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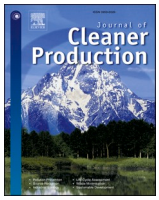

Enhanced anaerobic digestion of food waste using purified lactonic sophorolipids produced by solid-state fermentation of molasses and oil waste: A circular approach

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ABSTRACT

In the present work, the effect of pure lactonic sophorolipids (SL) on the anaerobic digestion of biowaste was investigated. For this purpose, crude SL were produced from organic waste (a mixture of molasses and winterization oil cake) through solid-state fermentation (SSF) in a 22L pilot-scale aerobic bioreactor using the yeast *Starmerella bombicola* as SL-producing microorganism. The crude material extracted from the SSF exhaust solid contained several forms of SL and it was purified using High-Performance Liquid Chromatography (HPLC) and characterized by means of Liquid Chromatography-Mass Spectrometry (LC-MS) yielding a high-purity C18 lactonic SL (>99%). This pure SL was used to enhance the batch anaerobic digestion of source-selected biowaste, mainly composed by kitchen waste. The best dosage of SL was in the range of 0.02–0.04 g SL/g TS, with an increment of methane yield of 41% in NmL/g VS. The presence of SL did not significantly alter the structure of the microbial community or general biodiversity. In summary, we propose a circular approach for waste valorisation in which different organic waste streams are combined in a biorefinery-like configuration, where the solid-state fermentation is used to produce SL and results in an enhanced bioenergy production.

1. Introduction

Worldwide, near to 2 billion metric tons of municipal solid waste (MSW) are annually produced. Around 50% of this MSW is organic matter with most of this fraction being food waste. The disposal and recovery of this waste is a complicated and pressing issue, since an improper management of MSW can cause severe environmental and ecological impacts (Chen et al., 2016). Simultaneously, organic waste has a significant potential as feedstock for both energy generation and the obtention of bioproducts through several processes (Molina-Peñate et al., 2022).

Anaerobic digestion (AD) and composting are the most implemented technologies to treat the organic fraction of MSW (OFMSW, or biowaste). Anaerobic digestion of biowaste is well known and an increasing way to obtain locally-available renewable energy, although it also presents several challenges. For instance, the heterogeneity and variable composition of biowaste makes the process unstable and less effective (Komilis et al., 2017).

Interest in solid-state fermentation (SSF) has quickly grown for its ability to use organic solid waste as substrate for fermentation. Agricultural waste, such as crop residues and food processing by-products, are excellent candidates to be used as substrates for SSF, and thus to introduce them in a circular bioeconomy context. SSF has been reported as suitable for the production of a wide variety of bioproducts: aromas, bioplastics, enzymes, biosurfactants and biopesticides, among others (Oiza et al., 2022). However, the implementation of this technology presents some challenges, as the scale-up of the process is hampered by complex heat and mass transfer dynamics (Lopes Perez et al., 2019).

Biosurfactants are one of the most promising bioproducts obtained through SSF. The biosurfactants market has been steadily growing and it is expected to reach $6.06 billion by 2030 (Spherical Insights, 2022). Among all the typologies of biosurfactants, sophorolipids (SL) are very interesting because of their properties. SL are amphiphilic molecules with a wide range of uses depending on their structure. They have been used as foaming and emulsion agents, biocide molecules or anti-cancer agents (Gudiña et al., 2013; Van Renterghem et al., 2018; Gaur et al., 2019). SL are typically produced by submerged fermentation (SmF), although some lab-scale and pilot SSF trials have been recently

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published (Jiménez-Peñalver et al., 2018, 2020; Rodríguez et al., 2021). Some studies such as Hu et al. (2021), Kaur et al. (2019), investigate the production of SL from food waste. While these studies operate in submerged fermentation, they found out that this waste-stream could be utilized for SL production. In a circular economic approach, the possibility of producing SL from a biowaste stream and later using it to improve the treatment of the remaining waste stream is interesting. Their production from food waste was uncovered in these aforementioned studies, but their effect on anaerobic digestion of this waste for methane production was not investigated. In addition to this, a preliminary techno-economical analysis of SL production through SSF was published (Martínez et al., 2022).

The first biological step of anaerobic digestion is the hydrolysis of biopolymers (mainly polysaccharides, fats, and proteins), which can be challenging in the case of biowaste due to its complex composition. To overcome this problem, several alternatives have been tested: two-stage anaerobic digestion processes, the use of co-substrates, the use of additives, etc. to avoid that the hydrolysis step may be rate-limiting (Babu et al., 2021; Tyagi et al., 2018). Additives are one of the most interesting strategies among these examples, because they don't require additional on-site infrastructure and compared to co-substrates, they introduce less heterogeneity and can be chosen to improve specific aspects of the process, without altering the overall composition. Some of the most studied additives are biochar, nanoparticles, and surfactants (Romero-Güiza et al., 2016). In this framework, surfactants have been found to influence the AD process in various ways. They can enhance the efficiency of hydrolysis by disaggregating organic matter and increasing the waste digestibility (Zhou et al., 2015). In this process, a higher amount of volatile fatty acids (VFA) is released. However, traditional surfactants are not always biodegradable. For this reason, biosurfactants are a safer and greener alternative in a circular bioeconomy context. Most studies on biosurfactants use them to increase VFA production, although the complete AD is not studied (Luo et al., 2013; Romero-Güiza et al., 2016; Johnravindar et al., 2022). Biowaste is a complex substrate, where the limiting step is hydrolysis. Additives such as zeolite or nanoparticles are known to improve anaerobic digestion by helping in the methanogenesis, but do not contribute to hydrolysis. On the other hand, enzymes do a similar function as SL, and while they would be an alternative, extensive work has already been done on this field while little research has been done on the effect of biosurfactants. At the same time our evidence is that the use of enzymes for the treatment of organic waste is quite expensive and does not result in a positive economic performance (Molina-Peñate et al., 2024), so considering some alternatives is of interest in the field.

In the particular case of SL, their effect has been scarcely studied. In a recent study by Xu et al. (2019), organic waste supplemented by SL was demonstrated to enhance VFA production up to five times at a dosage of 0.1g SL/g VS. However, a crude extract of SL was used, and it was not further characterized. Moreover, the objective of this work did not involve reaching methanogenesis, which does not permit to extract conclusions on the methane yield. Regarding this, it is important to note that crude SL extract can contain non consumed fatty acids or fats carried over from the fermentation process. Although the profile of fatty acids varies on the substrate used for the fermentation process, most of them have an inhibitory effect on AD, although they can also be consumed for methane production (Zonta et al., 2013). Therefore, a consistent and reliable purification method is necessary to study the specific effect of SL, a non-studied question when SL come from SSF of wastes.

The objective of this work is to study the effect of SL on the performance of batch AD of biowaste. To achieve it, crude SL was produced from molasses and winterization oil cake through SSF at pilot scale and purified to obtain a high-purity lactonic SL. To the best of the authors' knowledge, this is the first study to obtain a completely pure lactonic SL from SSF, and to study its effects on AD. The final aim of the work is to propose a circular approach in which different organic waste streams are combined in a biorefinery scheme, resulting in an improvement of bioenergy production.

2. Materials and methods

2.1. Solid-state fermentation

2.1.1. Materials

Organic waste used in the SSF process was obtained from local providers. Wheat straw used as bulking agent was obtained from the Veterinary Faculty of the Autonomous University of Barcelona (UAB). Sugar beet molasses (MOL) is a byproduct derived from the production of sugar refining and were gently provided by AB Azucarera Iberia S.L.U. (Madrid, Spain). Winterization oil cake (WOC) is the cake obtained after submitting sunflower oil to temperatures below 5 °C to crystalize waxes, and filtering with perlite. It was gently provided by Lípidos Santiga, S.A (Barcelona, Spain). The main properties of these materials are shown in Table 1. *Starmamella bombicola* ATCC 22214 was obtained from *Colección Española de Cultivos Tipo* (Valencia, Spain).

2.1.2. Solid-state fermentation

Fermentation was performed in a pilot 22 L reactor following the methodology explained in detail in Rodríguez et al. (2021). Briefly, this reactor consists of a stainless-steel cylinder, equipped with an air intake at the bottom and an air exhaust at the top (packed-bed configuration). Some images of the reactor are presented in Fig. S1 (Supplementary information) and a detailed scheme of this prototype is presented in Molina-Peñate et al. (2023). Fermentation was performed at 75% of water holding capacity (WHC). To achieve this, the straw and WOC were mixed in a ratio 1:1 (weight ratio) to provide porosity and to ensure aerobic conditions during fermentation, and autoclaved together at 121 °C for 20 min, while the MOL were diluted in the water required to achieve 75% of the WHC, and then autoclaved under the same conditions. A homogenous solid was obtained by mixing these components in a ratio 4:1 (WOC:MOL, total weight ratio) under non-sterile conditions. The solid was then inoculated with *S. bombicola* previously grown under sterile conditions according to Rodríguez et al. (2021) and introduced in the reactor to reach a viable cell concentration of around 10^8 CFU per gram of dry matter. The total initial mass of the fermentation was 3.3 kg. Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) were placed at different points to monitor the evolution of the process, as presented in Fig. S1 (Supplementary information). For 5–6 days, oxygen uptake rate (OUR) and temperature were on-line monitored, and a final sample was analysed to know the SL productivity (Rodríguez et al., 2021). The remaining fermented solid was collected, homogenized, packaged under a vacuum seal, and stored at −20 °C, prior to SL extraction and purification.

2.2. Purification and characterization of SL

2.2.1. Crude SL extraction

SL extraction was carried out following the procedure described by Jiménez-Peñalver et al. (2016) methodology with minor modifications. Briefly, the fermented solid material was dried at 80 °C, and two

Table 1
Main characteristics of the materials used in solid-state fermentation (MOC, molasses, WOC, winterization oil cake).

Property	MOC	WOC	Wheat straw
Dry matter (%, total weight)	82.9	95.6	94.4
Organic matter content (%, dry matter basis)	87.1	77	95.5
Sugar content[a] (%, dry matter basis)	12	–	<1
Fat content[b] (%, dry matter basis)	<1	44	<1
pH (aqueous extract)	5.50	7.51	–

[a] Data obtained from the provider.
[b] Obtained as extractable matter with n-hexane.

consecutive extractions were performed with ethyl acetate at a ratio 1:10 (vol/wt) for 1h at 200 rpm at room temperature. The resulting liquid extracts were mixed and vacuum-evaporated at 40 °C to obtain a solid SL crude. A final wash with n-hexane was performed to remove residual fatty acids. N-hexane was volatilized at room temperature. The solid SL crude extracts were stored at −20 °C.

2.2.2. SL purification

All reagents used for SL purification were of analytical grade and supplied by Merck Life Science S.L. (Madrid, Spain). This method is based on Daverey and Pakshirajan (2010). Adsorption chromatography was carried out using a 60 cm × 6 cm column packed with 60 Å, 230–400 mesh silica. First, 500 mL of the mobile phase (5% methanol in chloroform) passed through the sample. Afterwards, three more extractions were performed with 10%, 15% and 20% of methanol, of 500 mL each. Purified lactonic SL were collected in phases 7–12, of a total of 24 of 100 mL each.

2.2.3. Lactonic C18:1 SL characterization

High-Performance Liquid Chromatography (HPLC) methodology was adapted from Van Renterghem et al. (2018). A Phenomex nucleopore C18 column (3 μm 120 Å 100 × 4.6 mm) was used with milliQ water and acetonitrile, both with 0.1% of formic acid. 10 μL of SL purified sample were injected to the column. The working flow was of 1.4 mL/min and the chromatographic steps were: 10 min at 30% of acetonitrile, followed by a slow ramp to reach 90% of acetonitrile after 55 min and, finally, 10 min at 30% of acetonitrile to recondition the column. Column temperature was maintained at 40 °C. Ultraviolet detection was performed at a wavelength of 198 nm.

SL were identified by a Liquid Chromatography-Mass Spectrometry (LC-MS) system. The same HPLC method was used for UV detection, with a 10:1 splitter before the MS detector. The LC-MS was set in negative mode and run with an ESCI source type, a 3500 V capillary set, a 4-bar nebulizer, a dry gas flow of 8 mL/min and a 220 °C dry heater. The scanning range used was 50–1000 m/z.

2.3. Batch anaerobic digestion of biowaste with sophorolipids

2.3.1. Materials

Biowaste coming from the source-selected organic fraction of municipal solid waste was used as substrate for AD experiments. It was gently provided by the municipal waste treatment plant of Granollers (Barcelona, Spain). Inoculum for AD experiments was obtained from the full-scale anaerobic digester (3000 m^3) of the same plant, which works under mesophilic conditions at a hydraulic retention time of 15 days and wet mode (dry matter under 5%). The details of the plant can be found elsewhere (Abad et al., 2019). Table 2 shows the main properties of biowaste, and inoculum obtained from the plant.

2.3.2. Batch experiments

The experimental set up consisted of glass serum bottles (250 mL) containing inoculum and biowaste. The ratio of inoculum to biowaste volatile solids (VS) content was set to 2:1. The total volume used was 150 mL, adding distilled water when necessary. All reactors were purged with nitrogen for 4 min after sealing to ensure an anaerobic environment. Controls containing microcrystalline cellulose as substrate were used to check the inoculum activity. The reactors were maintained at 37 °C, with intermittent mixing once a day. Blank samples with only inoculum were used to assess the baseline production (subtracted from the rest of experiments) and controls using only biowaste were also carried out to be compared to those dosed with SL. For simplification, the purified lactonic SL used in the experimental section will be referred as PLSL. Its dosage in AD experiments was selected according to the values found in literature for the production of VFA using SL (Xu et al., 2019) or using other biosurfactants as rhamnolipids or plant derived biosurfactants (Zhou et al., 2015; Johnravindar et al., 2022), since no data related to SL was found for AD. The doses selected where: 0.1, 0.2 and 0.4 g PLSL/g TS (total solids). Controls with only inoculum and PLSL (no biowaste) were also performed in triplicate to evaluate the potential production of biogas if these compounds were anaerobically degraded.

Samples from all triplicates were taken at 20 days with a volume of 0.5 mL. This change in volume was considered for the biogas yields.

Table 2
Main characteristics of the materials (inoculum and digestate from the full-scale waste treatment plant) used in anaerobic digestion experiments.

Property	Inoculum	Biowaste
Total solids (g/L)	33.3 ± 0.8	14.2 ± 0.5
Volatile solids (g/L)	22.0 ± 0.7	6.4 ± 0.2
pH	5.52	7.96

2.3.3. Biogas and methane measurement

Details on biogas and methane measurement can be found elsewhere (Casals et al., 2014; Barrena et al., 2023). Specifically, biogas composition was analysed by gas chromatography (GC Agilent 7820), featuring a thermal conductivity detector (TCD) and two interconnected columns (Agilent G3591-81136 and G3591-80017) activated using synthetic air. A 100 μL sample was extracted from the headspace reactors valve using a gas-tight syringe and subsequently injected to the gas chromatograph. Nitrogen served as the carrier gas. The injector and detector temperatures were set at 200 °C and 250 °C, respectively. Initially, the oven temperature was maintained at 70 °C for 2 min, followed by a ramp of 20 °C per minute until reaching 200 °C. Quantification of methane content was conducted using an external standard of 100% methane (Carburos Metálicos, Barcelona, Spain).

2.3.4. Modified Gompertz model

The prediction of cumulative methane yield per g VS (CMY) was modelled according to Eq. (1), corresponding to a modified Gompertz model. Pm is the maximum methane generation in mL/g VS, λ is the lag phase in days, and Rm stands for velocity or maximum methane yield per day in mLg^{-1} VS d^{-1}. Finally, e is Euler's number (≈2.718) and t is the time in days.

$$CMY = P_m \cdot \exp\left\{ -exp\left[\frac{R_m \cdot e}{P_m}(\lambda - t) + 1\right]\right\} \quad (1)$$

2.3.5. Statistical analysis

Each PLSL dose test was performed in quadruplicate samples while the control and blank experiments were performed in triplicate. Statistical analysis was based on one-way ANOVA ($p < 0.05$ confidence) with the Tukey test. Significant differences were analysed using the software package Minitab 16 (Minitab Inc.).

2.3.6. Microbial community analysis

Samples from all triplicates were taken at 20 days and final day (80). These triplicate samples were pooled together and used for DNA extraction using the Soil DNA Isolation Plus Kit (Norgen Biotek, Canada). DNA extracts were tested for concentration and quality using a NanoDrop spectrophotometer.

Sequencing was performed by the Genomic Service of the Universitat Autonoma de Barcelona. The variable regions V3–V4 of the prokaryotic 16S rRNA gene sequences were analysed, giving 460 bp amplicons in a two-round PCR protocol. First amplification was done with the specific primers, forward (5′ TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGCCTACGGGNGGCWGCAG) and reverse (5′ GTCTCGTG GGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATC C). Sequencing adapters and dual index barcodes were added to the amplicon by a limited-cycle PCR using Nextera® XT DNA Index Kit, FC-131-1002 (Illumina, San Diego, CA, USA) for sequencing and pooled together in the MiSeq sequencer with the MiSeq® Reagent Kit v2 (500

cycles) MS-102-2003. Sequencing analysis was carried out with the 16S Metagenomic App at the BaseSpace platform (Illumina, Inc, USA) with the 16S Metagenomic App. The algorithm used was a high-performance implementation of the Ribosomal Database Project (RDP) Classifier described in Wang et al. (2007).

Principal Component Analysis and Alpha diversity indices of the different microbial communities were obtained from the EzBioCloud microbiological research platform (https://www.ezbiocloud.net).

3. Results and discussion

3.1. SL production through SSF at pilot scale

Fig. 1 shows the temperature and OUR profiles of the SSF and the SL yield obtained. As previously observed, the combination of a hydrophilic highly biodegradable waste as MOC with a lipid-enriched waste as WOC is an optimal mixture for SL production using *S. bombicola* (Jiménez-Peñalver et al., 2016). Thus, after 5–6 days of fermentation the production of crude SL was of 0.165 g/g dry matter, which is similar but slightly lower than that previously reported of other experiments carried out with the same procedure at the same scale (Rodríguez et al., 2021) and using 0.5 L bioreactors (Jiménez-Peñalver et al., 2016, 2018). Therefore, the absence of negative effects was confirmed when scaling-up this SSF process up to 22L. This is notably promising given that scaling-up is challenging in packed-bed reactors due to a limited heat and mass transfer and the feasibility of the process should be confirmed using full-scale reactors (Oiza et al., 2022).

During fermentation, OUR followed a typical evolution related to the biodegradation of the organic waste, reaching maximum values from 3.6 to 4 mg O_2 g^{-1} DM h^{-1} in the period from 48 to 60 h (Fig. 1). The internal temperature of the reactor is presented in Fig. 1 as the average of the sensors inside the reactor (Fig. S1, Supplementary information), and showed a maximum difference of temperature between the surroundings of the bioreactor and the fermentation solid below 10 °C. The quite similar temperature, together with the absence of temperature peaks due to material self-heating, are also positive for the yeast growth and SL production. From this part, it can be concluded that this bioreactor can produce significant amounts of crude SL in a similar time and yield to those of lab-scale reactors (Rodríguez et al., 2020), which opens the possibility of having enough crude SL to be further purified, characterized, and used for anaerobic digestion enhancement, as explained below.

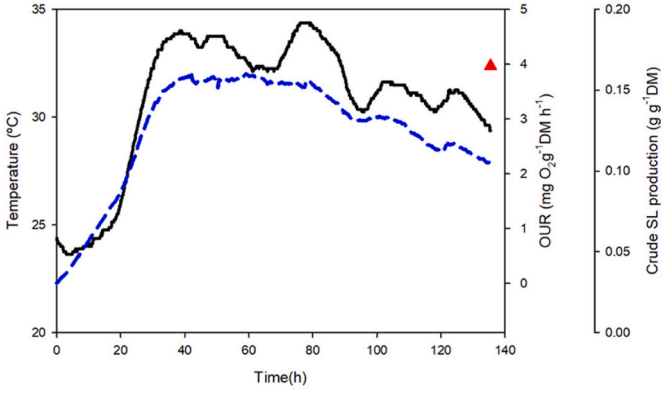


Fig. 1. Evolution of the solid-state fermentation process along time. OUR (black continuous curve) is the oxygen uptake rate. Temperature (blue discontinuous curve) is presented as the average value of all the points measured inside the bioreactor. The red triangle is the final crude sophorolipid yield. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Extraction and characterization of sophorolipids

3.2.1. Extraction of crude SL

First crude extraction to assess the performance of the fermentation was carried out using the conditions explained in detail in (Jiménez-Peñalver et al., 2026). However, this extraction method is optimized for small amounts of fermented solids. In order to increase the amount of extracted crude, the extraction process was scaled up by a factor of 10 up to 100 g of fermented solid maintaining all the conditions. Two extraction ratios were tested, 1:5 and 1:10 solids:solvent ratio. The efficiency of the crude SL extraction was reduced in a 14% when using the lower ratio. This it is not a critical loss of efficiency and would reduce solvent requirements 50%, therefore it should be studied at full scale since downstream processes can be a limiting factor for the viability of the overall SSF process (Kosaric and Vardar-Sukan, 2015; Catalan et al., 2019).

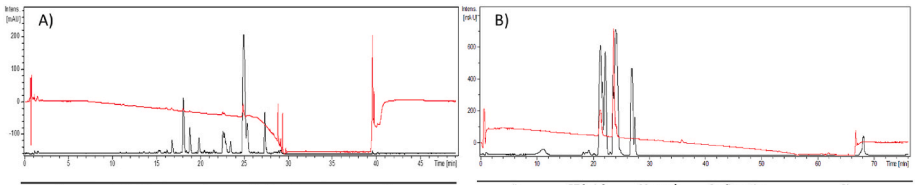
In fact, there is an evident lack of literature regarding the purification of SL produced by SSF, especially in relation to amounts of bioproduct beyond a milligram scale, being this one of the main challenges for the implementation of SSF and its evaluation in environmental and economic terms (Martínez et al., 2022).

3.2.2. Purification and characterization of lactonic SL

LC/MS analysis of the crude SL is presented in Fig. 2. It shows that a significant part of the produced SL does not present any UV absorbance. Specifically, absorption of sugars and lipids in the UV spectrum is weak or negligible. Since UV is the predominant method of SL quantification, this is highly relevant as they are composed of a sophorose group attached to a fatty acid (Li et al., 2023). This problem is common to other biosurfactants (Behrens et al., 2016). UV absorbance in SL depends exclusively on the number of double bonds in the acid chain and the number of bonds correlates linearly with the absorption. In fact, some studies on SL degradation only use UV to measure its disappearance (Xu et al., 2019). Moreover, commercially available standards as those from Biosynth and Cayman Chemicals provide their purity by UV relative area (Fig. 3). In Fig. 3, it can be observed that a wide range of SL are detected by LC/MS but they are not detected through UV. For all these reasons, SL quantification using only UV is not accurate enough. Other authors (Abdel-Mawgoud et al., 2014) have also expressed similar problems and propose LC/MS as an accurate and sensitive method for SL analysis. As observed in Fig. 2, there are several different peaks only visible through LC/MS. The first peak corresponds to acidic diacetylated C18:0 SL. The last peaks, appearing around 40 min, correspond to residual fatty acids not consumed during SSF.

During the purification process, 24 fractions of 100 mL were collected, most of them showing a honey-like colour similar to the crude. Six fractions out of these 24 were recovered presenting a whitish powder-like consistency. MS analysis of the combined sample containing only lactonic C18 SL is presented in Fig. 2B (and attached table). It can be seen that the UV spectra does not match with the LC/MS one, and only 2 out of 3 main peaks appear. These peaks correspond with m/z of 685, 687 and 689 in that order, which were identified as lactonic C18 diacetylated with saturations going from 2 to 0 in order of appearance. To our knowledge, this is the first study in which SL produced by SSF are purified and characterized at this level, and it demonstrates that a complex mixture of several forms of SL is obtained. The unique characteristics of each SL resulting from their different configurations, acidic or lactonic, length of the fatty acid chain, or number of saturations could interfere. For this reason, knowing the exact composition of the SL mix used is critical for further applications studies (Pal et al., 2023). In consequence, the use of crude SL should be carefully considered.

The quantification of the purity of these samples was difficult since there are no available standards with high level of purity. This hampers an accurate quantification of SL purified forms in anaerobic digestion environments. Since the lactonic diacetylated C18:0 does not absorb UV and due to the fact that UV quantification revealed a high purity of the



#	RT [min]	m/z	Configuration	SL
1	14.2	703.3	Acidic	Diacetylated C18:0
2	15.3	705.4	Acidic	Diacetylated C18:1
3	16.3	645.3	Lactonic	Monoacetylated C18:1
4	16.7	707.4	Acidic	Diacetylated C18:0
5	17.9	645.3	Lactonic	Monoacetylated C18:1
6	18.6	645.3	Lactonic	Monoacetylated C18:1
7	19.9	685.3	Lactonic	Diacetylated C18:2
8	22.4	661.3	Acidic	Monoacetylated C18:2
9	23.5	661.3	Acidic	Monoacetylated C18:2
10	24.8	687.3	Lactonic	Diacetylated C18:1
11	27.2	689.4	Lactonic	Diacetylated C18:0
12	36.3	981.6	Unknown	

#	RT [min]	Max. m/z	Configuration	SL
1	21.3	685.3	Lactonic	Diacetylated C18:2
2	23.8	687.3	Lactonic	Diacetylated C18:1
3	26.9	689.4	Lactonic	Diacetylated C18:0

Fig. 2. UV (red) and LC/MS (black) spectra for A) Crude sophorolipid and B) Purified sophorolipid (PLSL). In the respective tables, the retention time, mass and identification of the sophorolipids are presented. Note the differences found in the variety of sophorolipids when comparing crude and purified sophorolipid. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

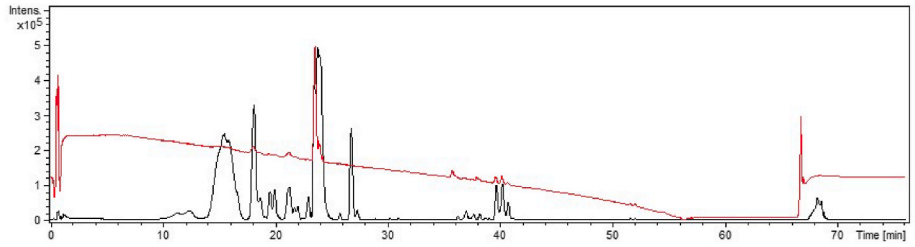


Fig. 3. UV (red) and LC/MS (black) spectra of Biosynth® commercial Lactonic Sophorolipid standard. Several different peaks are only visible through LC/MS. The first big peak corresponding to acidic diacetylated C18:1 sophorolipid. Last peaks, around 40 min, correspond to residual fatty acids. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

main homolog (lactonic diacetylated C18:1), it was assumed that the purity of the sample was >99% in lactonic SL. This pure SL will be named as PLSL, and it will be used as a potential additive for biowaste anaerobic digestion.

3.3. Batch anaerobic digestion performance

Purified C18 lactonic SL (PLSL) was added to the batch anaerobic digestion of biowaste at different dosages. The results are presented in Fig. 4. A strong initial inhibition in both biogas and methane production was observed in the samples containing PLSL. On the contrary, biowaste control performed as expected with no lag phase. After this initial long lag phase (around 22 days), the methane production quickly accelerated, which can only be related with the positive effect of PLSL.

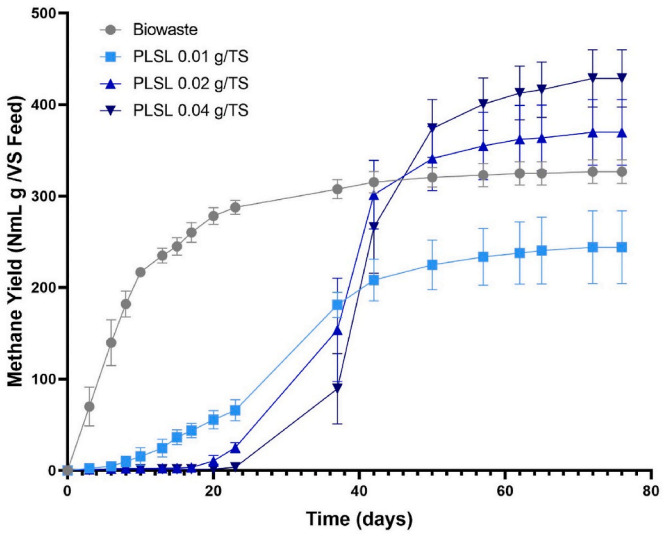


Fig. 4. Evolution of methane yield in the experiments amended with purified sophorolipid (PLSL) along time. Control with only biowaste is also presented. The different dosages of PLSL are referenced as 0.01, 0.02 and 0.04 according to their dosage per total solids of feed.

Regarding the methane yield, the increase observed when using different dosages of PLSL is presented in Fig. 5. To have a more accurate picture, Table 3 also shows the total methane yield and anaerobic digestion kinetic parameters obtained when adjusting the experimental data to the Modified Gompertz model. The lowest dose of PLSL (0.01 g PLSL/g TS) led to statistically similar but lower methane yield, while higher ones (up to 0.04 g PLSL/g TS) resulted in a 17–41% of improvement. The effect on the theoretical maximum production is similar. Meanwhile, the rate of methane production is greatly improved (Table 3), but with a much higher lag phase for higher dosages. This long lag phase could be caused by different reasons. As discussed above, SL are known to have antibacterial effect and to increase the release of VFA (Xu et al., 2019; Eras-Muñoz et al., 2022). Both facts could explain this inhibition. However, this negative effect could be highly reduced using adapted inoculum or continuous anaerobic digestion, where the adaptation of the microbial communities naturally occurs (see Table 4).

Incidentally, it is important to note that the PLSL controls (no biowaste) showed no increase in methane production when compared with the inoculum(Fig.S3). This indicated that PLSL was not being degraded or at least not used for biogas/methane production. Previous studies have confirmed SL biodegradability under aerobic conditions (Hirata et al., 2009; Lo and Lu, 2009), but no studies have analysed the anaerobic biodegradability. It has also been reported the antimicrobial activity of SL (Eras-Muñoz et al., 2022). Xu et al. (2019) reported that the addition of SL in an anaerobic process increased VFA production and supressed methanogenic activity. In our case the total inhibition of methanogens was not observed. These authors only specified the use of a

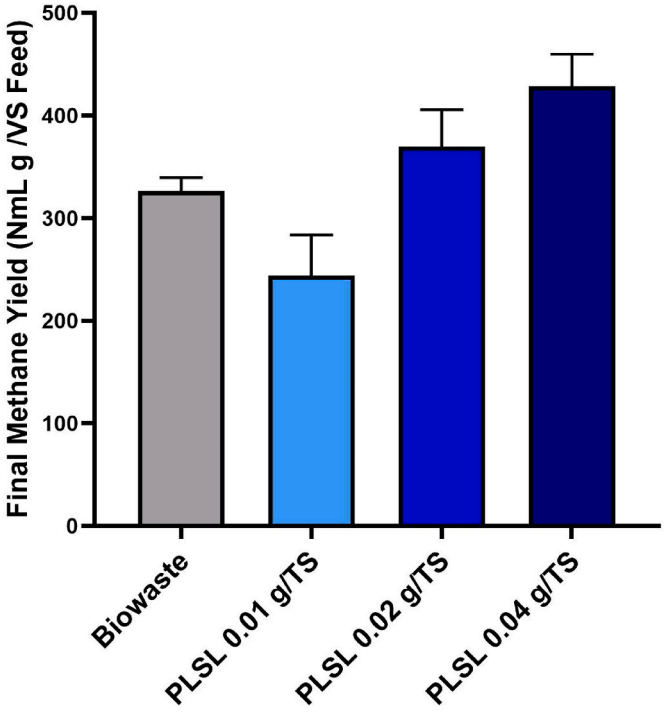


Fig. 5. Final accumulated methane yields. Different letters imply statistically significant differences. Control with only biowaste and experiments amended with several dosages of purified sophorolipid (PLSL) are presented.

commercial SL with 50% total solids and apparently consisting of diacetylated lactonic C18:1, however, as discussed for the previous standards, the commercial products can contain other forms of SLs (Fig. 3). The unknown composition could explain the differences between both studies. It is well known that SL properties and activity highly depend on their configuration (Bueno-Mancebo et al., 2024). In conclusion, any application of SL should be complemented with an accurate characterization of the different forms of this biosurfactant.

Sophorolipids enhance the solubilization of organic matter. This is especially relevant when working with complex polymeric organic matter such as biowaste that mainly consists of kitchen waste (Raj et al., 2022). Also, a light enrichment of the methane concentration in the biogas can be observed in samples with PLSL. Another secondary effect of the addition of PLSL is the reduction in the standard deviation values of the different replicates when compared to control experiments. Table S1 (Supplementary information) shows these values. Although the

Table 4
Adjustment of the cumulative methane production and the kinetic parameters derived from the modified Gompertz model. The percentage of improvement is related to the anaerobic digestion of only biowaste.

Treatment	Maximum methane yield (NmL/g VS)		Methane production rate (NmL g^{-1} VS d^{-1})		Lag phase (d)
	Value	Increase (%)	Value	Increase (%)	Value
Biowaste	247	–	19	–	0
PLSL 0.01	165	−33	8	−55	22
PLSL 0.02	290	17	35	82	34
PLSL 0.04	350	**41**	34	77	36

Table 3
Effects and mechanisms of surfactants and biosurfactants on AD (SDS, Sodium dodecyl sulfate; SDBS, Sodium Dodecyl benzene Sulfonate; RL: Rhamnolipids; WAS, Waste Activated Sludge; FOG, Fat, Oil, and Grease; APG, Alkyl Polyglucoside).

Surfactant chemistry	Specific type	Effect	Mechanism	Reference
Biosurfactant	Rhamnolipids	Increased hydrolysis	Higher biocompatibility, which allows highly active hydrolysis and further interaction in AD	He et al. (2019)
Biosurfactant	Rhamnolipids	Improved hydrolysis efficiency	Increased solubility of particles by reduction of surface tension and/or forming micelles	Luo et al. (2013)
Biosurfactant	Rhamnolipids	Enhanced methane generation	Increased SCFA production, higher methane concentration in biogas	Wang et al. (2021)
Biosurfactant	Sophorolipids	Increase of SCFA production	Accelerated solubilization of sludge cells and the biodegradability of the released organics and inhibition of methanogens	Xu et al. (2019)
Biosurfactant	Saponin	Lower effect on metabolic and species diversity	Theorized to be their non-ionic characteristic compared to ionic ones such as rhamnolipids and surfactin	He et al. (2019)
Biosurfactant		Increased bacteria-surface interaction and mobility	Lowering surface tension	Volkering et al. (1997)
Both	SDS, SDBS, RL	Improved SCFA yield	Changes in microbial composition	(Guan et al., 2017)
Both	SDS, SDBS, RL	Enhanced hydrolysis and acidification	Higher protein and carbohydrate solubility and enzymatic activity. Changes in microbial communities	Zhou et al. (2015)
Surfactant	SDS	Protein denaturation	Unfolding of protein tertiary structure	Bhuyan, 2009
Surfactant	SDS	Inhibition of methanogens	Destruction of microbial protein structure and accumulation in the environment of toxic byproducts	Feitkenhauer (2003)
Surfactant	SDS	Improved hydrolysis and acidification	Accelerated WAS solubilization, increase of protein and carbohydrate concentrations and improved enzymatic activity	Jiang et al. (2007)
Surfactant	SDBS/APG	Stimulating biogas production	Alteration of microbial communities' structure and enhancement of anaerobic co-digestion	Sun et al. (2019)
Surfactant	General	Increased enzymatic hydrolysis	Increased solubility helps the liberation of trapped enzymes and extracellular polymeric substances	Guan et al. (2017)
Surfactant	General	Enhancing hydrolysis	Separation of large sludge particles and releasing encapsulated enzymes, higher substrate for acidogenesis	He et al. (2019)
Surfactant	Anionic-Biodegradable (not specified)	Improving FOG solubility	Improvement in solubility of FOG in water, enhancing the biomethane production	Skripsts et al. (2022)
Surfactant	SDBS/APG	Stimulating biogas production	Alteration of microbial communities' structure and enhancement of anaerobic co-digestion	Sun et al. (2019)
Surfactant	Not specified	Removal of contaminants	Solubilization via micelles, emulsification of liquid pollutants, and enhanced transport	Volkering et al. (1997)
Surfactant	Not specified	Enzyme modulators	At low dose surfactants increase enzymatic activity and inhibit at higher doses	Guan et al. (2017)
Surfactant	Tyloxapol	Increase of PAHs biodegradability	Increased accessibility to heavy PAHs and their degradation	Bernal-Martinez et al. (2007)

reasons for this enrichment are not clear, this phenomenon has also been observed when using other additives for the enhancement of anaerobic digestion such as biochar and nanoparticles (Barrena et al., 2023, Parra-Orobio et al., 2023).

3.4. Effects of SL on anaerobic digestion

In an attempt to unveil the mechanism by which purified SL enhance the AD process of biowaste, a rigorous literature research was performed. In general, it can be stated that the underlying mechanisms regarding the effects of surfactants or biosurfactants on AD are not understood. Scopus® database shows only 130 papers in the last 10 years with the keywords: "surfactant" and "anaerobic digestion". Among these studies, only 32 contain "biosurfactant" and 2 contain "sophorolipid". These numbers point out the existing gap of knowledge regarding the effects and mechanisms that these molecules have on AD. The complex and diverse nature of biowaste as AD feedstock implies a high level of difficulty for interpreting the mechanism by which biosurfactants affect the AD process. Actually, surfactants have numerous effects on the process. Table 3 presents the most relevant existing literature about the effects and mechanisms of surfactants and biosurfactants on AD. The phenomena described more often are organic matter solubilization, enzyme enhancement and hydrolysis enhancement. All of them are closely related to each other and affect acidogenesis and the final biogas yield. The only publication to date on SL and anaerobic digestion is focused on the effect of these compounds on the production of short chain fatty acids (SCFA) from waste activated sludge (Xu et al., 2019), without reaching the methanogenesis step. To our knowledge, no other studies beyond this work have been published using SL as additive in AD, in this case using biowaste as substrate. However, the metabolism and the active microbial populations in the stages of AD (acidogenesis and methanogenesis) are completely different, and the effect of SL cannot be compared without additional experimental data (Pecorini et al., 2023).

3.5. Effect of pure lactonic SL on the microbial communities

The change in the abundance of certain key microbes was observed. The overall structure of the general microbial community was unaffected by the addition of PLSL although the change in the abundance of certain key microbes was observed. This can be seen in Fig.S4, Shannon index for all samples is very similar, around 3.9, and lower than initial sample (4.43). At 20 days biowaste index was 3.7 while the average of PLSL samples was 3.9. At the 80 day the difference was even smaller, demonstrating that the presence of PLSL did not affect general biodiversity significantly. Samples clustered together mostly by time and secondly by PLSL dosage, both in the phylogenetic tree and in the PCoA (Fig. S5 and Fig. S6). Our findings agree with Xu et al. (2019) who also observed negligible effects of SL on microbial communities of anaerobic digesters. While these studies were performed on wastewater sludge, the results presented herein reveal a similar effect on the process involving biowaste. In Fig. S5 and Fig. S6, it can be seen how the PLSL samples cluster together. On top of that, the biowaste controls are closer to the initial sample too. This shows that, even if the broader structure of the microbial population is not changed, there is an effect of the PLSL at a deeper level.

In all samples, *Firmicutes* is the most abundant phylum, ranging from 72 to 53% of the total population (Fig. 6). This is similar to the characterization of the initial biowaste, from the same treatment plant, performed by Molina-Peñate et al. (2023). In their study *Firmicutes* composes up to 80% of the community. Biowaste controls showed the lowest levels, showing that this group growth is affected by the AD process. On the other hand, samples with PLSL were less affected by this change. The second most represented phylum is *Bacteroides* which distribution is inhibited in the samples containing PLSL. After 20 days the relative abundance is around 6% when adding PLSL compared to the 17% of the Biowaste control. This effect is overcome at the end of the process. If this effect is provoked directly by PLSL or if it is because of the step of the process the samples are in, is not clear. Other abundant phyla such as *Synergistetes* or *Proteobacteria* are not significantly affected by PLSL and even being more abundant in samples with PLSL at the 80-day point. *Tenericutes* phylum appears at the later stages of DA and mostly in samples containing PLSL. Some species of this phyla are hypothesized to be related to acetate and hydrogen production (Wang et al., 2020).

The most representative genera, as seen in Fig. 7, *Saccharofermentans*, *Sedimentibacter*, *Syntrophomonas*, *Clostridium III* and *IV*, *Caloramator* and, *Caldalkalibacillus* belong to the *Clostridia* class, which represents around 50% of all samples but its specific distribution is different in samples containing PLSL. *Clostridia* are known for their nitrogenase activity and hydrogen production. There is a clear evolution through time of the *Clostridia* class during the AD process. With the replacement of the genera *Saccharofermentans* and *Syntrophomonas* by *Caldalkalibacillus* and *Caloramator*. Even though *Caldalkalibacillus* has been found in anaerobic digesters, its specific role in the process has not been described. *Caloramator* on the other hand, like the most abundant species *Caloramator australicus* and *Caloramator fervidus*, are involved in fermentation of proteins and carbohydrates. Some *Caloramator* have been described as hydrogen producers and even as playing a direct role in interspecies electron transfer (Singh et al., 2023; Kabaivanova et al., 2022). *Proteinphilum* is only present in PLSL samples and is more

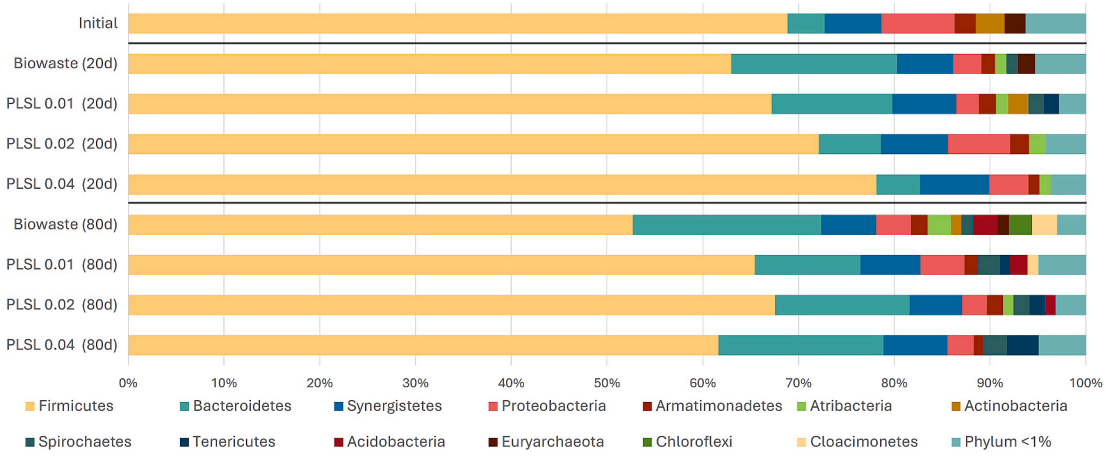


Fig. 6. Distribution of the microbial communities by phyla expressed as relative abundance. Changes in population composition can be seen through time and PLSL dosage.

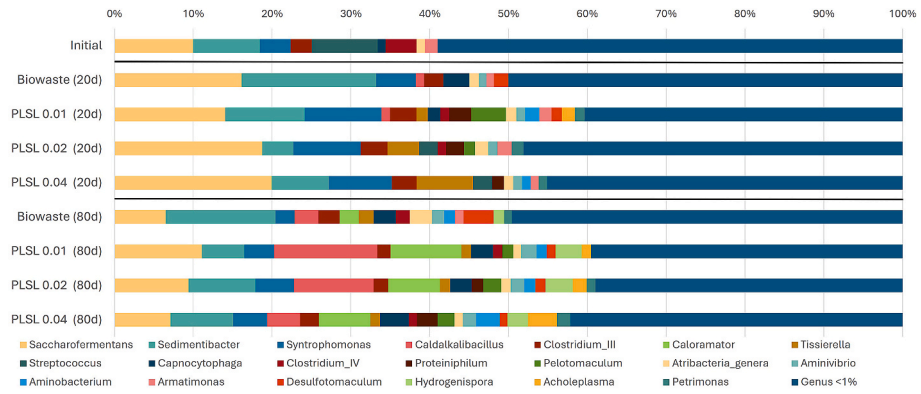


Fig. 7. Distribution of the microbial communities by genus expressed as relative abundance. Changes in population composition can be seen through time and PLSL dosage.

predominant in the 20 days sample. This genus role is important in the AD process, involved primarily in the degradation of polymers and their transformation into VFA (Hahnke et al., 2016)

Another interesting point is the absence of *Hydrogenospora* in the initial and intermediate samples and its enrichment in all final PLSL. From 1.4% in the final biowaste to around 3.5% in the PLSL samples. *Hydrogenospora* has a potential role in the hydrogenotrophic processes of methane production. (Barrena et al., 2021).

These results shed some insights to the changes in bacterial populations in the anaerobic reactors and their changes through time and PLSL dosage. Yet, more work is needed to truly understand the effects of microbial communities' changes and their effect on AD, and the effect of different additives on the populations. Biowaste is a complex material of variable composition which greatly affects the starting microbial communities and therefore AD. For these reasons it is difficult to establish quantitative relationships between the effects of SL, the communities, and the final AD production.

Some interesting investigations such as Hu et al. (2021), Kaur et al. (2019), Xu et al. (2019) have studied the effects of SL in the SCFA production, or the production of SL directly from food waste. Both show promising results but leave open the question of the effect this SL would have on AD process and the microbial communities, which this paper investigated.

4. Conclusions

In this work, a circular approach for organic waste management in a biorefinery-like configuration is presented to take profit from different technologies. On one hand, molasses from the sugar refinery process and winterization oil cake from the purification of commercial sunflower oil were used as substrates for the production of active biosurfactants as SL through solid-state fermentation of *S. bombicola* under aerobic conditions. SL were obtained as a mixture of different compounds if extracted using traditional downstream methods. However, the application of column chromatography and mass spectrophotometry resulted in the purification and identification of a pure lactonic (C18) SL. These purified PLSL forms (lactonic diacetylated C18:0, 1 and 2) were successfully applied in the anaerobic digestion of source-selected organic fraction of municipal solid waste, with an increase of 41% in the methane yield, although a long adaptation process of microbial anaerobic communities to PLSL was necessary. While PLSL had an impact on the microbial community's composition, the deeper meaning of these changes is still unclear. Further experimental research should be focused on unveiling the mechanisms by which SL improve AD performance in terms of biogas yield, which would permit to reduce the observed lag phase time, and to design effective strategies of SL enhanced-AD through continuous processes. In the framework of circular bioeconomy, in a moment when AD is being worldwide implemented as renewable energy source, a sustainability assessment to evaluate the entire process in environmental and economic terms would be also necessary.

CRediT authorship contribution statement

Nicolás Oiza: Writing – original draft, Validation, Investigation. **Javier Moral-Vico:** Writing – review & editing, Supervision, Data curation. **Antoni Sánchez:** Writing – review & editing, Supervision, Formal analysis. **Teresa Gea:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2024.143062.

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