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Valorisation of digestate from biowaste through solid-state fermentation to obtain value added bioproducts: A first approach

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Abstract

Digestate from biowaste was assessed as a potential source of bioproducts of commercial and industrial interest through solid-state fermentation. The targeted bioproducts were hydrolytic enzymes (cellulases and proteases from autochthonous microbiome), biosurfactants (sophorolipids produced from *Starmella bombicola*) and biopesticides (produced from *Bacillus thuringiensis*). Low cellulase production was observed within the range of 0.5-1.5 FPU g^{-1}DM while protease production showed two discrete peaks of 66±8 and 65±3 U g^{-1}DM at 3.5 and 48h, respectively. Low sophorolipids production was also obtained, with a maximum yield of 0.02 g g^{-1}DM using hygienised digestate supplemented with external sugar and fat sources. Biopesticides produced by *B.thuringiensis* were successfully at 72h of operation, reaching a maximum spore production of 8.15±0.04 (10^7) CFU g^{-1} DM and 2.85±0.22(10^7) CFU g^{-1} DM using sterile and hygienised digestate, respectively. These biopesticides could contribute to the substitution of chemically produced pesticides, moving towards a sustainable digestate management in a circular economy scheme.

Keywords: solid state fermentation, digestate, valorisation, bioproducts, biopesticide.

1. Introduction

In the last years, the EU has developed legislation that affected the waste management of organic materials. The Landfill Directive 199/31/EC aims to divert waste disposal from landfills and the Waste Directive 2008/98/EC aims to develop a new waste management hierarchy that promotes the use of wastes as secondary raw materials. In this context, anaerobic digestion (AD) is a widely used technology that can produce biogas, which contributes to the substitution of fossil fuels, providing a highly efficient method for resource recycling that allows to close the production cycle. However, the sustainability of AD processes will depend on the ability of the plant operator to properly manage the digestate remaining after the AD process (Dahlin et al., 2015).

Digestate is a heterogeneous material produced in large amounts during the anaerobic digestion process (Dahlin et al., 2017; Monlau et al., 2015). Nearly 95% of the digestate produced in Europe can be used as organic fertiliser reducing the needs of for chemically produced fertilizers (Fuldauer et al., 2018; Saveyn & Eder, 2013). Digestate obtained from source selected biowaste is conventionally sent to composting or to direct soil application, however its use is currently limited due to nutrient content, quality of the digestate (Paavola & Rintala, 2008), possible land contamination and, in some countries, the lack of fertile land (Fuldauer et al., 2018). In this sense, a more sustainable approach is proposed by the DECISIVE project (www.decisive2020.eu) in terms of digestate management, considering this waste as a feedstock for further biotransformation through solid-state fermentation in a circular economy context.

Solid-state fermentation (SSF) is defined as a process that takes place in a solid matrix in absence or near absence of free water (Thomas et al., 2013). SSF is an attractive technology that allows the use of solid substrates for biotransformation into value-added bioproducts (El-Bakry et al., 2015). Hydrolytic enzymes (El-Bakry et al., 2015), biosurfactants (Jiménez-Peñalver et al., 2016; Vishal and Aniruddha, 2012), aromas (Martínez et al., 2017), biopesticides (Ballardo et al., 2017; Monlau et al., 2015) or bioplastics (Castilho et al., 2009) have been successfully produced by SSF using different organic wastes. Conventionally, these bioproducts are produced in established and highly controlled conditions using specific microorganisms, defined substrates and carried out under sterile conditions which imply high operational costs. In order to reduce these costs, authors have developed a SSF

process carried out using non sterile and heterogeneous solid substrates for the production of hydrolytic enzymes (Abraham et al., 2003; Cerda et al., 2016; Cerda el al., 2017a), biopesticides (Ballardo et al., 2017) and biosurfactants (Jiménez-Peñalver et al., 2016). These authors reported robust and reproducible processes working at laboratory, bench and pilot scale (Abraham et al., 2017; Ballardo et al., 2017; Cerda et al., 2017b; Martínez et al., 2018). A first approach to biowaste digestate valorisation via SSF has been recently proposed by Mejias et al (2018) for cellulase and xylanase production using *Trichoderma reesei*, which is a well-known cellulase producer. However, these authors found low productivities mainly due to the poor nutritional quality of digestate. Thus, it seems interesting to further assess the potential of digestate as a source for the production of different specific bioproducts. Developing bio-based products represents economic and environmental benefits such as the replacement of chemically produced or fossil fuels derived materials, which is of great relevance in the circular economy concept.

In this context, the main goal of this work is to assess the potential of digestate without any pretreatment as a source of value added bioproducts by means of solid-state fermentation. A screening of bioproducts with industrial interest was carried out to achieve this goal. This first screening will provide information for the potential selection of the most suitable bioproduct to be obtained from digestate and to be further optimised. The selected targeted bioproducts were hydrolytic enzymes (cellulases and proteases), biosurfactants (sophorolipids) and biopesticides (derived from *Bacillus thuringiensis*). To the best of our knowledge, this is the first work targeting biowaste digestate as a source of value added bioproducts within a biorefinery concept.

2. Materials and Methods

2.1 Raw material

Digestate was kindly provided by a biowaste treatment plant (Granollers, Spain). This material was obtained from the anaerobic digestion of source selected biowaste. The process consisted in a mechanical pretreatment to reduce impurities followed by a mesophilic wet anaerobic digestion followed by a solid/liquid separation stage carried out using a screw press. Initial characterization of the digestate is presented in Table 1. Given the variability of biowaste digestate, standard deviation in

this table corresponds to the average of more than 10 samples analysed during a year and collected from the municipal waste treatment plant. In this plant, digestate is further composted.

Table 1

Once digestate was received in the facilities, the material was subjected to an hygienisation process as required by the European Regulation 142/2011. This process consisted in maintaining the residue for 1h into a previously heated oven at 70ºC. In order to avoid moisture losses, digestate was covered during the hygienisation process. After this process, the material remained stored in a cold room at 4ºC for a maximum of 15 days.

2.2 Experimental set-up

This setup consisted of 12 lines, each one able to carry out 3 independent fermentations using specific reactors (0.45 L). These reactors were cylindrical polyvinylchloride packed-bed reactors (13x7cm) each one provided with one mass airflow-meter with the exhaust gases outlet connected to carbon dioxide and oxygen sensors. The reactors were submerged in thermostatic baths in order to keep the temperature stable in a fixed value. The temperature depended on the process carried out and the optimum temperature of the specific microorganisms.

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

2.3 Bioproducts

Digestate was assessed as a source for the production of selected bioproducts through solid-state fermentation. In all experiments, raw digestate was used without any pretreatment as the main objective of this work is to evaluate the possibilities of digestate as obtained. Two of the main concerns when working with digestate are the low porosity of the material and the high moisture content (Paavola & Rintala, 2008). For these reasons, sponge cloth (Spontex®) pieces were added as

bulking agent in all fermentations. Sponge cloth is an inert material that provided adequate porosity and moisture levels to the solid mixture hence promoting the oxygen transfer through the solid matrix (Rodríguez-Couto, 2012; Ruggieri et al., 2009).

The targeted bioproducts were: hydrolytic enzymes (cellulases and proteases), biosurfactants (sophorolipids) and biopesticides derived from *Bacillus thuringiensis* (Bt). Hydrolytic enzymes are bioproducts obtained from a wide spectrum of microorganisms (El-Bakry et al., 2015) and often by the autochthonous microorganisms present in the waste (Abraham et al., 2013; Cerda et al, 2017a). The latter studies showed that it is possible to obtain hydrolytic enzymes using non sterile substrates thus reducing sterilisation related operational costs. Therefore, in the present study the potential of hydrolytic enzymes production from autochthonous microorganisms was assessed using hygienised digestate as the substrate. Different cases are those related to biopesticides and biosurfactants: these are specific bioproducts that require the inoculation of specific microorganisms (Ballardo et al., 2017; Jiménez-Peñalver et al., 2016). Therefore, the effect of the sterile and hygienised digestate was assessed. The main objective of these activities was to study the potential synergetic/antagonist effect that the autochthonous microorganisms may have on the externally added strain. Experimental conditions were selected on the basis of previous studies where we observed that some combinations did not produce a significant amount of bioproducts. This is the case, for instance, of sophorolipids production with no sterilisation.

A complete experimental methodology is detailed in the following section for each bioproduct.

2.3.1 Hydrolytic enzymes production using autochthonous microbiome

a) Procedure

Fermentations were performed using hygienised digestate as the substrate and sponge cloth (Spontex®) as the bulking agent in a ratio of 95:5 (w/w). The fermentations were carried out in triplicates in 0.45 L reactors with a total weight of 120 g of material in each reactor. The processes were performed at 37ºC using a fixed airflow of 20 mL min^{-1} during 96 h as previously reported by Abraham et al. (2013).

Sampling was performed at 0, 24 and 48h of fermentation by removing 3g of the content of each reactor. During the process, protease and cellulase activity and biological activity measured as sOUR were continuously monitored in addition to routine analysis, among them dry matter, moisture content and pH.

b) Specific analytical methods

Extraction for cellulase determination was done by mixing fermented solids with 0.05 M citrate buffer (pH 4.8) in a ratio 1:15 (w/v), shaking for 30 minutes. Extract was then centrifuged at 10000 rpm during 10 min and filtered to 0.45 µm. Supernatant was used for cellulase assay. Total cellulase activity was measured using filter paper assay (FPase), recommended by IUPAC (Ghose, 1987). Results were expressed as FPU g^{-1} DM, where one unit of FPase (FPU) was defined as the amount of enzyme that releases 1 µmol of reducing sugars from Whatman filter paper per minute.

Extraction for protease determination was carried out by mixing the fermented solid with 50 mM HCl-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). Supernatant was used as crude enzyme extract. Alkaline protease activity was determined using a method previously described by Alef and Nannipieri (1995). One unit of protease activity was defined as 1 µg of tyrosine released under the assay conditions.

2.3.2 Biosurfactants production using *Starmella bombicola* as inoculum

a) Inoculum preparation

Starmerella bombicola ATCC 22214 was obtained from the American Type Culture Collection (Manassas, USA). The microorganism was preserved at −80ºC according to the provider's recommendations, and a new agar slant was cultivated when needed. *S. bombicola* growth conditions for inoculum preparation were performed according to the methodology presented by Jimenez-Peñalver et al. (2016).

b) Procedure

Digestate is a material with low sugar (0.18%) and fat content (5%) (Table 1), which are the two main mandatory substrates for an efficient sophorolipids production as reported by Jimenez-Peñalver et al, (2016) and Vishal and Aniruddha, (2012). In order to tackle the nutritional requirements, external sugar and/or fat sources were considered using glucose and oleic acid as model substrates. Additionally, considering the high operational costs associated to a sterilization process,the use of sterile (121ºC, 20 min) and hygienised digestate (70ºC, 1 h) was also assessed. Specifically, in a first stage four combinations were assessed using sterile digestate: i) digestate (D), ii) digestate supplemented with glucose 1% (DG), iii) digestate supplemented with oleic acid 10% (w/w) (DA) and iv) digestate supplemented with glucose 1% and oleic acid 10% (w/w) (DGA). Digestate sterilisation process was performed in an autoclave at 121ºC for a period of 20 min.

In a second stage, the most successful combinations from the first stage were tested using hygienised digestate: i) digestate supplemented with glucose 1%(w/w) (DG_s) and ii) digestate supplemented with glucose 1%(w/w) and oleic acid 10% (w/w) (DGA_s). Digestate hygienisation was performed by maintaining the material at 70ºC in an oven for a period of 1h.

All experiments were carried out in triplicates in 0.45 L reactors according to the methodology described by Jimenez-Peñalver et al. (2016) and are summarised in Table 2. A total of 100 g of mixture were assessed under controlled conditions of airflow (20 mL min^{-1}) and temperature (30ºC) for a period of 5 days. In all experiments, sponge cloth (Spontex®) was added in a 5% (w/w) ratio as the bulking agent. Sampling was performed at the end of the fermentation by removing 3g of the content of each reactor. Specific and routine measurements were performed with the collected samples.

Regarding mixing, some authors (Jimenez-Peñalver et al. 2016) showed that intermittent mixing at 3, 5 and 7 days increased sophorolipids production in 25% compared to static conditions, when using different wastes. Hence, this strategy was implemented in the present work, selecting 96h instead of 72h due to the lag phase observed in our system.

Table 2

c) Specific analytical methods

Sophorolipids were extracted from the fermentation mixture according to Jimenez-Peñalver et al. (2016). Sophorolipids yield is defined as grams of sophorolipids per g of total dry mass of fermentation. In the text, the 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 & Melvin, 1953).

2.3.3 Biopesticides production using *Bacillus thuringiensis* as inoculum

a) Inoculum preparation

B. thuringiensis var. Kurstaky NRRL HD-73 (CECT 4497) (Bt) used in this study was acquired from the "Colección Española de cultivos tipo" (Valencia, Spain). The strain was rehydrated and stored at -80 ºC in cryovials with 10% (v/v) glycerol according to provider's recommendation. Bt growth conditions for inoculum preparation were carried out according to the methodology presented by Ballardo et al. (2017).

b) Procedure

Ballardo et al (2017) have already proven that *B. thuringiensis* is able to grow in a complex matrix of different substrates (soy fibre, OFMSW, among others) without any nutrient supplementation, and then no supplementation was planned for biopesticide production experiments.

Fermentations were carried out in 0.45 L reactors using a mixture of digestate as substrate and sponge cloth (Spontex®) as the bulking agent (BA) in a 5% (w/w) ratio. The total weight of the inoculated solid matrix was 120 g of mixture per fermentation. Due to the nature of the substrate two sets of experiments were performed: one using sterile digestate and another without sterilization. Bt-containing inoculum with a concentration of 10^7 CFU mL^{-1} was added in a 10% (w/w) ratio to the solid matrix. These fermentations were carried out in triplicates at 30ºC using a constant airflow of 20 mL min^{-1} during 96 h. Samples of 3 g of each reactor were collected at 0, 12, 24, 48 and 96 h of the fermentation time to ascertain the evolution of viable Bt cells and spores.

c) Specific analytical methods

Viable cell count and spore count were performed according to Ballardo et al. (2016). Briefly, viable cells were extracted from the solid phase using Ringer solution in a 1:9 (w/v) ratio stirred at 130 rpm for 20 min. Serial dilutions were prepared, plated in Petri dishes and incubated at 30ºC for 20h. After that, manual counting of viable cells was performed. For spore count, the diluted sample was maintained at 80ºC at 10 min and then placed in a cold bath for 5 min. After that, the same procedure for viable cells was followed. All the counts were done in triplicate and were expressed as colony forming units per gram of dry matter (CFU g DM^{-1}). Images of sporulated and vegetative forms of Bt were observed by using an OLYMPUS optical microscope BH-2 with an objective of x100. Bt cells and spores were visually identified. In previous experiments, Ballardo et al. (2016) gram stain was used to differentiate and identify Bt in the mixture, and malachite green was used as a differential stain for bacterial endospores. After a large number of experiments, it was noted that these last staining procedure was not necessary to identify Bt cells and spores.

2.4 Dynamic respiration index

Dynamic respiration index (DRI) is an index that reflects the stabilisation of organic wastes. This parameter was determined according to the methodology described by Ponsá et al. (2010). Briefly, DRI can be calculated from oxygen and air flow data from a given time through Equation (1) derived from a steady state mass balance:

$$DRI = \frac{(O_{2,i} - O_{2,o}) \times F \times 31.98 \times 60 \times 1000^{a}}{1000^{b} \times 22.4 \times DM} \qquad \text{Equation (1)}$$

where, DRI is the dynamic respiration index expressed in mg O_2 g^{-1} DM h^{-1}; $(O_{2,i} - O_{2,o})$, the difference in oxygen content between airflow in and out the reactor at a given time; F is the volumetric airflow under normal conditions, mL min^{-1}; 60, the conversion factor, min h^{-1}; 1000^{a}, the conversion factor, mg g^{-1}; 1000^{b}, the conversion factor mL L^{-1}; 22.4, the volume occupied by one mol of ideal gas under normal conditions, L; DM, the dry matter of sample loaded in the reactor, g.

DRI when is measuring the biological activity of a sample in a specific moment of fermentations is named as specific oxygen uptake rate (sOUR) with the same units.

2.5 Routine methods

Moisture content, total and volatile solids, pH and electrical conductivity were determined according to standard procedures (Composting Council, 2001). Cellulose, hemicellulose and lignin content were determined by the method of Van Soest et al. (1991) using the Ankom 200 Fiber Analyzer incubator (Ankom Technology, Macedon, NY).

2.6 Statistical analysis

All experimental data has been analysed using pairwise comparisons were based on the Tukey test ($p < 0.05$). Statistics were performed with MINITAB ™ V17. If some experimental value exceeded a 95% of confidence, it was marked with an asterisk in Figures.

3. Results and Discussion

3.1 Hydrolytic enzymes production

Results of digestate characterisation (Table 1) showed in that it contains significant amounts of protein and lignocellulosic material, hence it was hypothesized that digestate could have potential as a source for proteases and cellulases production.

The average sOUR profile obtained in the SSF for hydrolytic enzymes production is presented in Figure 1. The initial biological activity observed in the fermentations reflected that digestate presented a considerable content of aerobically biodegradable organic matter (Barrena et al., 2006). Maximum sOUR was found at 10h of fermentation with a value of 2.6±0.2 mg O_2 g^{-1} DM h^{-1}. After that peak, biological activity decreased to a value of near 2.2 mg O_2 g^{-1} DM h^{-1} which remained stable until the end of the fermentation. This is a typical behaviour of organic wastes under aerobic biodegradation that never reach a sOUR near to zero.

Figure 1

Cellulase production ranged between 0.5-1.5 FPU g^{-1}DM, which is in the lower range reported at laboratory scale at similar conditions (Cerda et al., 2017a) and even lower when compared with SSF using sterile substrates or adding a single strain (Dhillon et al., 2012; El-Bakry et al., 2015).

Figure 1 also shows that there is no clear correlation between maximum biological activity measured as sOUR and maximum cellulase activity. This is not in agreement with the reported by Cerda et al. (2017a), who 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 any other relevant parameter, indicating a highly complex process with different microorganisms and/or metabolites working together in a synergetic or antagonistic manner (Cerda et al., 2017a; Eichorst et al., 2013).

Digestate presented a significant initial cellulase activity when compared with the rest of the fermentation (1.57±0.52 FPU g^{-1}DM). This is probably associated with the previous anaerobic digestion process. The produced enzymes could remain immobilized in the solid substrate and hence, obtaining a high initial enzymatic content in the digestate.

In general, there is a consistent decline in cellulase production, followed by a slight recovery at the end of the fermentation, which could indicate shifts in the microbial population or even in the substrate structure (Cerda et al., 2017b; López-González et al., 2014). This can be explained by a change between anaerobic conditions (in which is obtained the digestate) and aerobic conditions (in SSF). However, this should be confirmed by specific microbial analysis and characterisation.

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 such as proteins, lipids or carbohydrates and therefore do not imply an improvement of the cellulase production. The low cellulase production can be attributed to the fact that during the fermentation the pH achieved values between 8-9 and cellulase production conventionally occurs at acid pH (4-6) (Dhillon et al., 2012; El-Bakry et al., 2015; Li et al., 2013). Another aspect to take into consideration is the extraction ratio of cellulases. Several options for a further study on cellulase production can be assessed such as the optimisation of the extraction ratio or even the extracting agent (Pirota et al., 2013), the adaptation of the native microorganisms using a sequential batch operation strategy (Cerda et al., 2017a) or the

inoculation with specific strains. Another unexplored alternative could be direct enzyme extraction followed by stabilization of the exhausted solid by means of composting as proposed by Marin et al. (2018).

Figure 1 also shows the protease production profiles. Two discrete enzymatic peaks were observed, 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. Again, as proteases are produced by anaerobic and aerobic microorganisms it is possible that going from one condition to another one can be reflected as two peaks of proteases production. A similar production profile was reported by Novelli et al. (2016), observing many protease activity peaks when using several fungi strains as mixed inoculum. These authors attributed this profile to underlying adaptations in the molecular and physiological machinery of the microbiome present in the substrate mixture.

The obtained values of proteases production are in the lower range of those reported in literature using different substrates. Abraham et al. (2013) used the same SSF configuration for protease production using soy fibre, 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 is likely that the pH observed during the fermentation (8.7-9) promoted protease production as stated by several authors (Abraham et al., 2013; El-Bakry et al., 2015; Soares et al., 2005). Moreover, proteases hydrolytic capacity is also boosted by alkaline conditions. The release of protease to the solid media at suitable hydrolytic conditions could have induced the hydrolysis of other enzymatic components, such as the produced cellulases, hence affecting cellulase production yield.

Summarizing, in this first screening digestate was not found suitable as a substrate for cellulase and protease production using only autochthonous microbiome. Further optimisation must be performed in order to increase the production yields. One of the difficulties of using digestate as a substrate is the low biodegradable matter content, therefore, one alternative to boost microbial activity can be the use of a biodegradable co-substrate such as the organic fraction of municipal solid wastes.

Another interesting alternative to be assessed is the development of different operational strategies such as working in a sequential batch configuration thus allowing the adaptation of the autochthonous

microbiome as reported by Cerda et al. (2017a). Another approach would be to work with specific strains to overproduce, by instance, proteases, as successfully reported by El-Bakry et al. (2016).

3.2 Biosurfactant production

In a first set of experiments the incorporation of external fat and sugar sources were considered. The addition of glucose (G) and oleic acid (A) to sterile digestate was carried out under specific conditions summarized in Table 2. *S. bombicola* was inoculated in all cases since the beginning of the experiment. In all fermentations, pH slightly increased from 8.8 to an average value of 9.2±0.2. It is known that pH drops to acidic values when *S. bombicola* metabolises the nutrients and synthesizes the sophorolipid. Due to the high buffering capacity of digestate, lowering the pH prior to fermentation was discarded since high amounts acidic solutions would be required.

Figure 2a shows the sOUR profiles of the first set of fermentations, using respectively D, DG, DA and DGA. A lag phase was present in all fermentations, however, this was of particular relevance in those fermentations using an external fat source (DA and DGA). After 96 hours, mixing was performed, generating an increase of sOUR in the DA from 1 to 1.5 mg O_2 g^{-1} DM h^{-1}. Mixing increased the bioavailability of substrates to the yeast and, therefore, more oxygen and fats were consumed (Jiménez-Peñalver et al., 2016).

Figure 2

Figure 2a shows positive results in terms of biological activity when external sources of fats and sugars were added. sOUR of DGA fermentation presented a substantial increase, where the three substrates were used, achieving a maximum value of 3.8 mg O_2 g^{-1} DM h^{-1} at 76 h of operation. After that peak, sOUR showed a slow decrease, reaching at the end of the fermentation a value of 3 mg O_2 g^{-1} DM h^{-1}. In spite of the good results, obtained in terms of biological activity, no sophorolipids production was observed. This fact can be attributed to different aspects of the fermentation such as the pH observed during the process (Thomas et al., 2013). *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 (Göbbert et al., 1984). Additionally, in order to attain the proper growth and sophorolipids production, this yeast requires a high initial sugar concentration (100 g L^{-1} or more)

which was not provided in the presented fermentations (Rosa & Lachance, 1998; Vishal and Aniruddha, 2012). Therefore, the addition of an external sugar source was considered. In this sense, there is no consensus among reported research on the subject, observing that while Vishal and Aniruddha, (2012) reported positive effects, Jimenez-Peñalver et al. (2016) obtained no improvements on sophorolipids production. It is also possible that the sterilization process may have some impact in the substrate structure that made the substrate more accessible for the microorganisms present during the fermentation. It also has to be considered that sterilization of large amounts of solids is a complex process that may only reduce biodiversity but not completely eliminate microorganisms initially present. For that reason, it is possible that the surviving microorganisms would be able to thrive in the substrate, competing *S. Bombicola* thus hindering the production process. However, in our experience, the use of short times (2-5 days) of fermentation makes very difficult to observe the growth of opportunistic microorganisms, especially when the inoculation of a specific strain is performed. Additional fermentations were carried out using hygienised digestate at the conditions described in Table 2. These results are presented in Figure 2b.

The highest sOUR in both fermentations was found at 20h of operation with values of nearly 3.4 mg O_2 g^{-1} DM h^{-1} for DGA_s and DG_s. After that time, both fermentations decreased their biological activity. Although sOUR peak was similar for both conditions, showing similar easiness of biodegradability, DGA_s kept higher sOUR values showing a higher microbial activity due to a higher biodegradable OM content (Ponsá et al., 2010). The only positive result obtained from these series of experiment was in DGA fermentation, where the sophorolipid production yield was 0.020 g g^{-1} DM, which is a low value when compared with that reported by Jimenez-Peñalver et al., (2016). These authors reported a yield of 19.1 g g^{-1} DM using winterization residue and molasses as the substrate and *S. Bombicola* as the inoculum. Moreover, the obtained yield using digestate supplemented with sugar and fat sources was lower than that obtained by Vishal and Aniruddha, (2012) which observed 38 g g^{-1} DM using wheat bran supplemented with oleic acid and glucose. The overall results of this part of the work indicate that sophorolipids production using digestate as the main substrate is not a suitable option without exhaustive optimisation. These optimisations must meet the nutritional requirements for the proper *S. Bombicola* growth (acid pH) and sugar and fat

content for sophorolipids production. A potential alternative to achieve these goals could be the use of residual streams derived from a productive process, thus promoting waste valorisation and circular economy.

3.3 Biopesticide production

Figure 3 shows the results of the SSF carried out using digestate as sole substrate under sterile (Figure 3a) and non-sterile conditions (Figure 3b) for biopesticide production using Bt.

In fermentations using sterile digestate (Figure 3a), the maximum sOUR was obtained at nearly 9h of operation reaching a value of 0.80±0.08 mg O_2 g^{-1} DM h^{-1} and decreased afterwards until the end of the process. Bt inoculation generated a rapid start-up of the process, generating 2-fold increase on the biological activity reflected as sOUR when compared with sterile digestate non inoculated with Bt (data not shown). Also, the differences among the triplicates were minimal (s.d<5%), which indicates that the process is reliable and reproducible as reported by other authors in similar processes (Abraham et al., 2017; Cerda et al., 2017b).

Figure 3

Figure 3a also shows the viable cell and spore counts during the fermentations. Viable cell count in all replicates showed an initial increase in the first 48h until values of 8.49±2.12 (10^8) CFU g^{-1}DM. After that period, viable cell count decreased until values of 9.09±0.81(10^7) CFU g^{-1}DM. These results are very positive, because they reflect the ability of Bt of not only surviving in a complex solid matrix based on digestate, but to thrive and achieve an almost 3-fold increase the initial viable cell count. It is likely that the decrease in viable cell count is related to changes in the solid environment, highly attributed to a depletion of substrate, hence leading to the bacteria to create their resistant form, i.e spores. Digestate has a low content of readily metabolizable compounds, then it seems plausible to assume that in a non-sterile fermentation this material would be quickly depleted. Martinez et al. (2017) showed that when using a high soluble sugar content substrate (30% d.b) such as sugar beet molasses in a non-sterile SSF, the sugar content was reduced in nearly 80% in 24h.

In this sense, the fermentations started with no spore presence in the solid matrix, being only detected from the first sampling at 24 h of fermentation, reaching a maximum at 72 h with a value of

8.15±0.04 (10^7) CFU g^{-1}DM. After that period, spore count remained almost stable until the end of the fermentation. One hypothesis to explain why spore count remains stable at 72 h could be that a steady-state may be reached, where part of the spores produced can germinate if a favourable microenvironment is created in the reactor, while other viable cells start the sporulation process due to stressful situations in other non-favourable microenvironment.

Other studies using different substrates working at similar conditions observed a final cell count of 6.6 $\cdot 10^{10}$ CFU g^{-1} DM (Devi et al., 2005), $1.0 \cdot 10^{10}$ CFU g^{-1} DM (Zhuang et al., 2011) and $6.2 \cdot 10^{11}$ CFU g^{-1} DM (Ballardo et al., 2016), which are higher than those obtained in the present study using digestate as the substrate. On the other hand, spore counts reported by the same authors ranged between 10^7-10^9 CFU g^{-1} DM, which indicates that the results obtained in this work are in the middle range of those reported in other publications. It has to be considered that the substrates used by Devi et al., (2005) and Ballardo et al., (2016) were wheat bran and soy fibre, respectively. These materials present low variability in their composition and were (in the case of Devi et al., (2015)) supplemented with external carbon and nitrogen sources.

This is of great importance considering that it is during the sporulation stage that Bt is able to produce the insecticidal crystal (Cry or Cyttoxins)(Bravo et al., 2011). In this context, it can be stated that digestate presents a limited amount of nutrients that provokes Bt sporulation in a short time.

Figure 3b presents the results obtained using non-sterile digestate. Biological activity observed in this case increased faster and was overall higher than under sterile conditions, which is likely related to the higher presence of microbial populations in the reactors. Maximum sOUR was found at 4h with an average value of 2.53±0.83 mg O_2 g^{-1} DM h^{-1}. 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 and higher deviation among replicates.

In contrast with that observed in the experiments using sterile digestate, Bt was not able to thrive onto the hygienised digestate in the same magnitude than when using sterile digestate. In this sense, a maximum of cell growth was observed at 24h with a viable cell count of 1.11±0.10 (10^8) CFU g^{-1} DM, representing a 1.34-fold increase of the initial cell count. After that moment, viable cells decreased until the end of the fermentation until values of 6.02±0.56 (10^7) CFU g^{-1} DM. As for the

spore production, the fermentation started with no spore content detected in the digestate and only became accountable after the first 24h of operation, when the viable cell started to decrease. Spore counts reached its maximum at 72h of fermentation, with a value of 2.85±0.22 (10^7) CFU g^{-1} DM. Previous research performed at the same conditions using soy fibre and biowaste as substrates, presented a higher viable cell count of $3.8 \cdot 10^{11}$ CFU g^{-1} DM (Ballardo et al., 2016) but a spore count of $2.1 \cdot 10^7$ CFU g^{-1} DM (Ballardo et al., 2017), which is in the range of that obtained in the present work. In is possible that the substrates used by these authors had more available nutrients and hence providing a more suitable environment for the growth of Bt. The same authors confirmed that the final cell and spore counts 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 (biowaste) the final viable cell counts obtained were $9.9 \cdot 10^5$ CFU g^{-1} DM, $1.1 \cdot 10^7$ CFU g^{-1} DM and $2.5 \cdot 10^7$ CFU g^{-1} DM, respectively (Ballardo et al., 2017). All these values are lower than the observed in the present work, showing that unfavourable conditions for Bt growth promote its sporulation.

More interestingly, from the present study it can be stated that it is possible the use of digestate as a sole substrate for biopesticide production. Bt was able to grow and to sporulate onto the solid matrix using hygienised digestate at a controlled temperature. This will be of great importance looking towards the development of the productive process and the assessment of its associated costs.

The use of digestate as a substrate for bioconversion into a value-added product will contribute to close the organic matter cycle, hence optimising its management into a circular approach. The positive outcomes obtained in this work open new alternatives for the valorisation of digestate as a substrate for biopesticide production. Further studies have to be performed in order to validate these results at a larger scale (bench or pilot) to assess the process technical, economic and environmental feasibility. Moreover, different biopesticide production could be assessed by using other microorganisms such as other bacteria or even fungi.

4. Conclusions

Digestate was assessed as a substrate for bioproducts via SSF, approaching a new waste management strategy. In spite several production strategies have been tested, hydrolytic enzymes and biosurfactant production reported low production yields. Further studies are required to enhance productivities. The best results were obtained for Bt-produced biopesticide. This microorganism was able to grow and to sporulate using hygienised digestate (non-sterile) as a sole substrate. Spore production obtained was in the middle range of the values reported in other references. Further studies will be performed for optimisation, scale -up and field tests on biopesticide action.

E-supplementary data for this work can be found in e-version of this paper online

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References

1. Abraham, J., Gea, T., Komilis, D., Sanchez, A. 2017. Reproducibility of solid-state fermentation at bench-scale: the case of protease production. Global NEST J., 19(2), 183-190.

2. Abraham, J., Gea, T., Sánchez, A. 2013. Potential of the solid-state fermentation of soy fibre residues by native microbial populations for bench-scale alkaline protease production.Biochem. Eng. J., 74, 15-19.

3. Alef, K., Nannipieri, P. 1995. 7 - Enzyme activities A2 - Alef, Kassem. in: Methods in Applied Soil Microbiology and Biochemistry, (Ed.) P. Nannipieri, Academic Press. London, pp. 311-373.

4. Ballardo, C., Abraham, J., Barrena, R., Artola, A., Gea, T., Sánchez, A. 2016. Valorization of soy waste through SSF for the production of compost enriched with *Bacillus thuringiensis* with biopesticide properties. J. Environ. Manage., 169, 126-131.

5. Ballardo, C., Barrena, R., Artola, A., Sánchez, A. 2017. A novel strategy for producing compost with enhanced biopesticide properties through solid-state fermentation of biowaste and inoculation with Bacillus thuringiensis. Waste Manage., 70, 53-58.

6. Barrena, R., Vázquez, F., Sánchez, A. 2006. The use of respiration indices in the composting process: a review. Waste Manage. Res., 24(1), 37-47.

7. Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M. 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem.Mol. Biol., 41(7), 423-431.

8. Castilho, L.R., Mitchell, D.A., Freire, D.M. 2009. Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation. Bioresour. Technol., 100(23), 5996-6009.

9. Cerda, A., Gea, T., Vargas-García, M., Sánchez, A. 2017a. Towards a competitive solid state fermentation: cellulases production from coffee husk by sequential batch operation and role of microbial diversity. Sci. Total Environ.589, 56–65.

10. Cerda, A., Mejías, L., Gea, T., Sánchez, A. 2017b. Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: The consistency of the process and the microbial communities involved. Bioresour. Technol.243, 1059-1068.

11. Composting Council, U.S. 2001. Test Methods for the Examination of Composting and Compost, Edaphos International. Houston, U.S.

12. Dahlin, J., Herbes, C., Nelles, M. 2015. Biogas digestate marketing: Qualitative insights into the supply side. Resour. Conserv. Recycl., 104, 152-161.

13. Dahlin, J., Nelles, M., Herbes, C. 2017. Biogas digestate management: Evaluating the attitudes and perceptions of German gardeners towards digestate-based soil amendments. Resour. Conserv. Recycl., 118, 27-38.

14. Devi, P.S., Ravinder, T., Jaidev, C. 2005. Cost-effective production of *Bacillus thuringiensis* by solid-state fermentation. J. Invertebr. Pathol., 88(2), 163-168.

15. Dhillon, G., Kaur, S., Brar, S., Verma, M. 2012. Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation. Ind. Crops Prod., 38(0), 6-13.

16. Eichorst, S., Varanasi, P., Stavila, V., Zemla, M., Auer, M., Singh, S., Simmons, B., Singer, S. 2013. Community dynamics of cellulose-adapted thermophilic bacterial consortia. Environ. Microbiol., 15(9), 2573-2587.

17. El-Bakry, M., Abraham, J., Cerda, A., Barrena, R., Ponsá, S., Gea, T., Sánchez, A. 2015. From Wastes to High Value Added Products: Novel Aspects of SSF in the Production of Enzymes. Crit. Rev.Environ.Sci. Technol., 45(18), 1999-2042.

18. El-Bakry, M., Gea, T., Sánchez, A. 2016. Inoculation effect of thermophilic microorganisms on protease production through solid-state fermentation under non-sterile conditions at lab and bench scale (SSF). Bioprocess Biosyst. Eng., 39(4), 585-592.

19. Fuldauer, L.I., Parker, B.M., Yaman, R., Borrion, A. 2018. Managing anaerobic digestate from food waste in the urban environment: Evaluating the feasibility from an interdisciplinary perspective. J. Clean.Prod., 185, 929-940.

20. Ghose, T. 1987. Measurement of cellulase activities. Pure Appl. Chem., 59(2), 257-268.

21. Göbbert, U., Lang, S., Wagner, F. 1984. Sophorose lipid formation by resting cells of *Torulopsis bombicola*. Biotechnol. Lett., 6(4), 225-230.

22. Jiménez-Peñalver, P., Gea, T., Sánchez, A., Font, X. 2016. Production of sophorolipids from winterization oil cake by solid-state fermentation: Optimization, monitoring and effect of mixing. Biochem. Eng. J., 115, 93-100.

23. Li, C., Yang, Z., He Can Zhang, R., Zhang, D., Chen, S., Ma, L. 2013. Effect of pH on cellulase production and morphology of *Trichoderma reesei* and the application in cellulosic material hydrolysis. J. Biotechnol., 168(4), 470-477.

24. López-González, J., Vargas-García, M., López, M., Suárez-Estrella, F., Jurado, M., Moreno, J. 2014. Enzymatic characterization of microbial isolates from lignocellulose waste composting: Chronological evolution. J. Environ. Manage., 145, 137-146.

25. Marín, M., Artola, A., Sánchez, A. 2018. Optimization of Down-Stream for Cellulases Produced Under Solid-State Fermentation of Coffee Husk. *Waste Biomass Valori.* DOI: https://doi.org/10.1007/s12649-018-0327-5.

26. Martínez, O., Sánchez, A., Font, X., Barrena, R. 2018. Enhancing the bioproduction of value-added aroma compounds via solid-state fermentation of sugarcane bagasse and sugar beet molasses: Operational strategies and scaling-up of the process. Bioresour. Technol., 263, 136-144.

27. Martínez, O., Sánchez, A., Font, X., Barrena, R. 2017. Valorization of sugarcane bagasse and sugar beet molasses using *Kluyveromyces marxianus* for producing value-added aroma compounds via solid-state fermentation. J. Clean. Prod., 158, 8-17.

28. Monlau, F., Sambusiti, C., Ficara, E., Aboulkas, A., Barakat, A., Carrere, H. 2015. New opportunities for agricultural digestate valorization: current situation and perspectives. Energ. Environ. Sci., 8(9), 2600-2621.

29. Novelli, P.K., Barros, M.M., Fleuri, L.F. 2016. Novel inexpensive fungi proteases: Production by solid state fermentation and characterization. Food Chem., 198, 119-124.

30. Paavola, T., Rintala, J. 2008. Effects of storage on characteristics and hygienic quality of digestates from four co-digestion concepts of manure and biowaste. Bioresour. Technol., 99(15), 7041-7050.

31. Pirota, R.D.P.B., Miotto, L.S., Delabona, P.S., Farinas, C.S. 2013. Improving the extraction conditions of endoglucanase produced by *Aspergillus niger* under solid-state fermentation. Bra. J. Chem. Eng., 30, 117-123.

32. Ponsá, S., Gea, T., Sánchez, A. 2010. Different Indices to Express Biodegradability in Organic Solid Wastes. J. Environ.Qual., 39(2), 706-712.

33. Rodríguez-Couto, S. 2012. A promising inert support for laccase production and decolouration of textile wastewater by the white-rot fungus *Trametes pubescesns*. J. Hazard. Mater., 233-234, 158-162.

34. Rosa, C.A., Lachance, M.A. 1998. The yeast genus *Starmerella gen. nov*. and *Starmerella bombicola* sp. nov., the teleomorph of *Candida bombicola* (Spencer, Gorin & Tullock) Meyer &Yarrow. Int. J. Syst .Bacteriol., 48, 1413-1417.

35. Ruggieri, L., Gea, T., Artola, A., Sánchez, A. 2009. Air filled porosity measurements by air pycnometry in the composting process: A review and a correlation analysis. Bioresour. Technol., 100(10), 2655-2666.

36. Saveyn, H., Eder, P. 2013. End-of-waste criteria for biodegradable waste subjected to biological treatment (compost & digestate): Technical proposals, Publications Office of the European Union. Brussels.

37. Scott, T.A., Melvin, E.H. 1953. Determination of Dextran with Anthrone. Anal. Chem., 25(11), 1656-1661.

38. Soares, V.F., Castilho, L.R., Bon, E.P., Freire, D.M. 2005. High-yield Bacillus *subtilis* protease production by solid-state fermentation. Appl. Biochem. Biotechnol., 121-124, 311-319.

39. Thomas, L., Larroche, C., Pandey, A. 2013. Current developments in solid-state fermentation. Biochem. Eng. J., 81, 146-161.

40. Soest, P., Robertson, J., Lewis, B. 1991. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. J. Dairy Sci., 74(10), 3583-3597.

41. Vishal, J., Aniruddha, P. 2012. Solid State Fermentation (SSF) for the Production of Sophorolipids from *Starmerella bombicola NRRL Y-17069* using glucose, wheat bran and oleic acid. Curr. TrendsBiotechnol. Pharm., 6(4), 418-424.

42. Zhuang, L., Zhou, S., Wang, Y., Liu, Z., Xu, R. 2011. Cost-effective production of *Bacillus thuringiensis* biopesticides by solid-state fermentation using wastewater sludge: Effects of heavy metals. Bioresour. Technol., 102(7), 4820-4826.

Table 1. Average digestate characterisation. Standard deviation corresponds to the average of more than 10 samples analysed during one year.

Parameter	Units	Digestate (n=10)
Moisture	% (wb)	75.60±5.11
Dry Matter	% (wb)	24.40±5.11
Organic Matter	% (db)	63.00±1.62
pH (1:5)	-	8.31±0.07
Carbon	% (db)	34.14±2.38
Nitrogen	% (db)	4.32±0.33
Hydrogen	% (db)	2.89±0.25
Sulphur	% (db)	0.33±0.14
C/N ratio	-	11.85±0.70
Dynamic respiration index	$mgO_2\ g^{-1}DM\ h^{-1}$	1.38±0.38
Protein	% (db)	16.95 ± 0.50
Hemicellulose	% (db)	10.06±0.90
Cellulose	% (db)	10.42±1.25
Lignin	% (db)	17.54±1.96
Soluble sugars	% (db)	0.18±0.16
Fats	% (db)	5.05±0.04

Table 2. Experimental description of solid-state fermentation trials for sophorolipids production.

Operational Conditions	**Solid mixture for SSF**
Experiment 1: Sterile digestate	
Airflow: 20 mL min^{-1}	Substrate: Digestate
Temperature: 30°C	Digestate + Glucose 1% (DG)
Time: 5 days	Digestate+OleicAcic 10% (DA)
Sterile digestate: 121ºC, 20 min	Digestate+Glucose1%+OleicAcid 10% (DGA)
	Inoculum: *S. Bombicola*
Experiment 2: Hygienised digestate	
Airflow: 20 mL min^{-1}	Substrate: Digestate + Glucose 1% (DG_s)
Temperature: 30°C	Digestate + Glucose 1% + Oleic Acid 10% (DGA_s)
Time: 5 days	Inoculum: *S. Bombicola.*
Hygienised digestate: 70ºC, 1h	

Figure captions

Figure 1. Operational profiles of SSF carried out in triplicates using digestate as substrate in a 0.45 L reactor for hydrolytic enzymes production. Cellulase (circles) and protease (squares) production and sOUR profiles (s.d<10%) are presented. Experimental points with an asterisk indicates high deviation ($p>0.05$).

Figure 2. Average sOUR profiles during SSF carried out in triplicates using 0.45 L reactors for sophorolipids production. Figure 3a shows the effect of the addition of external sugar and/or fat sources to sterile digestate using: digestate (D), digestate and glucose (DG), digestate and oleic acid (DA) and digestate with glucose and oleic acid (s.d<5%). Figure 3b shows the effect of the use of hygienised digestate (non-sterile) supplemented with: glucose (DG_s) and using glucose and oleic acid (DGA_s) (s.d<5%).

Figure 3. sOUR and viable cell (circles) and spore (triangles) count profiles obtained in 0.45 L SSF reactors using a) sterile and b) non-sterile digestate as substrate for biopesticide production. Experimental points with an asterisk indicates high deviation ($p>0.05$).

Figure 1.

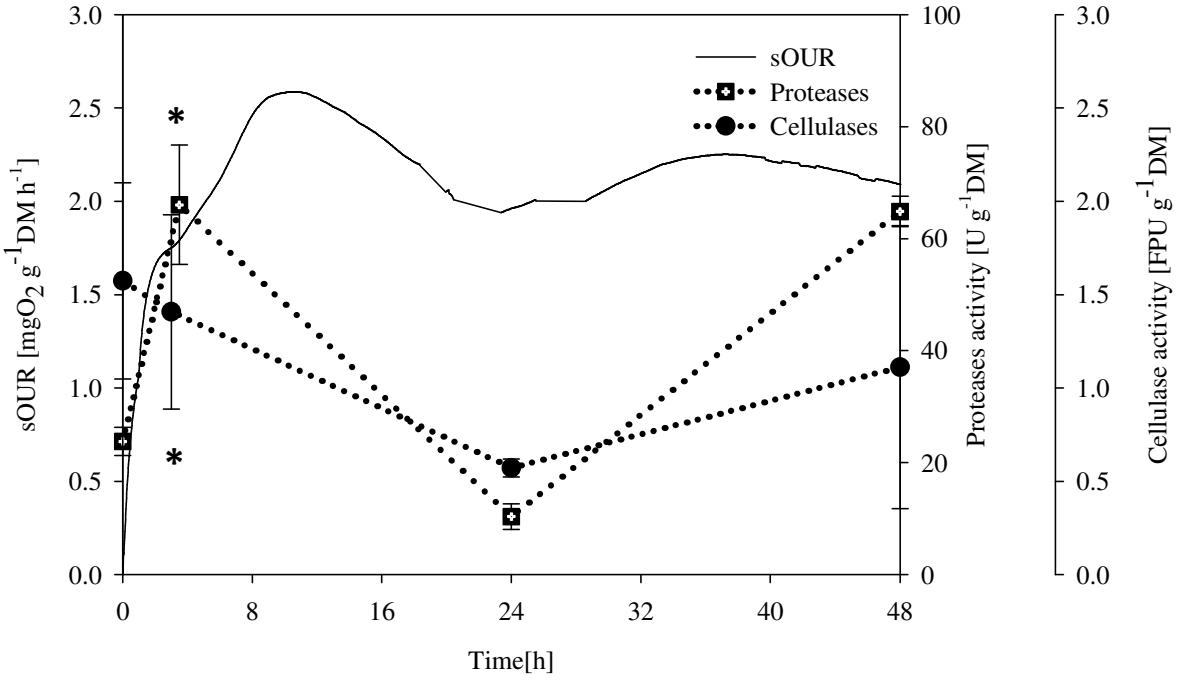

sOUR
Proteases
Cellulases
sOUR [mgO2 g-1DM h-1]
Proteases activity [U g-1DM]
Cellulase activity [FPU g-1DM]
Time[h]
0
8
16
24
32
40
48
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
*
*

Figure 2.

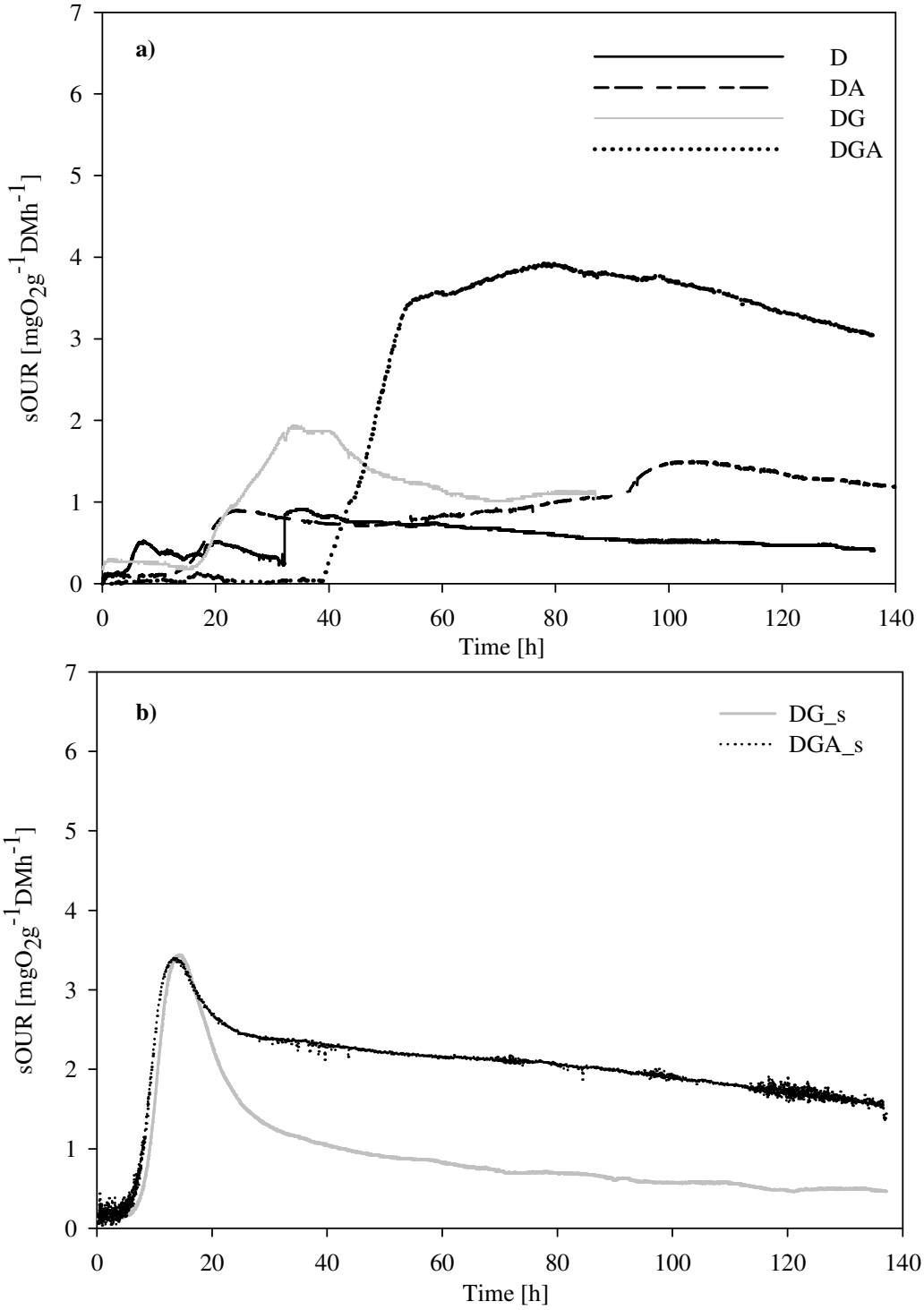
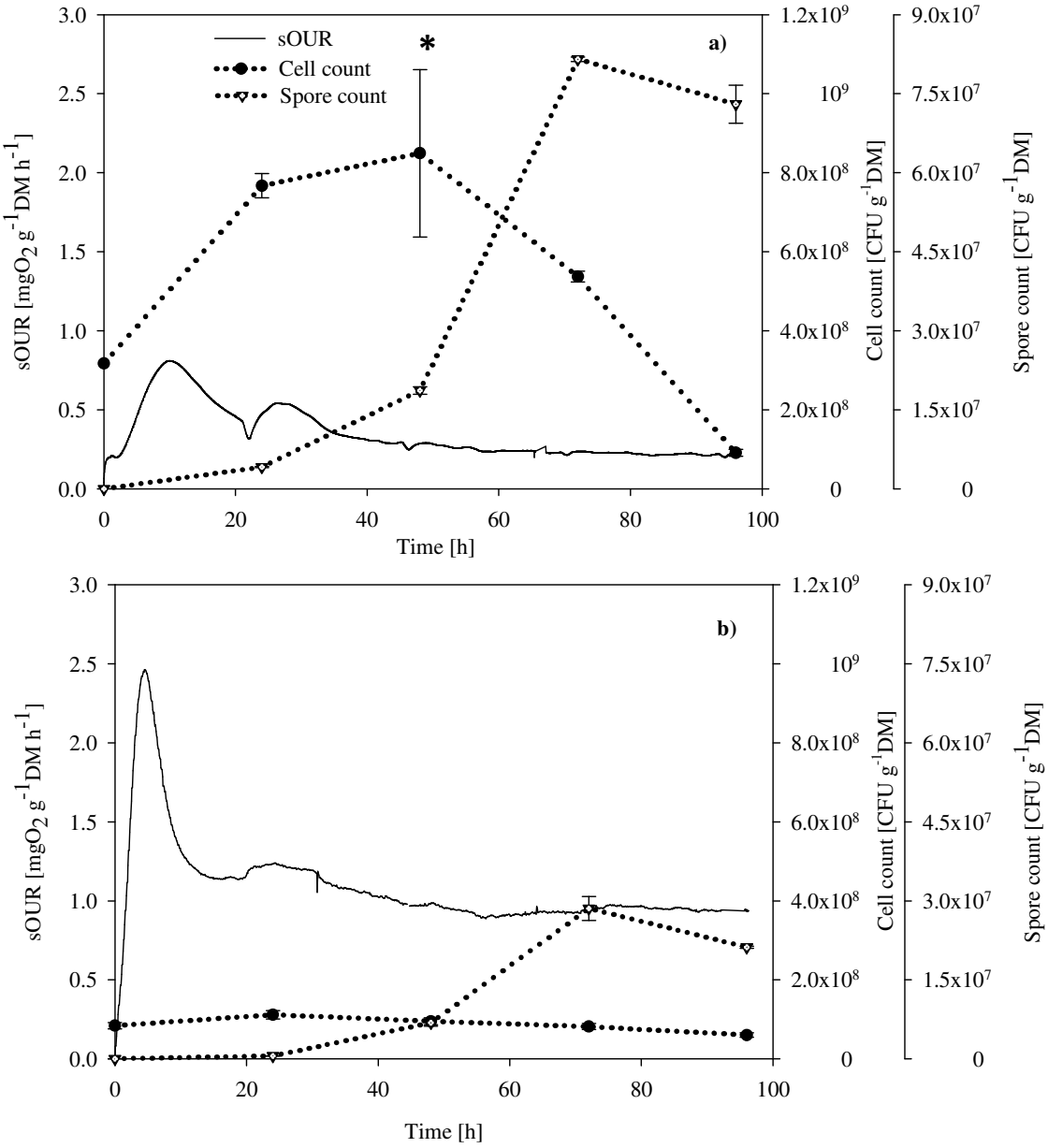

a)
D
DA
DG
DGA
sOUR [mgO2g-1DMh-1]
Time [h]
b)
DG_s
DGA_s
sOUR [mgO2g-1DMh-1]
Time [h]

Figure 3.


a)
sOUR
Cell count
Spore count
*
sOUR [mgO2 g-1DM h-1]
Cell count [CFU g-1DM]
Spore count [CFU g-1DM]
Time [h]
b)
sOUR [mgO2 g-1DM h-1]
Cell count [CFU g-1DM]
Spore count [CFU g-1DM]
Time [h]