR Specac Golden Gate) which avoids the use of the KBr pellets. The infrared spectrum was recorded from 600 to 4000 cm^{-1}.

4.4.3 Results of sophorolipids production

4.4.3.1 Effect of the addition of a fat source to digestate in SLs production.

In this set of experiments the incorporation of an external fat source was considered. The addition of olive oil (O) to the DG was carried out under specific conditions (Table 3). In both experiments an increase on the pH was observed, from 8.8 to 9.3 (only DG as substrate) and to 9.1 (olive oil and digestate as substrates, DGO) (data not shown).

Figure 13 shows the sOUR profile of two different fermentations, using respectively only DG or DGO. A lag phase was present in both fermentations of nearly 15h and 24h for DGO and DG, respectively.

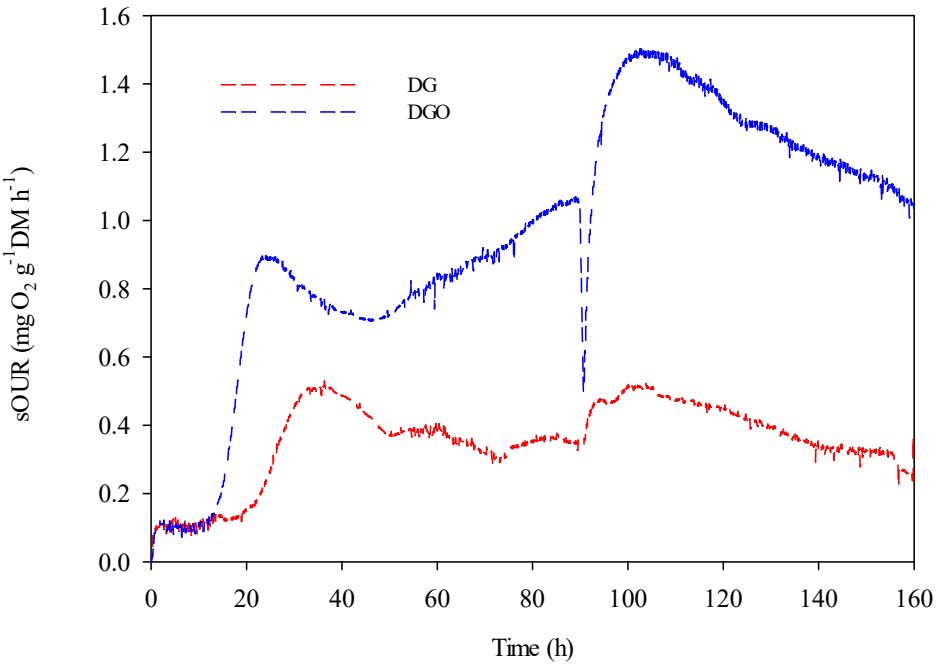

FIGURE 13. AVERAGE SOUR PROFILES DURING SSF IN 0.45L REACTORS ASSESSING THE ADDITION OF AN EXTERNAL FAT SOURCE (DGO) (S.D<5%).

After 96 hours, mixing was performed, generating an increase of sOUR in both reactors, but especially in the DGO fermentations. The latter, increased from 1 to 1.5 mgO_2 g^{-1}DM h^{-1}. Mixing increased the bioavailability of substrates to the yeast and, therefore, more oxygen and fats were consumed (Jimenez-Peñalver *et al;* 2016). In these fermentations, there was no SLs production in any case. This can be due to the type of substrate used for SLs production, which is probably not suitable for the microorganism to produce sophorolipids. *S. Bombicola* cultivation

is associated with a strong pH drop for optimal sophorolipid production, where the pH should be kept at 3.5 after a spontaneous decrease (Goobert *et al.,* 1984). Additionally, in order to attain the proper growth and SLs production, this yeast requires an initial high sugar concentration (100 g L^{-1} or more)which was not provided in the presented fermentations (Rosa and Lanchance, 1998). In any case, the incorporation of an external fat source resulted in an increased biological activity and hence in the next set of experiments the addition of an external sugar source was considered (Table 3).

4.4.3.2 Effect of the addition of a sugar source to digestate in SLs production.

In Figure 14 the complete sOUR profile of the SSF carried out at a lab scale at the conditions described in Table 3is presented (Experiment 2). Results showed positive results when external sources of fats and sugars were added.

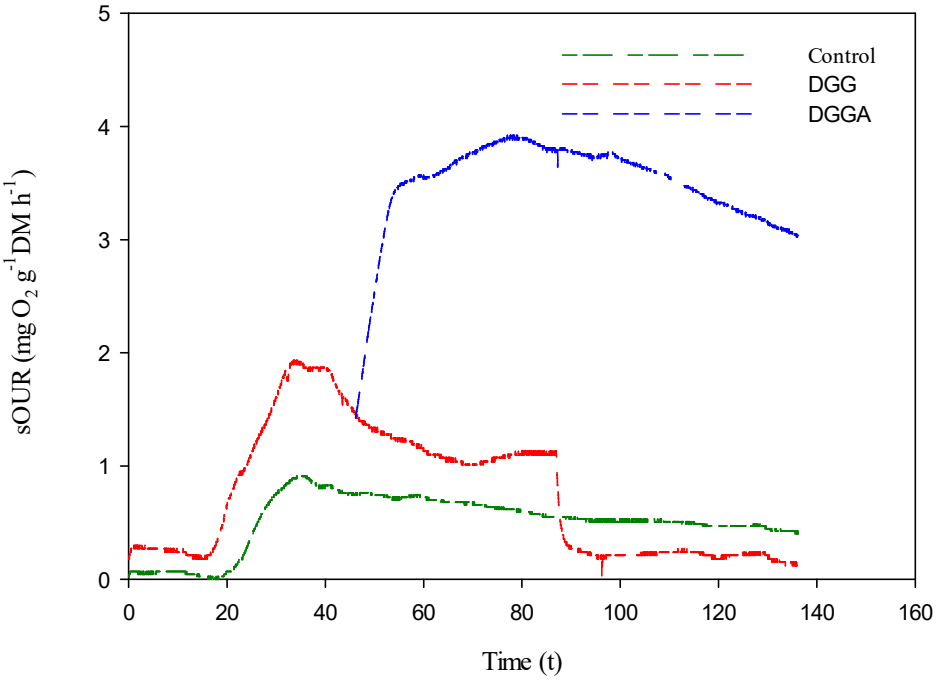

FIGURE 14. AVERAGE SOUR PROFILES OBTAINED IN A DG SSF IN 0.45L REACTORS ASSESSING THE ADDITION OF EXTERNAL GLUCOSE (DGG) AND FAT SOURCES (DGGA) (S.D<5%).

sOUR of DGGA fermentation presented a substantial increase when the three substrates were used, achieving a maximum value of around 4mg O_2 g^{-1} DM h^{-1} at 60h of operation and then, until the end of the fermentation the sOUR remained at this value. During this fermentation, there was a malfunction of the data acquisition system which led to an absence of sOUR values during the first 40h of fermentation.

Despite the positive results on biological activity enhancement, SLs were not produced once again. It can be attributed to the operational conditions which provided a hostile environment for the growth of *S. Bombicola*. Sugar content of the digestate was measured and the results showed a composition of 0.18% of total sugar and 0%of D-glucose which makes it a not ideal substrate for SLs production.

4.4.3.3 Effect of the sanitation of digestate in SLs production.

Considering the previous negative results obtained using sterile DG, it was hypothesized that the sterilization process may have some impact in the substrate structure that made the substrate more accessible, not only for *S. Bombicola*, but to other opportunistic microorganisms that used this substrate for their growth. Then, a last set of experiments were carried out, performing a sanitation process to DG at the conditions described in Section 3.1.1. These results are presented in Figure 15.

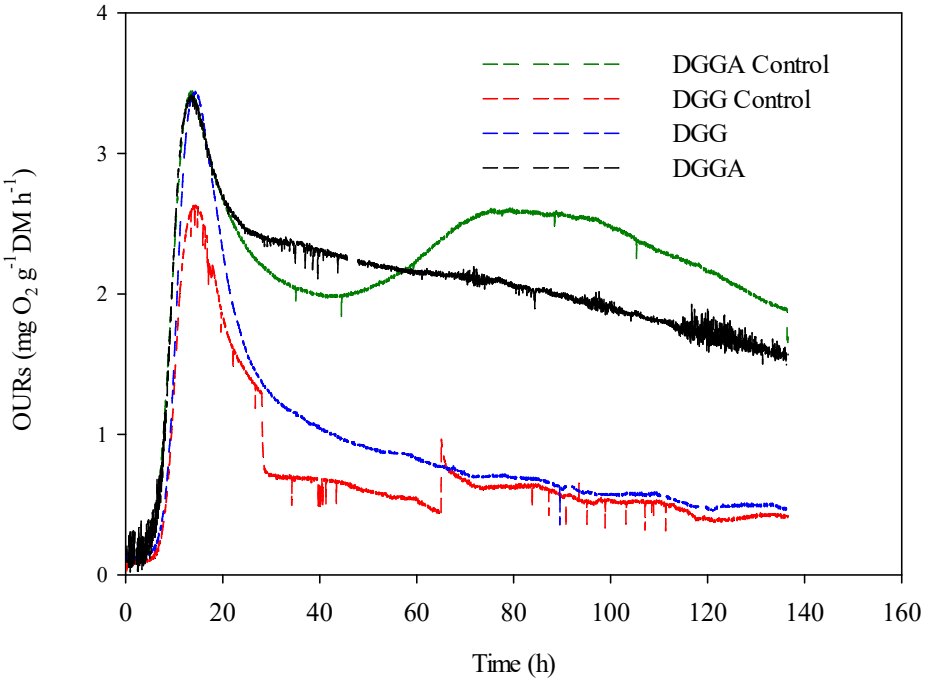

FIGURE 15. AVERAGE SOUR PROFILES USING SANITIZED DG AS A SUBSTRATE IN A SSF CARRIED OUT IN 0.45L REACTORS AND ASSESSING THE ADDITION OF EXTERNAL GLUCOSE (G) AND FAT SOURCES (A) (S.D<5%).

It can be observed that two controls were performed: DGG control (digestate and glucose) and DGGA control (digestate, glucose and oleic acid) (Figure 15). Inoculation of *S. bombicola* increased sOUR in the DGG experiments, however no significant effect was observed in DGGA experiments. The highest sOUR value in the fermentations supplemented with fats and sugar sources (DGGA) was 3.4 mg O_2 g^{-1} DM h^{-1}, obtained at 20h of operation. At the same time DGG fermentation also achieved its biological activity peak, with a value of nearly 2.5 mg O_2 g^{-1} DM h^{-1}. After that time, both fermentations decreased their biological activity.

The yield of sophorolipids was 0.020 g g^{-1}DM, which is a low yield when compared with that reported by Jimenez-Peñalver *et al*., (2016) which worked at similar conditions and obtained a yield of 19.1 g g^{-1}DM. These results indicate that even supplying external fat and sugar sources, DG is nor a suitable substrate for SLs production.

After SLs extraction, the product was analyzed by FTIR in order to identify the functional groups and their relation with the SLs structure. A full description of the functional groups associated with the SLs structure and the frequency where are obtained in the FTIR method is presented in Table 4. Results are presented in Figure 16. The spectrum revealed a broad band at 3396 cm^{-1}, which corresponds to the O-H stretch. Asymmetrical stretching and symmetrical stretching of methylene were observed at 2923 $cm^{-1}$and 2853 cm^{-1}, respectively. The C=O absorption band at 1741 cm^{-1} may include contributions from lactones, esters or acids. The stretch of the C-O band of C(=O)-O-C in lactones appeared at 1173 cm^{-1}.

TABLE 4. FREQUENCIES OF THE FTIR SPECTRA WITH THE CORRESPONDENT STRETCH, FUNCTIONAL GROUP AND STRUCTURE.

Frequency (cm^{-1})	Stretch	Functional group	Structure
3396	O-H	-OH	Acids and glucoses
2924	ν_{as} CH_2	-CH_2-	Fatty acid chain moiety
2854	ν_s CH_2	-CH_2-	Fatty acid chain moiety
1742	C=O	-C(=O)-O-X (X=H or R)	Lactones, esters and acids
1456	C-O-H	-COOH	Acids
1367	δ_s CH_3	R-O-C(=O)-CH_3	Acetyl group
1235	C=O	R-O-C(=O)-CH_3	Acetyl esters
1165	C-O	R´-C(=O)-O-R´´	Lactones
1034-1075	C-O	C-O-H	Glucoses (sophorose moiety)
721	C=C	-HC=CH-	Fatty acid chain moiety

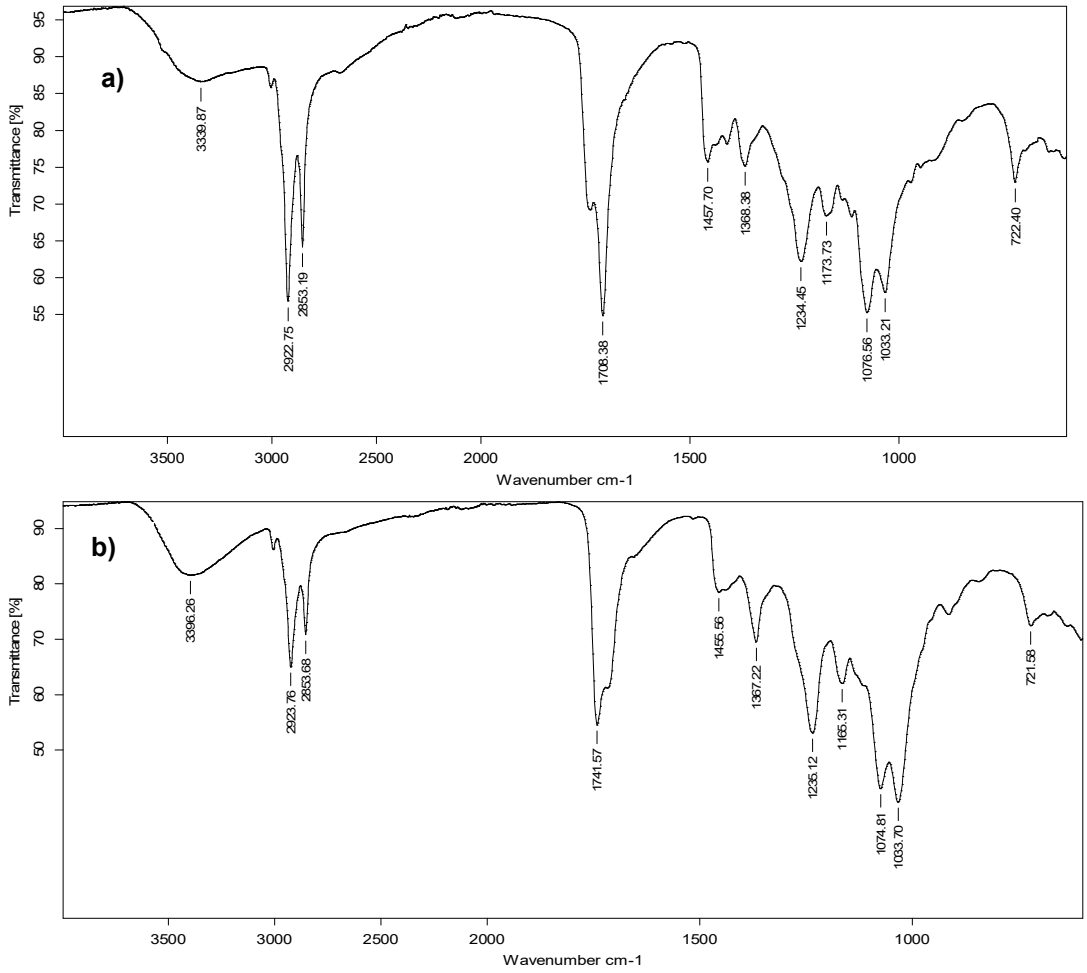

FIGURE 16. SPECTRA OF THE SL PRODUCED BY FTIR (A) PRODUCED IN THE FERMENTATIONS CARRIED OUT WITH DG AND EXTERNAL SUGAR AND FAT SOURCES (DGGA) AND B) THE REPORTED BY JIMENEZ-PEÑALVER *et al.* (2016).

The C=O absorption band at 1234 cm^{-1} of acetyl esters and the band at 1368 cm^{-1} for the symmetrical bending of the methyl groups of the acetyl esters indicate that the SL mixture contains acetylated sophorose moieties. The band

at 1455 cm^{-1}corresponds to the C-O-H in-plane bending of carboxylic acid (-COOH) and may indicate the presence of small quantities of unused fatty acids that were left after hexane washings or the contribution of acid SLs. The spectra also show the absorption for C=C at 721 cm^{-1}. Finally, a C-O stretch from C-O-H groups of sugars was observed at 1033–1074 cm^{-1}.The results of this work were compared with the results of the structural SLs characterization reported previously by Jiménez-Peñalver *et al*. (2016*)*. The presence of functional groups associated with the SLs structure and the similarities with the FTIR spectra reported by Jiménez-Peñalver *et al*. (2016*)* confirmed the presence of SLs.

4.4.3.4 Food waste and recycled cooking oil as substrate for SLs production.

In light to the low SL yield of the fermentations using digestate, the next step was to assess food waste (FW) as a substrate for SLs production. Also apple pomace (AP) was assessed as a specific, vegetable and rich in sugar fraction of food waste. Apple pomace and food waste were used as substrates and the results are presented in Figure 17. sOUR reported high values (5 mg O_2 g^{-1} DM h^{-1}) when using AP while FW achieved 3mg O_2 g^{-1} DM h^{-1}. In sOUR profile of AP it is possible to observe several peaks related to the mixing performed during reactors' sampling.

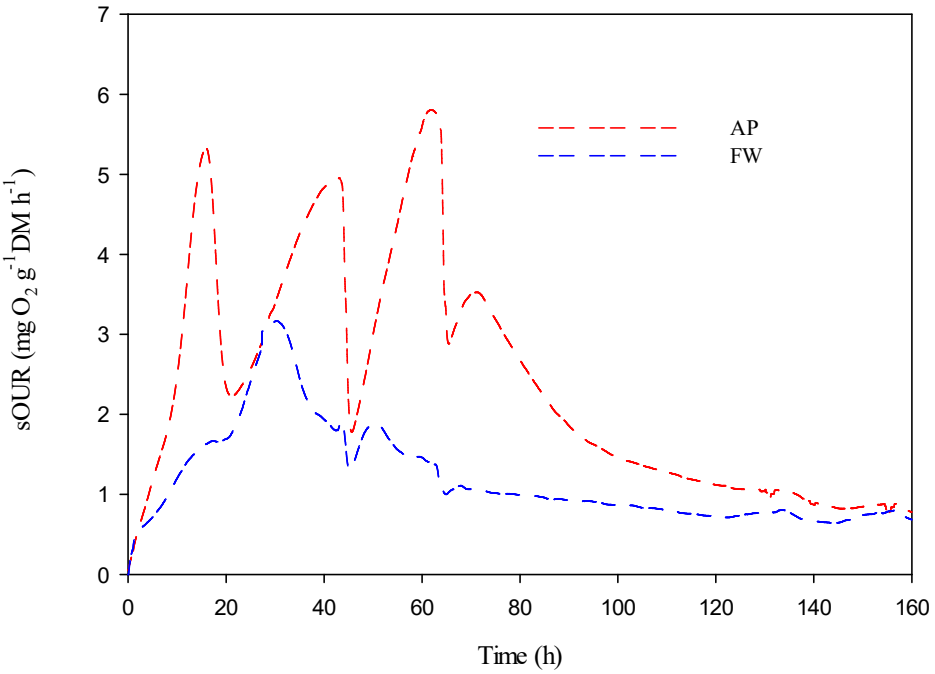

FIGURE 17. AVERAGE SOUR PROFILES USING NON-STERILE APPLE POMACE (AP) AND FOOD WASTE (FW) AS SUBSTRATES IN A SSF CARRIED OUT IN 0.45L REACTORS (S.D<10%).

As mentioned in section 4.4.1 (Table 3), these experiments were performed in triplicates. Regarding to the reproducibility of SSF using FW and AP as the substrate, it was observed that the sOUR profile was very similar among the three reactors (s.d<10%) (data not shown).

SLs extraction and quantification were carried out after 48h of process and at the end of the fermentation. AP fermentations yielded 0.020 g g^{-1}DM at 48h and 0.018 g g^{-1}DM at the end of the fermentation. In FW fermentations, SL yields were 0.031g g^{-1}DM and 0.025g g^{-1}DM at 48h and at the end of the fermentation, respectively. SLs yields showed no improvement when compared with the fermentations using DG as the substrate and therefore the valorisation of the wastes proposed in the DECISIVE project for SLs production is not viable.

4.4.3.5 Effect of an acid pretreatment on the digestate and the sugar release.

In the previous sections, it has been proven that DG was not a suitable substrate for SLs production. Despite that fact, when a sugar and fat source were included SLs were produced. In this sense it was expected that after the performance of the acidic pre-treatment, soluble sugars were released and they could be potentially used for SLs production. However, the best results were obtained by using 5% of H_2SO_4 observing two effects. The pre-treated

DG showed an acid pH of 6.5 which is suitable for yeasts growth and SLs production. In spite, the soluble sugar content was near 0.23% which made this pre-treatment not useful to hydrolyze the complex sugars present in the DG.

These results also provide information for a possible bioethanol production process. If it is not possible to release soluble sugars the bioethanol production is not feasible, and therefore stronger pre-treatments and intensive research on that process would be required, which is beyond the scope and the philosophy of the DECISIVE project.

4.5 Enzymatic cocktail

The anaerobic digestion of solid waste has an inconvenient the relative low rate of biodegradation due to this limiting solid (mainly composed of lignocelluloses) hydrolysis step (Mata-Alvarez et al., 2000). Since extracellular enzymes carry out the hydrolysis, some authors have investigated with positive results the direct addition of hydrolytic enzyme to enhance this stage for anaerobic digestion.

In this work, the effect of introducing: i) OFMSW which was aerobically pretreated over a short period, and ii) an enzymatic extract obtained from the same aerobically pretreated OFMSW, in the anaerobic digestion (AD) process of raw OFMSW has been studied and published in Martínez-Valdés et al. (2016). The aim to use the liquid extract from pre-treated OFMSW is to use it as an enzymatic cocktail in digestion experiments replacing the solid material. paper

4.5.1 Experimental procedure

OFMSW obtained from a municipal composting plant was aerobically degraded in lab-scale experiments using the system described in Section 3.2.1.1. The degradation process of an 11-day aerobic pre-treatment step was followed by measuring the sOUR and the enzymatic activities. The experiments were performed under near-adiabatic conditions with continuous aeration at a minimum rate of 100 mL min^{-1}. The reactors included a data acquisition system with a PLC (programmable logic controllers), which allowed data reading every minute. Particularly, PLC system read the values of oxygen, airflow and temperature, which are connected to a personal computer, and it enables on-line complete monitoring. The oxygen was regulated by means of airflow manipulation in the exhaust gas to maintain the system in favourable aerobic conditions (oxygen content above 12 %).

The monitoring of the process was done to quantify the extent of aerobic degradation as well as the time of the maximum biological activity. This aided in establishing the optimum time of treatment. The pre-treated material removed at that optimum time was used as a co-substrate during the anaerobic experiments that followed. The co-substrates were either the same pre-treated material, in its solid form, or a liquid extract obtained from that aerobically pre-treated solid waste.

The aerobically treated OFMSW and the corresponding treated extract were used as co-substrates with simulated raw OFMSW (S-OFMSW) in the subsequent anaerobic digestion (AD) experiments. Simulated OFMSW was used to overcome the inherent heterogeneity of the raw OFMSW that commonly leads to a large variance among replicate runs. The composition of S-OFMSW (on a wet weight basis) was: 17% cooked pasta, 7% bread, 15% salad components, 17% tomatoes, 17% apples, 17% oranges, 7% cooked meat and 1% napkins as suggested by the Agencia de Residus de Catalunya (2006). Control runs with S-OFMSW, solid treated OFMSW and the extract from treated OFMSW were performed to allow the comparison between the Biochemical Methane Potential (BMP) of the OFMSW with and without the use of the selected co-substrates. BMP from raw OFMSW from a composting plant was also determined with the aim to quantify the loss of biogas potential during aerobic treatment.

4.5.2 Specific analytical methods

The amylase enzyme activity was quantified through the release of reducing sugars using starch as substrate in 50 mM citrate buffer at a concentration of 0.5% as described in Omemu *et al.* (2005). 800 L of corn starch and 200 L of enzymatic extract were incubated at 60ºC for 1 h. The protease activity was determined using a modified method described by Alef and Nannipieri (1995). One mL aliquot of enzyme extract was added to 5 mL of casein solution at 2 % and was incubated at 50ºC under stirring for 2 h. Furthermore, enzymatic activity was reported as (U g^{-1} DM),

where one unit (U) is the amount of enzyme that in an enzymatic reaction catalyzes the conversion of 1 µmol of substrate per minute. All analyses were performed at least in duplicate.

4.5.3 Results of enzymatic cocktail production

Many authors have assessed the use of extracellular enzymes to improve the AD process, with positive results and obtaining an enhancement in biogas production (Mata-Alvarez *et al.,* 2014).

In this part of the report, the effect of introducing: i) OFMSW which was aerobically pre-treated over a short period, and ii) an enzymatic extract obtained from the same aerobically pre-treated OFMSW, in the anaerobic digestion (AD) process of raw OFMSW has been studied. The aim of using the liquid extract from pre-treated OFMSW is to use it as an enzymatic cocktail in digestion experiments.

4.5.3.1 Aerobic degradation process as a pre-treatment of AD experiments

Evolution of the degradation aerobic process

Temperature and sOUR profiles obtained during the aerobic degradation process are shown in Figure 17. The temperature profile is typical for aerobic degradation processes peaking at maximum of 72ºC on day 5. sOUR showed an initial peak presumably as a consequence of the presence of readily biodegradable compounds at the beginning of the process. The maximum sOUR and the peak of temperature were achieved at the same time, as is common in composting experiments (Puyuelo *et al*., 2010).

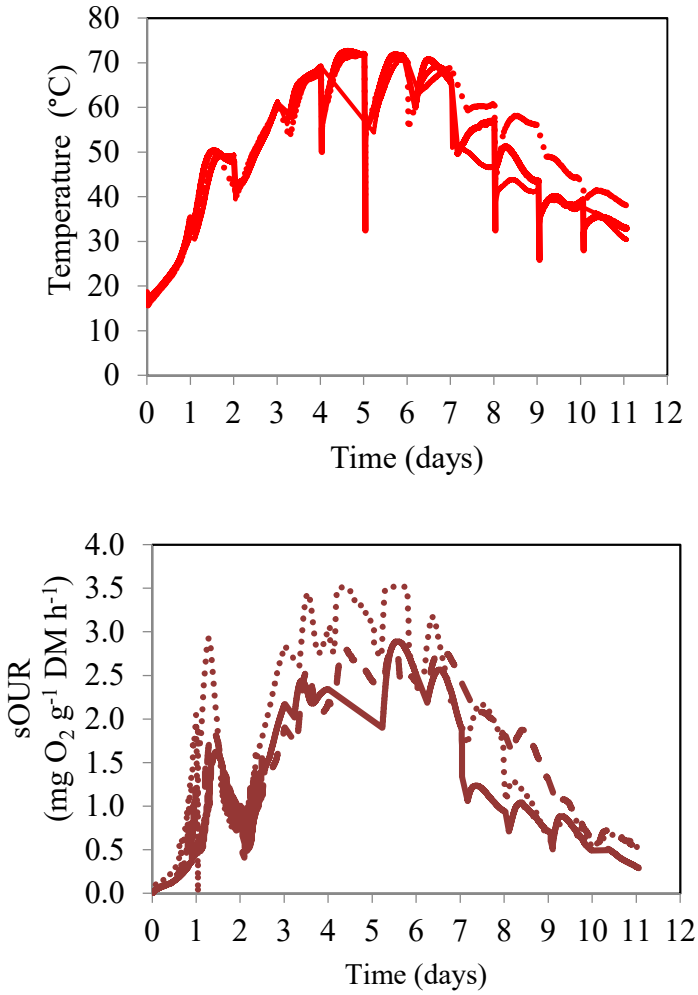

FIGURE 17. EVOLUTION OF AEROBIC BIODEGRADATION PROCESS OF THE RAW OFMSW IN A 10 L REACTOR (RESULTS ARE FROM TRIPLICATES); TEMPERATURE PROFILE (TOP) AND OXYGEN UPTAKE RATE (OUR) PROFILE (BOTTOM)

In Figure 18 the evolution of different parameters during the aerobic degradation of OFMSW is shown. pH decreased slightly at the beginning of the process, thereafter increased progressively until 8.8 on day 5 and remained at this value until the end of the process (Figure 18a).

Reducing sugars (Figure 18b) observed a maximum at around day 4 and remained constant after day 7. This is expected, since, during the aerobic stage, carbohydrate monomers, proteins and lipid monomers are consumed, since they are soluble and readily biodegradable. Amylase activity (Figure 18c), which initially had an activity of 2.8 U g^{-1} DM, decreased after day 1 to 0.2 U g^{-1} DM and, thereafter, increased progressively up to 2 U g^{-1} DM on day 6.

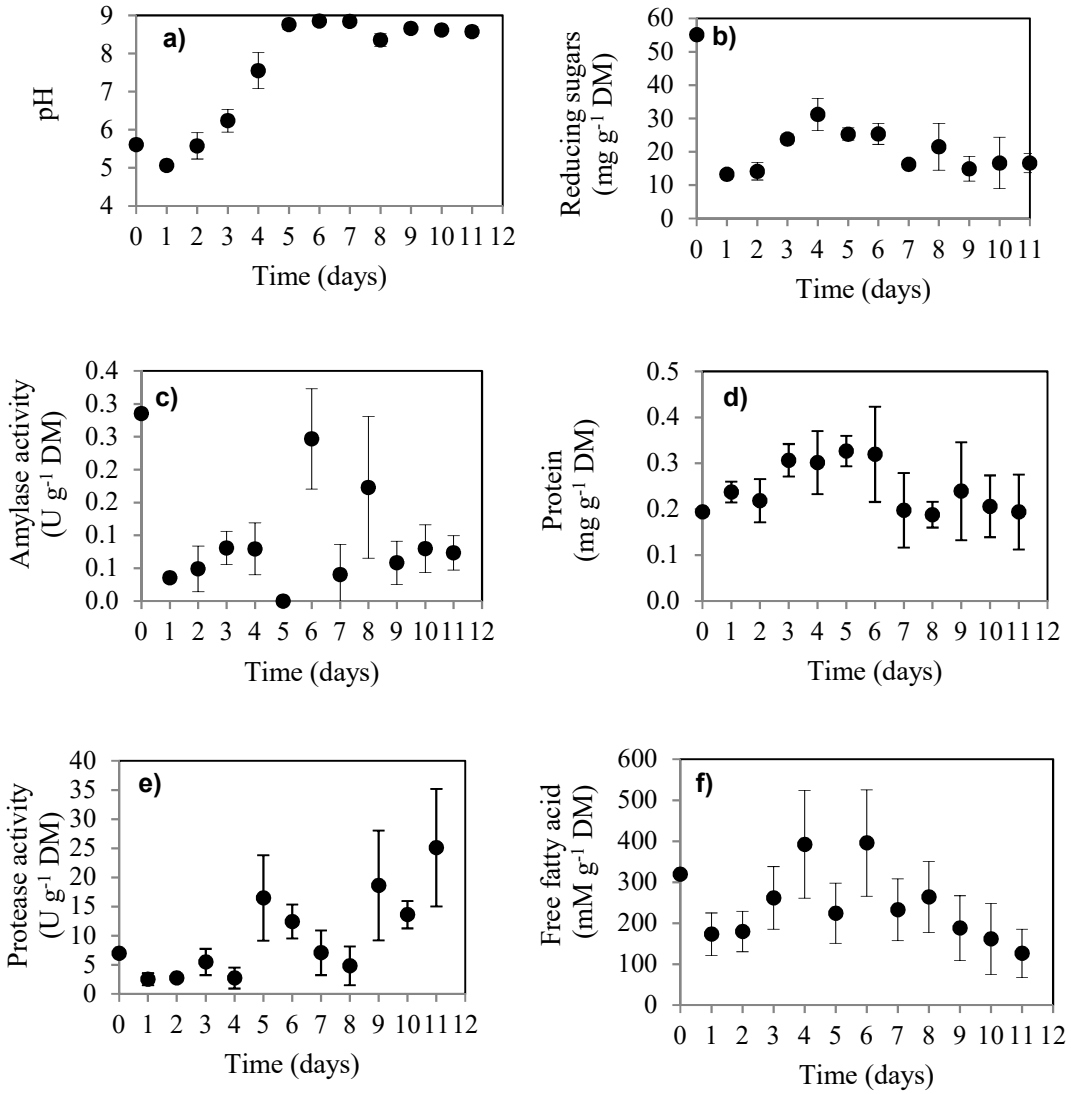

FIGURE 18. EVOLUTION OF DIFFERENT PARAMETERS DURING THE AEROBIC DEGRADATION OF OFMSW A) PH; B) REDUCING SUGARS; C) AMYLASE ACTIVITY; D) TOTAL PROTEIN, E) PROTEASE ACTIVITY AND F) FREE FATTY ACIDS. ERROR BARS REPRESENT THE STANDARD DEVIATION OF TRIPLICATES.

The proteolytic activity (Figure 18e) showed the first peak on day 5 reaching 16.4 U g^{-1} DM. However, an additional increase was observed after day 8 reaching values up to 25.4 Ug^{-1} DM. Free fatty acids also peaked on days 4-6 reaching concentrations up to 400 mMg^{-1}DM (Figure 18f). The increase in the concentration of soluble protein (Figure 18d) and free fatty acids can be related to the increase of metabolic activity during the aerobic degradation of OFMSW (Tejada *et al.*, 2009).

Therefore, it can be observed that the maximum concentration of reducing sugars and the enzymatic activities are in agreement with the maximum sOUR achieved. Maximum enzymatic activity has been related to the maximum metabolic activity, which can be measured indirectly through the oxygen uptake rates (Puyuelo *et al*., 2010).

Selection of the optimal time to aerobically pre-treat OFMSW before its use in the anaerobic digestion experiments

Figure 18 shows that a peak of metabolic activity was reached on the 5th day based on most of the parameters recorded (sOUR and enzymatic activities). Also, an important change in the pH of OFMSW was observed at day 5. At the beginning, pH was acidic (4.74), but after pre-treatment, an increase in pH in the range of 6.35 to 7.29 was achieved, approaching optimal values for AD. This fact can positively affect the anaerobic digestion process, since the acid generation during the AD process, commonly attributed to the presence of readily degradable organics, will be limited.

As a conclusion, the 5th day of aerobic degradation of OFMSW was selected as the appropriate time to remove material from the aerobic process and to use it in the anaerobic digestion process of S-OFMSW as a co-substrate. In addition to the solid, liquid extracts were obtained from that 5day aerobically pre-treated OFMSW for use in the AD experiments.

4.5.3.2 Anaerobic digestion experiments

Methane potential of the OFMSW

Methane production during the AD test of the different assays is shown in Figure 19.The parameters obtained after fitting the methane production experimental data to the Gompertz model, methane potential (P), the maximum rate of methane production (R_{max}) and the lag time (λ), are shown in Table 5. It is important to highlight that S-OFMSW had a maximum methane potential of 507 NL_{CH4} kg^{-1} VS and OFMSW (used in aerobic degradation experiments) of 518 NL_{CH4} kg^{-1} VS, being both statistically similar (at $p<0.05$) as shown in Table 5. This result validates the use of S-OFMSW in the AD assays, indicating that the simulation of OFMSW was close to reality. On the other hand, the R_{max} of the S-OFMSW and OFMSW were statistically different at $p<0.05$, being 75 and 54 $NLCH_4$ kg^{-1} VS d^{-1} respectively. This difference can be attributed to the potential presence of slowly biodegradable matter in raw OFMWS that, as commented, was collected already in a mixture with bulking agent that has a high lignocellulosic content.

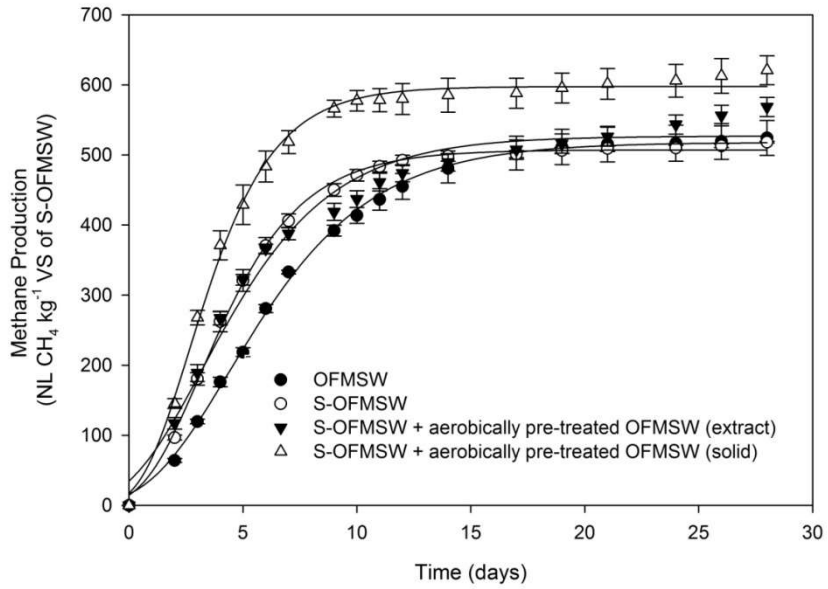

FIGURE 20. NET CUMULATIVE METHANE PRODUCTION DURING THE ANAEROBIC DIGESTION EXPERIMENTS (RESULTS ARE EXPRESSED PER MASS OF VS OF S-OFMSW OR OFMSW INCLUDED IN THE MIXTURE). THE SOLID CURVED LINE REPRESENTS THE GOMPERTZ MODEL FIT.

Methane potential after co-substrate addition

According to Table 5, if S-OFMSW is co-digested with the solid aerobically pre-treated OFMSW (50%), the maximum methane potential (expressed per kg VS of S-OFMSW basis) is increased by 19%. No such significant increase was, however, observed when the extract from the aerobically pre-treated OFMSW was used as co-substrate. These results are in agreements to the findings of Fisgativa (2016) on the impact of aerobic pretreatments of FW on AD performance.

Although no improvement was observed after adding the liquid extract, the increase of the maximum production rate (R_{max}) by almost 40% after the use of the pre-treated solid clearly indicated an improvement of the AD. Accordingly, an incomplete enzyme extraction from the pre-treated solid can explain the fact that this improvement was not observed when the extract was used directly. Also, this improvement could be due to a synergistic effect observed in an anaerobic co-digestion process (Mata-Alvarez *et al*., 2014).

TABLE 5. ACTUAL METHANE PRODUCTION (PREAL) AFTER 30D AND CALCULATED MAXIMUM METHANE POTENTIAL (P), MAXIMUM METHANE PRODUCTION RATE (RMAX) AND LAG PHASE (Λ) ESTIMATED BY THE FITTING OF THE GOMPERTZ MODEL TO THE DATA.

Test	P_{real} (NL CH_4 kg^{-1} VS of S-OFMSW)	P_{model} (NL CH_4 kg^{-1} VS of S-OFMSW)	R_{max} (NL CH_4 kg^{-1} VS of S-OFMSW day^{-1})	λ (day)
Control AD experiments				
S-OFMSW	518 ± 1.5^{b}	510 ± 2^{b}	74 ± 3^{b}	0.65 ± 0.11^{a}
OFMSW	524 ± 30^{b}	522 ± 27^{b}	53 ± 1^{c}	0.86 ± 0.12^{a}
Pre-treated OFMSW	430 ± 33^{c}	413 ± 35^{c}	42 ± 6^{d}	1.05 ± 0.3^{a}
AD experiments				
S-OFMSW + aerobically pre-treated OFMSW after 5 days *(liquid extract)*	568 ± 17^{b}	538 ± 14^{b}	55 ± 2^{c}	0.2 ± 0.41^{a}
S-OFMSW + aerobically pre-treated OFMSW after 5 days *(solid material)*	620 ± 16^{a}	602 ± 14^{a}	103 ± 4^{a}	0.54 ± 0.14^{a}

Different letters indicate statistically different means at $p < 0.05$. For the OFMSW control experiment, the results are expressed in $NLCH_4$ kg^{-1} VS of OFMSW.

Combination of aerobic and anaerobic treatment for OFMSW

Despite the benefits of aerobic pre-treatment prior to anaerobic digestion, it is important to consider the potential loss of biogas yield due to the loss of organic carbon during the aerobic OFMSW pretreatment step. In this work, due to the aerobic degradation, the biogas production of the pre-treated OFMSW was 18% lower than that generated with raw OFMSW. This loss could be considered an important loss of biogas potential, but it is important to note that only a fraction of the OFMSW will be pre-treated prior to AD. So, when the anaerobic co-digestion was performed using S-OFMSW with pre-treated OFMSW in a ratio 1:1 (w/w), there was an increase in the methane potential of approximately 20% in both the real data and the model estimates, compared to the S-OFMSW alone, and the maximum methane production rate also increased by 39%. As commented, this is probably attributed to

reasons related to co-digestion effects, such as the improvement of the balance of nutrients and the positive synergisms established in the digestion medium. In this sense, it seems to be a good compromise to direct one part of the OFMSW to aerobic pre-treatment for later use in the co-digestion of raw OFMSW. Thus, the combination of aerobic and anaerobic treatments could be an effective mode to apply the benefits of a short aerobic pre-treatment. However, the ratio in the co-digestion of aerobically pre-treatment OFMSW versus OFMSW should be optimised.

5. Conclusions

The research performed by UAB, in search for possibilities for the valorisation of biowaste, was focused on the production of the following bioproducts: Biopesticide, protease, cellulase and sophorolipids, using digestate as substrate; and enzymatic cocktail from OFMSW.

Protease, cellulase and sophorolipids production showed low productivities using digestate as the substrate when compared to literature and therefore they were discarded as potential bioproducts. Even when OFMSW was used as substrate, productivities were very low.

Despite the single enzyme production was discarded, the enzymatic cocktail production presented acceptable enzymatic activities. Aerobic treatment prior to anaerobic digestion showed an increase in the degradation rate and on the methane potential when pre-treated material is co-digested with fresh OFMSW.

Production of bioethanol was not assessed as insufficient amount of soluble sugars was present in the digestate and no sugars could be obtained by cellulase hydrolysis from digestate under mild conditions.

Biopesticides production provided the most interesting and successful results of this study. It was observed that there were no significant differences between the experiments carried out under sterile and non-sterile conditions at a lab scale, which is of great importance for the further scale-up assessment that it is included in the DECISIVE project. In addition, bench scale-up was performed with good results in terms of the growth of *Bacillus thuringiensis*. However it must be taken in consideration that growth issues were observed when the operation temperature increased until the thermophilic range. This aspect has to be further optimized for a proper reactor design or operational strategies have to be developed to allow performing an SSF process under a mesophilic temperature regime.

Some national regulations state that the digestate requires a sanitation stage previous to its use (by instance, in a fermentation process). This sanitation showed no significant differences among the productivities obtained for each assessed bioproduct (with and without sanitation). This pretreatment of the raw materials should be further discussed and agreed among the DECISIVE partners in order to finalize the setup for the following pilot activities, always considering the national regulations applied to these materials.

Annex I

21/7/2017 DECISIVE_BD_Caracterizaciones.xlsx

NÚMERO MUESTRAS	PROCEDENCIA2	FECHA DE RECOGIDA	CÓDIGO MUESTRA	CÓDIGO SAQ	REF.MUESTRA	Valores2	%C	%H	%N	%S	% H	% MS	% MO	% HEMICELULOS	% CELULOSA	% LIGNINA
1	Ecoparc2 , Montcada i Reixac	03/11/2016	ECO20161103	16AE241	ECO2 10/10/16	Media	28	3,4	2,1	0,5	82,7	17,3	63,6	15,49	9,32	11,26
1	Ecoparc2 , Montcada i Reixac	03/11/2016	ECO20161103	16AE241	ECO2 10/10/16	St.Desv.	8,6	7,9	5,8	14,7	1,2	1,2	6,3			
2	Ecoparc2 , Montcada i Reixac	22/11/2016	ECO20161122	17AE004/1	ECO PRS/COMP	Media	28,27	3,49	2,46	0,48	84	16	54,8	11,09	13,01	13,70
2	Ecoparc2 , Montcada i Reixac	22/11/2016	ECO20161122	17AE004/1	ECO PRS/COMP	St.Desv.	3,4	7,1	6,2	4	1	1	4,6			
3	Ecoparc Granollers (Barcelona)	30/11/2016	GRA20161130	17AE004/2	GRA 3011 2019	Media	38,8	5,03	2,99	0,4	75,1	24,9	71,8	9,41	12,85	20,35
3	Ecoparc Granollers (Barcelona)	30/11/2016	GRA20161130	17AE004/2	GRA 3011 2020	St.Desv.	2,5	4,2	3,2	7,8	0,3	0,3	1,3			
4	Ecoparc2 , Montcada i Reixac	16/01/2017	ECO20170116	17AE082/5	ECO16012017	Media	33,11	4,12	2,71	0,49	81,0	19,0	52,8			
4	Ecoparc2 , Montcada i Reixac	16/01/2017	ECO20170116	17AE082/5	ECO16012017	St.Desv.	1,2	0,4	3,6	2,2	1,1	1,1	2,3			
5	Ecoparc Granollers (Barcelona)	16/01/2017	GRA20170116	17AE082/1	GRA16012017	Media	33,45	4,35	3,07	0,50	75,0	25,0	23,9			
5	Ecoparc Granollers (Barcelona)	16/01/2017	GRA20170116	17AE082/1	GRA16012017	St.Desv.	1,00	5,50	1,70	3,10	0,6	0,6	2,0			
6	Ecoparc Granollers (Barcelona)	30/01/2017	GRA20170130	17AE014/4	4 Support	Media	35,86	4,36	3,28	0,24	74,5	25,5	78,7	9,34	9,95	15,33
6	Ecoparc Granollers (Barcelona)	30/01/2017	GRA20170130	17AE014/4	4 Support	St.Desv.	0,7	0,6	0,8	20,4	0,9	0,9	15,6			
7	Ecoparc Granollers (Barcelona)	06/02/2017	GRA20170206	17AE022	GRA/ARG/COMP	Media	35,09	4,33	3,10	0,10	70,7	29,3	61,3	9,71	10,56	17,81
7	Ecoparc Granollers (Barcelona)	06/02/2017	GRA20170206	17AE022	GRA/ARG/COMP	St.Desv.	0,62	0,06	0,04	0,00	0,4	0,4	1,0			
8	Ecoparc Granollers (Barcelona)	20/02/2017	GRA20170220	17AE034	GRA20022017	Media	35,18	4,59	2,86	0,15	88,6	11,4	76,7	9,93	9,94	19,2
8	Ecoparc Granollers (Barcelona)	20/02/2017	GRA20170220	17AE034	GRA 20022017	St.Desv.	1,08	0,24	0,19	0,03	0,9	0,9	3,3			
9	Ecoparc Granollers (Barcelona)	13/03/2017	GRA20170313	17AE046	GRA 13032017	Media	*33,38*	*4,11*	*2,88*	*0,34*	72,8	27,2	61,3	11,77	9,34	15,78
9	Ecoparc Granollers (Barcelona)	13/03/2017	GRA20170313	17AE046	GRA 13032017	St.Desv.	*0,63*	*0,04*	*0,21*	*0,08*	0,6	0,6	1,0			
10	Ecoparc Granollers (Barcelona)	24/03/2017	GRA20170324	17AE082/2	GRA24032017	Media	30,75	3,96	2,53	0,41	73,3	26,7	65,5	10,2	9,87	16,77
10	Ecoparc Granollers (Barcelona)	24/03/2017	GRA20170324	17AE082/2	GRA24032017	St.Desv.	1,10	1,8	0,9	12,5	0,2	0,2	0,5			
11	Ecoparc Granollers (Barcelona)	08/05/2017	GRA20170508	17AE082/3	GRA31032017	Media	32,53	4,06	2,65	0,44	74,4	25,6	63,7	11,44	11,65	21,25
11	Ecoparc Granollers (Barcelona)	08/05/2017	GRA20170508	17AE082/3	GRA31032017	St.Desv.	2,20	0,8	6,1	4,4	0,3	0,3	1,5			
12	Ecoparc Granollers (Barcelona)	31/05/2017	GRA20170531	17AE091	GRA20170531	Media	32,23	4,06	2,65	0,41	76,2	23,8	64,1	9,75	9,9	14,95
12	Ecoparc Granollers (Barcelona)	31/05/2017	GRA20170531	17AE091	GRA20170531	St.Desv.	0,88	0,02	0,01	0,00	0,4	0,4	0,4			

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