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Alternative extraction strategies for biosurfactants produced by solid-state fermentation from organic waste

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ABSTRACT

This study investigates alternative extraction techniques in solid-state fermentation of organic substrates. Extracting from a solid matrix is a significant downstream processing challenge, as most technologies are adapted from those used in submerged fermentation without accounting for the solid matrix's intrinsic properties. In this study, extraction of sophorolipids (SL) through conventional ethyl acetate solvent extraction (maceration) was compared to pressurized liquid extraction (PLE) and ultrasound-assisted extraction (UAE). SL are especially relevant not only as potential substitutes to some traditional surfactants but also because of their interesting antimicrobial and anti-cancer properties. All strategies used both ethyl acetate and ethanol to reveal the effect of the solvent on the processes. In this study, PLE with ethyl acetate achieved the highest crude SL yields (0.443 g crude SL/g dry fermented solid). UAE yielded results similar to traditional extraction but reduced the time from 2 h to 30 min; while ethyl acetate generally resulted in higher total crude extracts, ethanol improved SL extraction across all methods. PLE using ethanol achieved the highest SL yields with high purity (0.063 g final extract/g dry fermented solid and 62 % SL content). Afterwards, supercritical fluid extraction with CO_2 (SFE-CO_2) was employed to selectively remove fatty acids and oils from the fermented solids as a pretreatment step before SL extraction. This improved SL recovery, and by performing a sequential ethanol PLE after SFE-CO_2 in the same cell, a 308 % increase in extracted SL yield was achieved compared to traditional solvent extraction with ethyl acetate. This approach also showed itself to be the greenest one following the Path2green methodology and considering solvent consumption. This research shows that PLE, SFE, and UAE are promising, more sustainable, and efficient alternatives to traditional solvent extraction.

1. Introduction

As industries transition toward sustainable and circular bioeconomy models, the valorization of agro-industrial and food waste through solid-state fermentation (SSF) offers a promising technology for producing high-value bioproducts [33]. SSF is a fermentation process that uses solid or semi-solid substrates in the absence of free-flowing water, employing specific microorganisms to produce valuable bioproducts [32]. SSF enables the use of solid waste that is traditionally discarded or requires costly pretreatments, making it both economically and environmentally advantageous [18]. A wide range of bioproducts have been produced using this technology, including biopesticides, biosurfactants, enzymes, and aromas [23].

Among the biosurfactants gaining industrial attention, sophorolipids (SL), glycolipids primarily produced by the yeast *Starmerella bombicola*, stand out for their biodegradability, emulsifying capacity, and potential eco-friendly alternatives to petroleum-derived surfactants [9]. SL are produced mainly through submerged fermentation at commercial scale. The existence of consumer available products using this biological substitute for traditional chemical surfactants highlights their viability and market interest, being Holiferm (Manchester, United Kingdom) one of the main producers. SSF arises as a potentially complementary technology useful in the development of the bioeconomy that can contribute to new waste-based value chains for biosurfactant production [10,26].

While most studies on SSF processes focus on fermentation conditions, production yield, process optimization and substrate screening, an

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effective high-yield extraction of these compounds remains underexplored, being sometimes the bottleneck of the entire SSF process in environmental and economic terms [5]. Currently, solid-liquid extraction via agitation is the most widely used method [9,23]. In general, in SSF the solvents employed are water or ethanol. Other organic solvents are used, according to the compounds to be extracted, with ethyl acetate being the most common for SL.

In this framework, advanced extraction techniques like pressurized liquid extraction (PLE) and supercritical fluid extraction (SFE) are well-established in other fields, but little research has been conducted in their use in the extraction of biocompounds from fermented solids produced through SSF. This trend is evident in literature. In the Scopus® database (www.scopus.com), in the last 25 years, only 0.78 % out of the almost 8.700 available documents containing "solid state fermentation", include results such as "supercritical", "pressurized liquid", "SFE", "PLE" or "ultrasound". SFE and PLE reduce extraction time and solvent usage, enable the use of alternative solvents, and yield high-quality products (Herrero and Ibáñez, 2015). Supercritical fluids exhibit unique properties that combine the diffusivity of gases and the solvating power of liquids when operated above their critical points. Among these, carbon dioxide (CO_2) is the most widely used due to its low toxicity, solvent-safety, and environmentally friendliness [2]. PLE utilizes liquid solvents above their boiling points and controlled pressures, which enhances solubility and extraction efficiency. Temperature is a key factor in PLE, as it alters the physical and chemical properties of solvents, improving both selectivity and yield (Ibañez et al., 2003). Both PLE and SFE have been successfully applied to extract high-value compounds from a variety of raw materials, including plant biomass, food waste, and microbial cultures (Balvardi et al., 2015). Therefore, a significant research gap is found in the case of SSF exhaust solids, especially in the field of molecules such as biosurfactants, which interact in a hydrophilic-hydrophobic layer [19]. Developing greener and more efficient extraction methods is crucial for the real-world application of SSF technology and its final implementation as a complete process beyond fermentation. Downstream processes often represent the limiting factor in terms of economic and environmental viability, accounting for a big part of the total production cost and environmental impacts [20]. In this framework, exploring the application of alternative advanced extraction technologies in SSF could unlock new possibilities for sustainable and cost-effective production of high value bioproducts from discarded organic waste in the circular bioeconomy context.

Our goal was to identify promising techniques and operational windows before investing in exhaustive optimization, since those techniques have never been explored for SL from solid-state fermentation. To achieve this, SL were produced through SSF using winterization oil cake and molasses as substrates in a 22-L packed-bed pilot reactor with continuous aeration and parameters' monitoring. Afterwards, PLE, SFE and UAE were compared to traditional solvent extraction with ethyl acetate. Additionally, the effects of ethanol as a solvent for SL was compared against ethyl acetate in different extraction methodologies.

2. Materials and methods

2.1. Solid-state fermentation

2.1.1. Materials

The substrates utilized in the SSF process were sourced locally. Wheat straw was supplied by the Veterinary Faculty of the Autonomous University of Barcelona (UAB). Sugar beet molasses (MOL) was provided by AB Azucarera Iberia S.L.U (Madrid, Spain), while winterization oil cake (WOC) was kindly donated by Lípidos Santiga, S.A (Barcelona, Spain). *Starmarella bombicola* ATCC 22214 strain was acquired from the Colección Española de Cultivos Tipo (Valencia, Spain). For the extractions, 95 % purity hexane, absolute ethanol and 99.5 % ethyl acetate were used and acquired from Merk Millipore (Burlington, USA). For SFE, technical quality CO_2 was used (Carburos metálicos, Madrid, Spain).

2.1.2. Solid-state fermentation

The fermentation process was carried out in a pilot scale 22-L reactor, following the methodology described by Rodríguez et al. [26]. This bioreactor consists of a packed-bed stainless-steel vessel with continuous forced aeration. Wheat straw and winterization oil cake were mixed in a 1:1 ratio (*w*/w), autoclaved, and combined with autoclaved sugar beet molasses diluted in distilled water to achieve 75 % of the water holding capacity of the solid material. The solid mixture, prepared at a 4:1 ratio of winterization oil cake to molasses by total weight, was inoculated with *S. bombicola* to a cell concentration of approximately 10^8 CFU/g dry matter (DM). The fermentation process, with a total mass of 3.3 kg, was monitored over 5–6 days, with oxygen uptake rate and temperature measured at various reactor heights, as explained elsewhere [13]. The final fermented product was vacuum-sealed and stored at −20 °C until SL extraction.

2.2. Extraction

2.2.1. Sample preparation

The fermented solids were dried to avoid co-extraction with the inherent water present in the samples. For this purpose, the samples were dried in batches of 200 g. Drying was carried out in an oven at 80 °C for 72 h until constant weight. Dried samples were crushed manually since mechanical grinding resulted in fine powder, which is not advised for SFE. Crushed samples were sieved in 3 different sizes: >1 mm, 1–0.5 mm and < 0.5 mm using an automatic sieve shaker (B A 200 N CISA, Barcelona, Spain). Each sample was sieved 3 times for 2 min at 2 mm amplitude. Samples were bagged, vacuum-sealed and stored at −20 °C.

2.2.2. Solvent extraction

Solvent extraction by agitation followed the protocol developed by Jiménez-Peñalver et al. [16] with slight modifications. Briefly, 1 g of fermented solid was introduced in a 60 mL vial. Ethanol or ethyl acetate were utilized as solvents. The solvent to solid ratio was 1:10 (dry solid weight/ solvent volume). The mix was agitated at 180 rpm for one hour. The solvent was separated using paper filter and the procedure was repeated on the solid. Two extractions were performed in all procedures compared to maximizing SL recovery. These extractions were performed with triplicates of independent experiments.

2.2.3. Pressurized liquid extraction

PLE was performed using an Accelerated Solvent Extractor (ASE 200, Dionex Corporation, Sunnyvale, USA). 11-mL cells were used with Restek ASE 200 cellulose filters (Pennsylvania, USA), at the inlet and outlet. After the static stage of extraction, the cell and the tubing were rinsed using fresh solvent. 0.75 g of crushed sample was introduced with an equal volume of 4 mm glass beads to avoid sample agglomeration. Nitrogen was used as the pressurizing gas. For all experiments the pressure setting was 100 bar, and the time was 15 min. Three different temperatures were tested, 50, 100, and 150 °C with triplicates of independent experiments.

2.2.4. Ultrasound assisted extraction

Ultrasound assisted extraction was performed using an ultrasound bath (JP Selecta, Barcelona, Spain). This system was set up as a direct comparison with the traditional solvent extraction by changing orbital agitation with ultrasound assisted extraction. This was performed in 15 min, so as to evaluate the extraction efficiency directly to PLE extraction. For this, 1 g of fermented solid was placed in 60 mL vials with 10 mL of solvent; sonication was carried without temperature control. After this time, the solvent was removed, the solvent filtered through paper filter, and the fermented solid returned to the vial for the process to be repeated a second time. These extractions were performed with triplicates of independent experiments.

2.2.5. Supercritical fluid extraction

The SFE was performed using a Helix Speed SFE (Applied Separations, Allentown PA, USA). Briefly, 10 g of crushed and sieved solid samples were introduced in a 300 mL cell with a paper filter at the bottom and an equal volume of 4 mm glass beads as the inserted material. Pressurized CO_2 was heated to the selected extraction temperature + 10 %. A constant flow rate of CO_2 was established at 4 L/min, measured at room conditions after complete depressurization. Extraction was performed for 90 min. Fractions were collected for 15 min during the first hour and a final sample for the last 30 min, to study the kinetics of the extraction. SFE conditions were evaluated considering two key variables: temperature (40, 50, and 60 °C), and pressure (200, 250, and 400 bar). The selected temperature and pressure ranges were based on typical values used for extracting compounds from vegetable matrices, chosen to minimize the risk of compound degradation during extraction [27], and for lipid extraction [29]. These parameters are also since no references for extraction of biosurfactants produced through SSF were found. These extractions were performed with triplicates of independent experiments.

2.2.6. Sequential ethanol extraction in SFE cell

Sequential static ethanol extraction was performed from the SFE-CO_2 process by adding 1:10 (*w/v*) of solvent to the SFE extraction cell. The cell was then pressurized with CO_2, and the extraction was performed for 15 min at the same conditions as SFE-CO_2, 250 bar and 50 °C. Extracts dissolved in ethanol were collected after CO_2 depressurization. Solvent removal was done using a rotary evaporator.

2.2.7. Hexane washing of crude extracts

Crude fractions can be defined as direct extracts from the extraction processes after drying and before any further purification or separation of specific compounds. A washing step with hexane was performed on crude SL extracts with the aim to reduce and eliminate leftover fatty acids from the fermentation process. This step helps in analytical performance and allows us to compare it to relevant literature where it is commonly performed. The washing of these crudes involves the evaporation of the solvents from the previous extraction steps by rotary-evaporation at 40 °C. This was performed in the same vials from the extraction process. After drying, the crude was quantified gravimetrically before washing with 1:20 (*w/v*) of hexane (95 % purity, Merk Millipore) assisted with ultrasound bath. This washing was performed twice. The sample was let precipitate for one minute. Afterwards, the washed hexane was collected in previously weighed vial for its analysis. Both vials were evaporated under a nitrogen stream and rotary-evaporated to ensure hexane was completely removed and quantified gravimetrically again.

2.3. Analysis and characterization of sophorolipids in washed extracts

Samples were diluted to 1 g/L directly in the evaporation vial, when possible, as to avoid the heterogeneity of the samples affecting the analytical results.

High-Performance Liquid Chromatography (HPLC) was performed following the protocol by Oiza et al., [22]. Briefly, water and acetonitrile with 0.01 % formic acid were used as mobile phases. A 25-min gradient of 30–95 % B was used, maintaining 5 min additional at 95 % B. System was equilibrated at initial conditions between samples for 5 min. Evaporative light scattering detector (ELSD; Agilent 1260) and ultraviolet (UV) were used as detectors. ELSD optimal conditions were established through testing. Evaporation temperature was set at 60 °C, nebulization temperature at 40 °C, and gas flow rate to 1.5 ml/min.

Identification was conducted using a liquid chromatography-mass spectrometry (LC-MS) system. The same chromatographic method as previously described was employed. The LC-MS was operated in negative ionization mode with an ESI source type. The instrument settings included a capillary voltage of 3500 V, a nebulizer pressure of 4 bar, a dry gas flow rate of 8 mL/min, and a dry heater temperature of 220 °C. The mass scanning range was set from 50 to 1000 *m/z*.

Quantification of extracted SL was performed through UV, since ELSD presented challenges while employed for quantification. In this sense, the observation was that comparing crude or washed SL's extracts with pure samples, extracts presented some kind of suppression on the SL signal. Thus, purer samples such as commercial standards or in-house purifications, prepared to similar concentration to those expected in the real extracts, resulted in complete saturation of the SL peaks, while the signal was very low in the extracts. Different approaches were tested to uncover the underlaying problems, but no solution was reached. For this reason, UV was used at the end for quantification, following the standard method reported elsewhere [14,15,22]. On the other hand, we acknowledge the interest and the importance of further investigation in this subject.

Since no commercial pure standards are available for the main congener produced by *S. bombicola*, SL lactonic C18 diacetylated. The areas between minutes 5 and 15 were identified, by LC-MS, as only containing SL in both the mixed SL commercial standard and our extracts. This has also been discussed previously in Oiza et al. [22] and Ingham et al. [15]. Therefore, the total chromophore area at 198 nm between minutes 5 and 15 of this commercial standard containing a mixture of different SL was used as reference. Thus, the expressed SL content is not for a single compound but an approximation to total SL content.

2.4. Statistical analysis

Statistical analysis was performed using one-way ANOVA with a confidence level of $p < 0.05$, followed by the Tukey test to identify significant differences. The analyses were carried out using the JMP Pro 18 software package (JMP Statistical Discovery LLC. Cary, USA).

2.5. Environmental performance evaluation

To evaluate the sustainability of the extraction processes, the Path2Green tool was used, which provides a comparative assessment of extraction methods based on the twelve principles of green chemistry [3,6]. This framework considers multiple dimensions of process greenness, including biomass utilization, transportation requirements, raw material pre-treatment, solvent selection, scalability, purification steps, yield efficiency, post-treatment operations, energy consumption, application potential, repurposing opportunities, and waste management. Each principle contributes to an overall score, which is visually represented through a pictogram generated by the Path2Green Android application (available in the supplementary material of de Souza Mesquita et al. (2024)). This approach allowed us to benchmark the environmental performance of the studied extraction strategies in a systematic and transparent manner.

3. Results and discussion

The efficiency and sustainability of SL recovery from SSF relies heavily on the choice and optimization of extraction methods. To this aim, a series of experiments were conducted to optimize key process variables and evaluate the yield and selectivity of various extraction techniques, comparing them with conventional solvent extraction to determine their potential as eco-friendly alternatives.

3.1. SSF sample size optimization

Drying is an expensive step, especially on a commercial scale. In this study, water present in samples can result in co-extraction of non-targeted compounds. This is especially relevant for SFE, where water in samples has widely been reported to produce coextraction [2,31] and high-water content can lead to poor extraction yields [11]. Additionally,

drying has been reported to improve oil extraction efficiency when using physical extraction methods [28]. Besides, particle size is one of the key parameters influencing the solid-liquid extraction of solids since it has a direct influence on the mass transfer rate of the process. This is even more important when dealing with SFE technology since it can present some limitations regarding the possible compaction of the bed and, consequently, causing channeling, leading to uneven extraction [11]. To study these factors, three different particle sizes (>1 mm, 1–0.5 mm, and < 0.5 mm) were investigated using SFE under the following fixed conditions (250 bar, 50 °C, 4 L/min, 90 min). Results indicate that the largest particle size (>1 mm) yielded a total of 0.19 g of crude extract/g DM, 17 % lower than that achieved with the smaller particle size (0.23 g of crude extract /g DM). Since >0.5 mm and 0.5–1 mm resulted in similar extraction yields, the intermediate sizing was chosen for further experiments to avoid accidental clogging and compression of the solids leading to uneven extraction.

3.2. Traditional solvent extraction and solvent effect

Traditionally, SL have been extracted using ethyl acetate as solvent both in submerged fermentation [15] and SSF. A general scheme of this procedure is depicted in Fig. 1 [10].

However, there are no studies comparing the effects of solvent selection on SL content in SSF. Only one study was found comparing the effect of methanol and ethyl acetate on extraction yields of SL, but in this study, the SL profile was only characterized through Fourier Transform Infrared Spectroscopy only, and the yields compared indirectly by surface tension activity [25]. Additionally, methanol is a harsher solvent both environmentally and to the user's health. For these reasons, ethyl acetate was compared to ethanol in solvent extraction with agitation. Crude extract yields were higher with ethyl acetate than with ethanol (Table 1). However, analysis of the hexane-washed extracts (after removal of residual fatty acids) revealed that ethanol yielded slightly higher SL yields. Ethanol has a relative polarity of 0.654, while ethyl acetates polarity is 0.228. Both SL and fatty acids share an ester as a functional group. However, only SL have a hydroxyl functional group located in their sophorose. It is known that solvents present better solubility to molecules with similar functional groups. We can hypothesize that both factors could affect the selectivity of the solvents for SL or free fatty acids.

This can be attributed to the higher SL concentration observed in extracts obtained with ethanol (Fig. 2) and highlights the importance of analyzing SL content in extracts in front of only measuring the total yield in crude weight. The aim of this research is to maximize SL extraction, therefore, washed yields with high SL content results are prioritized over total crude yields.

Traditionally, SL have been extracted using ethyl acetate followed by a manual hexane wash. This washing step is aimed at eliminating all fatty acids and oils remaining from the fermentation process. As demonstrated by the previous results, this washing step is crucial; insufficient washing can result in a significant overestimation. Due to its honey-like consistency, SL crude extract can trap residual fatty acids if washing is not complete. In this study, this step was assisted by ultrasound, thus increasing the contact area between extract and solvent. This may explain why the SL yields obtained in this study were lower when compared to gravimetric results from previous works [10,16]. High amounts of residual fatty acids result in heterogeneous composition of the sample, making it difficult to obtain representative samples, and therefore hindering replicability and accuracy in the analysis. Furthermore, previous experiences have shown that high fatty acid concentrations hinder SL detection in analytical procedures. For these reasons, despite hexane washing not being a green and sustainable step, it was performed on all samples to obtain comparable results. In consequence, given that solvent selection significantly impacted SL extraction yields, both solvents were evaluated in further advanced extraction techniques.

3.3. PLE optimization

To evaluate the efficiency of PLE for crude recovery, different extraction temperatures were tested to determine their impact on total yield and compound selectivity using ethanol as extraction solvent. PLE

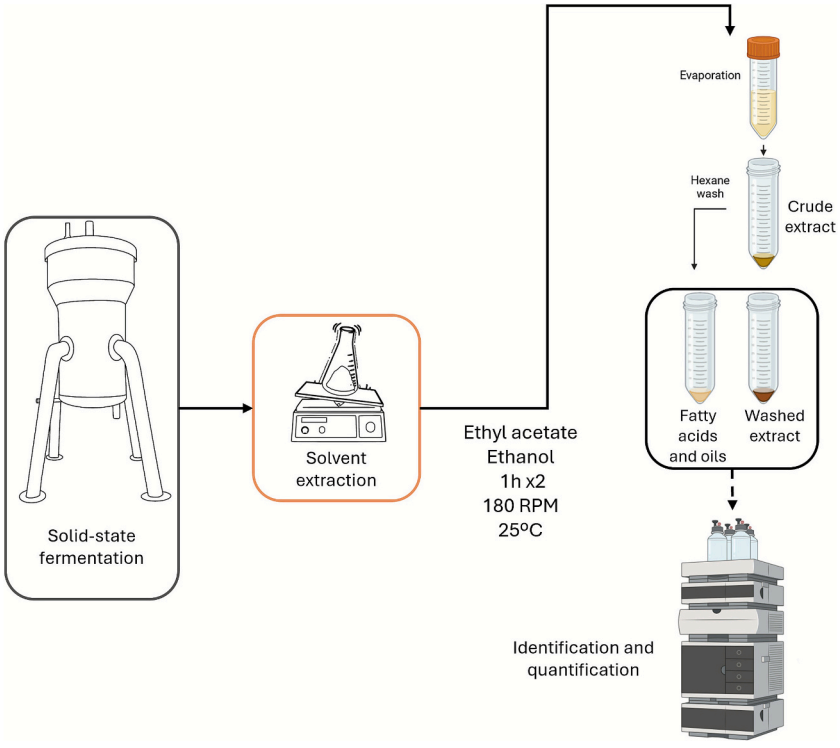

Fig. 1. Scheme of solvent effect comparing traditional solvent extraction through agitation.

Table 1
Results of sequential extractions and total yields before (crude) and after hexane washing (washed). Total SL extracted is calculated by the extraction yields and the SL content in the washed extracts. (DM: dry matter).

Stage	Solvent	First extraction (g/g DM)	SL Content	Second extraction (g/g DM)	SL Content	Total extract (g/g DM)	Total SL extracted (g/g DM)
Crude	Ethyl acetate	0.270 ± 0.006	–	0.078 ± 0.002	–	0.348 ± 0.007	–
	Ethanol	0.219 ± 0.003	–	0.082 ± 0.013	–	0.301 ± 0.013	–
Washed extracts	Ethyl acetate	0.024 ± 0.001	43 % ± 6.7	0.019 ± 0.002	42 % ± 1.1	0.043 ± 0.002	0.019 ± 0.006
	Ethanol	0.024 ± 0.000	59 % ± 3.4	0.015 ± 0.002	58 % ± 2.4	0.040 ± 0.002	0.023 ± 0.002

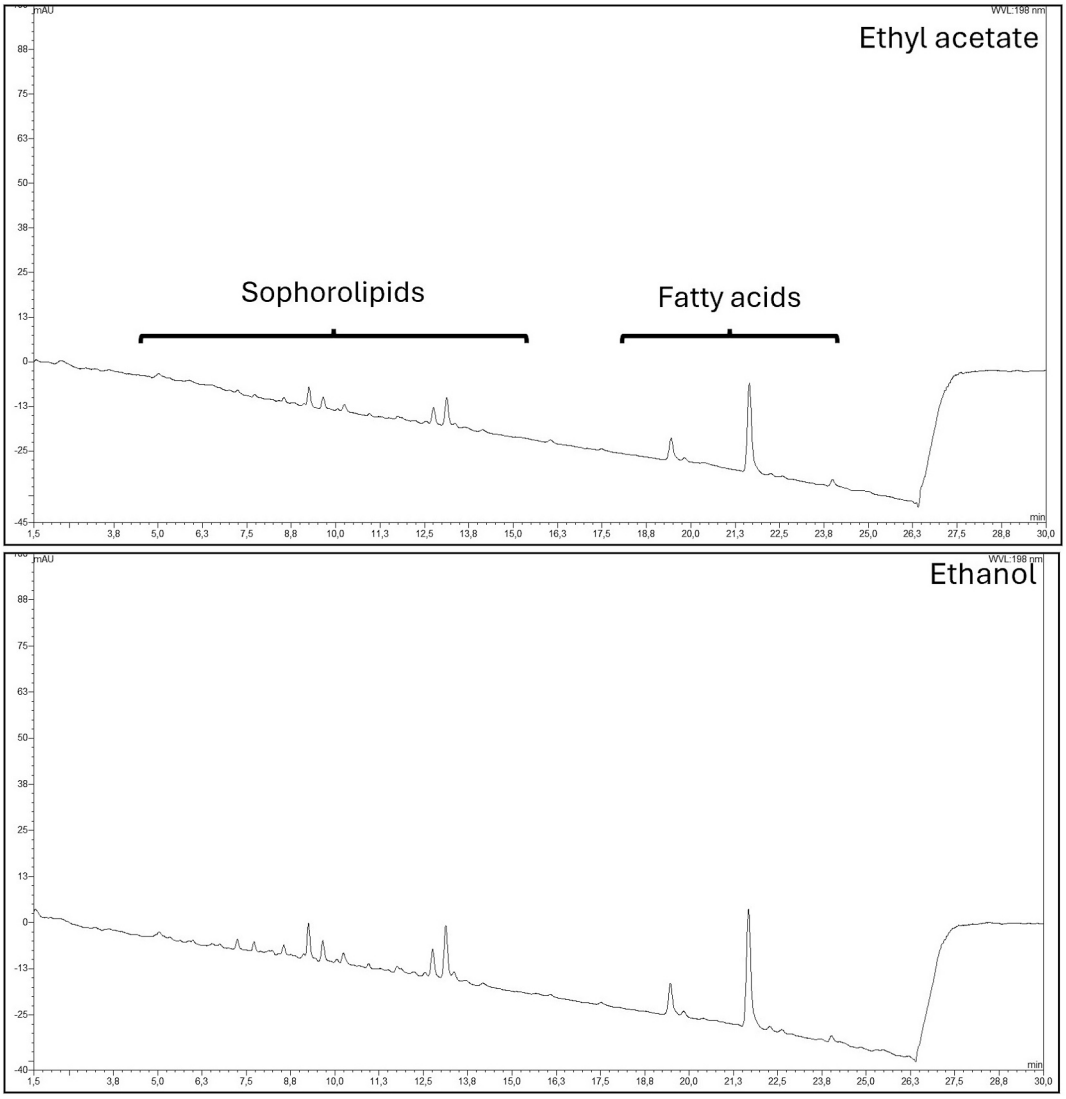

Fig. 2. Chromatograms corresponding to washed extracts obtained with ethyl acetate and ethanol solvents through agitation. All sophorolipids eluted between 5-15 minutes; fatty acids eluted between 18 and 25 minutes.

total crude yields increased with temperature, achieving 0.422 g crude extract/g DM at 150 °C, 0.375 g crude extract /g DM at 100 °C and 0.271 g crude extract /g DM at 50 °C. These results are significantly higher than those obtained through traditional solvent extraction of SL produced through SSF, for which SL extraction ranged from 0.15 to 0.2 g/g DM [10,22] using ethyl acetate as extraction solvent. Higher temperatures resulted in higher crude yields, this phenomenon being also described in the extraction of oils from other solid matrices (Balvardi et al., 2015). However, these crudes not only contain SL, but also impurities and mostly free fatty acids or residual oils from the fermentation process [14,15]. When analyzing these results through ELSD, the areas corresponding to SL were higher at 50 and 100 °C. However, at 50 °C the extraction resulted in a higher co-extraction of fatty acids. For this reason, 100 °C was selected as the optimum temperature condition for PLE.

3.4. Screening of extraction methods

A comparative assessment was conducted to evaluate the performance of traditional solvent extraction through agitation, PLE, and ultrasound-assisted extraction and two solvents (ethanol and ethyl acetate) in terms of crude yield and SL recovery as schematically presented in Fig. 3.

Traditional solvent extraction with ethyl acetate was used as a

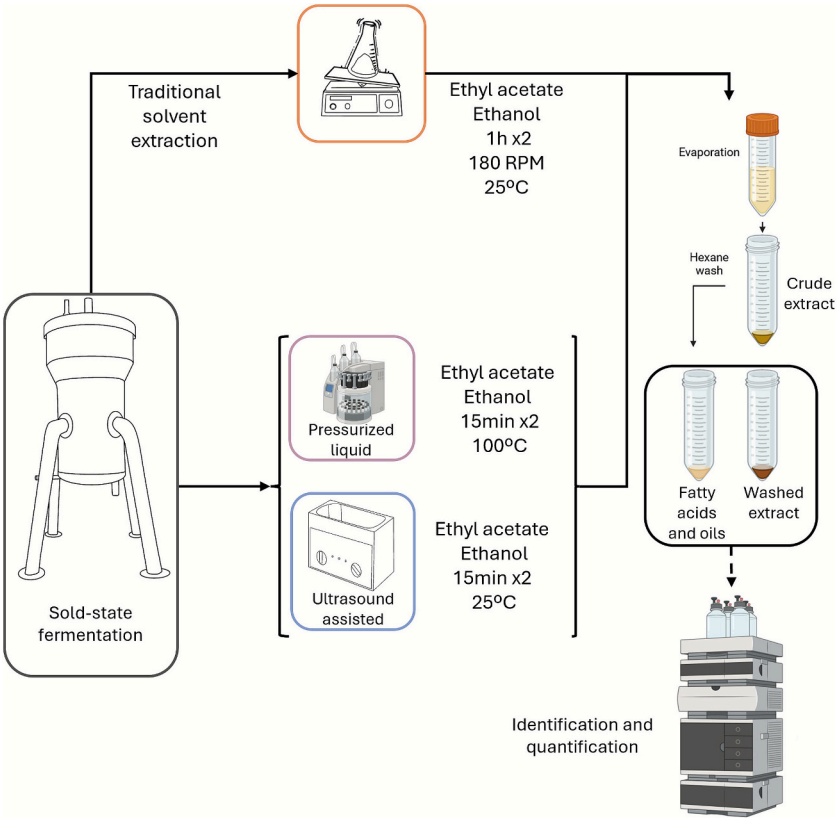

Fig. 3. Schematic of the alternative extractions and the conditions tested for each one. Traditional solvent extraction was previously explained (RPM-revolutions per minute).

control for the advanced techniques presented in Fig. 3, as it is the most employed methodology in the literature for the recovery of SL from SSF, as commented before [10,16]. Ultrasound-assisted extraction time was not systematically optimized but it was set to the same operation time as PLE to compare the competitiveness between both processes.

3.4.1. *Total crude extracted*

As shown in Table 2, the choice of the extraction method has a significant effect on crude production yields (adjusted *p*-value = 0.0004). PLE with ethyl acetate achieved the highest crude yield (0.443 g crude extract /g DM; Table 2) and was significantly higher (adjusted p-value = 0.0014) than that of traditional ethyl acetate extraction (0.348 g crude extract/g DM; Table 1). In contrast, ultrasound extraction using ethanol resulted in the lowest yield (0.269 g crude extract /g DM, Table 2) and performed significantly worse than traditional ethyl acetate extraction (adjusted p-value = 0.0099). Although ethyl acetate generally led to higher yields than ethanol, this difference was not statistically significant with 95 % confidence (adjusted p-value = 0.0651).

The efficiency of the first extraction step varied with the extraction method. PLE recovered the highest percentage of the total yield (82 % with ethyl acetate, 84 % with ethanol, respectively), followed by traditional extraction (78 % with ethyl acetate, 73 % with ethanol, respectively). Ultrasound extraction was the least efficient technique in the first step (77 % with ethyl acetate, 61 % with ethanol, respectively).

It is important to emphasize that, although no significant differences were observed in crude yields between conventional solvent extraction and PLE, the latter offers substantial advantages in terms of extraction time and solvent volume. This ultimately leads to reduced solvent and energy consumption for crude extract recovery, enhancing overall efficiency and sustainability of the biosurfactants recovery from SSF.

Table 2
Crude yields after different extraction procedures (pressurized liquid extraction-PLE and ultrasound-assisted extraction) with different solvents. Both consecutive extractions on the same material were recorded as well as the total crude extract.

Extraction method	Solvent	First extraction (g/g DM)	Second extraction (g/g DM)	Total extraction (g/g DM)
PLE	Ethyl acetate	0.362 ± 0.018	0.082 ± 0.006	0.443 ± 0.019
	Ethanol	0.327 ± 0.012	0.062 ± 0.004	0.389 ± 0.013
Ultrasound	Ethyl acetate	0.232 ± 0.006	0.070 ± 0.005	0.302 ± 0.008
	Ethanol	0.164 ± 0.027	0.105 ± 0.002	0.269 ± 0.027

3.4.2. *Effects of hexane washing on the extraction yields*

After hexane washing, approximately 9–15 % of the first extraction dry crude and 20–30 % of the second extraction dry crude remained as washed dry extracts. Total yields and SL content can be found in Table 3. PLE achieved the highest yield of washed crude with ethanol (0.039 g/g DM). PLE, independently of the solvent, performed better than traditional ethyl acetate extraction (Adjusted *p*-value 0.0001). Ultrasound extraction performed very similarly to traditional solvent extraction in 30 min compared to 2 h. In consequence, it can be concluded that the extraction method had a significant effect on washed extract yield (adjusted p-value <0.0001).

In the first PLE extraction, SL content with ethyl acetate was 37 % while with ethanol it was 68 %. This phenomenon was already observed in Section 2.2. However, ethanol showed higher SL selectivity when using PLE. This explains that although PLE with ethyl acetate achieved the highest yields of crude, ethanol achieved higher total SL yields. Solvent did have a significant impact on SL content of the washed

Table 3
Extraction yields after washing the crudes obtained in Section 3.3.1 with hexane to remove residual fatty acids. (pressurized liquid extraction-PLE and ultrasound-assisted extraction) with different solvents. Both consecutive extractions on the same material were recorded as well as the sophorolipid (SL) content.

Extraction method	Solvent	First extraction (g/g DM)	SL Content	Second extraction (g/g DM)	SL Content	Total washed extract (g/g DM)	Total SL extracted (g/g DM)
PLE	Ethyl acetate	0.038 ± 0.004	37 % ± 6.5	0.023 ± 0.003	36 % ± 2.9	0.060 ± 0.005	0.022 ± 0.002
	Ethanol	0.045 ± 0.001	68 % ± 7.7	0.018 ± 0.001	46 % ± 2.9	0.063 ± 0.001	0.039 ± 0.004
Ultrasound	Ethyl acetate	0.020 ± 0.001	45 % ± 1.8	0.014 ± 0.001	42 % ± 2.7	0.034 ± 0.001	0.015 ± 0.001
	Ethanol	0.025 ± 0.002	50 % ± 3.4	0.019 ± 0.000	60 % ± 2.3	0.044 ± 0.002	0.024 ± 0.000

extracts (Adjusted p-value 0.0001). Ethanol achieved higher SL content in all extractions, independently of the technology applied or the number of consecutive extractions.

At this point, it is important to mention that SL quantification is a complex issue. Different studies use different approaches to SL quantification, such as anthrone-thin layer chromatography [30], gas chromatography [21], or oil spreading [17]. However, these analyses are indirect measurements of SL content. Most in-depth studies on SL quantification have been carried in submerged fermentation samples, where SL quantification is performed in the fermentation broth or from precipitated SL [8,14,15]. When these analytical methods are applied to SL extracts produced from SSF, some inconsistencies appear. Our experience shows that the heterogeneous composition of SSF-produced SL extracts makes impurity removal highly challenging. These difficulties, in addition to the changes in hexane washing and the lack of pure SL standards containing a single congener, make it challenging to compare SL results across different studies. This challenge was also highlighted by Ingham et al. [15].

As PLE with ethanol showed the highest total SL yields and purity, it was selected as the optimum technique to study alternative methods for removing fatty acids from the extracts.

3.5. SFE-CO_2 optimization

The first step was to perform a kinetic study to determine the effect of time on extract yield and composition. Results of these extractions can be seen in Fig. 4. Higher pressure and temperature in SFE-CO_2 led to increased total crude yields, with the highest yield observed at 90 min, 400 bar and 60 °C (0.238 g/g DM). However, SL were detected using the highest-pressure conditions and primarily in the later extraction fractions of the kinetic study, indicating delayed release or lower solubility under supercritical conditions. At lower pressure conditions (200 bar) and intermediate conditions (250 bar), no SL were detected during extraction. The differences in the extract's profiles can be found in the Supplementary Information (Fig. S1).

Intermediate pressure conditions achieved a crude yield similar to that of high-pressure conditions (0.23 g/g DM) while low pressure conditions at 60 °C yielded 0.159 g/g DM and at 40 °C, 0.19 g/g DM. Because of this, the intermediate condition was selected as optimal due to its lower operational pressure and high fatty acid removal, without co-extracting SL. These intermediate conditions (250 bar and 50 °C) are similar to those used in other studies to extract oils and fatty acids [12]. The primary aim of SFE-CO_2 in this context was to act as a selective pretreatment to remove residual fatty acids, thereby preventing coelution with SL in subsequent steps. This approach offers an alternative to hexane washing, a method known to leave undesirable chemical residues and cause oxidative degradation of extracted oils [7,24]. This way a new product stream is recovered of fatty acids with no hexane contamination which could be revalorized or reused in the fermentation process.

The use of SFE on biosurfactants extraction has not been previously explored. However, it has been used for oil and fatty acids extraction with good results and high selectivity [4]. In submerged fermentation, hexane washing of the broth is performed before ethyl acetate extraction. This removes residual fatty acids before the SL extraction. In SSF, because of the volume of the solids, extracting from the fermented solids directly with hexane would require high amounts of this solvent. Hexane is a non-green solvent, with high toxicity and its use should be avoided

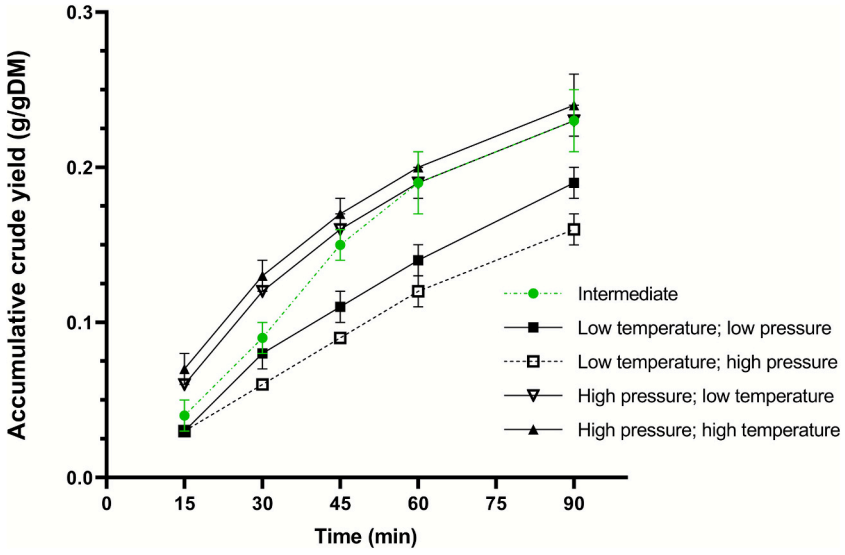

Fig. 4. Kinetic extraction through SFE-CO_2 from fermented solids. Intermediate (250 bar, 50ºC); low pressure (200 bar); high pressure (400 bar); low temperature (40 ºC) and high temperature (60ºC).

in large quantities. To circumvent this, supercritical CO_2 extraction (SFE-CO_2) is proposed for this step. To explore its potential, SFE-CO_2 was optimized by varying pressure and temperature to assess its efficiency in removing residual oils and fatty acids without altering SL integrity.

3.6. SFE-CO_2-ethanol

To reduce all extraction steps to a single system, a static ethanol extraction was performed immediately following SFE-CO_2 treatment in the same cell as presented in Fig. 5. Ethanol was added directly to the same extraction cell and processed under identical conditions (250 bar, 50 °C) for 15 min. The system was then gradually depressurized, and the solvent was collected.

The ethanol extract yielded 0.138 g crude extract/g DM; this crude was also washed with hexane to remove any residual fatty acids. After hexane washing 55 % of the sample remained much higher than the 9–20 % of the previous results. At this point it should be emphasized that SFE-CO_2 was performed to extract fatty acids while hexane washing of the extracted crudes was performed to compare to results from previous sections and to improve analytical results. The resulting washed SL had a content of 73 %, one of the highest obtained in this study. This approach resulted in a final recovery of 0.078 g washed extract/g DM, an 181 % increase over ethyl acetate solvent extraction, and 0.056 g of SL/g DM, representing a 308 % increase compared to traditional ethyl acetate extraction (0.019 g/g DM). This extraction used only one solvent extraction while the previous sections used two sequential extractions, reducing the solvent use by half. Additionally, this method also produced a clean fatty acid fraction without SL contamination, enabling potential revalorization, which was recovered using CO_2 instead of hexane. CO_2 is an environmentally friendly solvent, cheap and generally recognized as safe [11] and can be used directly to remove the fraction of fatty acids from the fermented solids. However, performing a direct extraction on the fermented solids to remove the fatty acids with hexane is not possible since it would require high volumes of hexane. In washing steps of the crude, hexane is added at a 1:20 (*w/v*) ratio. Since this is applied to the crude extracts, the volume needed is small. However, if this same ratio was to be applied directly to the fermented solid, as in the removal of the fatty acids before SL extraction, the volume use would be 10-fold. Besides, both hexane wash and solvent extraction with ethyl acetate are performed twice to ensure higher extraction yields, it can be assumed that a similar approach would be needed with a direct hexane extraction, thus increasing the volume needed even further. In this experiment, 10 g of dry fermented solid resulted in around 1.4 g of crude SL extract, after SFE-CO_2 removal of fatty acids. For this step, 28 mL of hexane were used, while if we keep all our previous assumptions, the needed volume for a direct extraction would be 400 mL. These values can be considered as an estimation on how the volume of hexane is reduced when using SFE-CO_2 removal of fatty acids.

To evaluate the efficiency of all these approaches, the solvent consumption to obtain one gram of SL was calculated. As seen in Table 4, ethanol extractions performed better than ethyl acetate, with values between 900 and 700 mL compared to over 1000 mL. The benefits of the proposed SFE system are made clear here, where the reduction in residual fatty acids, and the increase in purity of the washed extract result in the highest efficiency with a consumption of only 171 mL. This results in just a 15 % of the needed solvent to achieve 1 g of SL.

In conclusion, using only a single vessel as an extraction cell also improves operational times from an industrial application of this technology. By doing the ethanol extraction in the same vessel and under the same conditions as CO_2 extraction, there is no need for lengthy cooling/heating and depressurizing and repressurizing procedures. SFE-CO_2, followed by ethanol extraction, is proposed as a highly effective, environmentally preferable alternative to solvent intensive downstream processes. This process can be translated into a biorefinery concept, where two fractions are recovered. The washed SL fraction that can be valorized into multiple uses as stated before, and the oil and fatty acids fraction without hexane traces, that can be valorized in different ways, such as animal feed, some industrial applications, can be reused in the fermentation process as hydrophobic substrate. As waste, the CO_2 of the SFE-CO_2 and the ethanol of the extraction can be recaptured and reused,

Table 4
Extractive solvent needed to obtain 1 g of sophorolipid with each of the tested methodologies. These calculations take into account the sophorolipid purity in the washed extracts.

Extraction method	Solvent needed (mL)
Solvent extraction ethyl acetate	1080.8
Solvent extraction ethanol	855.9
PLE ethyl acetate	1358.0
PLE ethanol	764.7
Ultrasound ethyl acetate	1346.2
Ultrasound ethanol	842.1
SFE ethanol	171.2

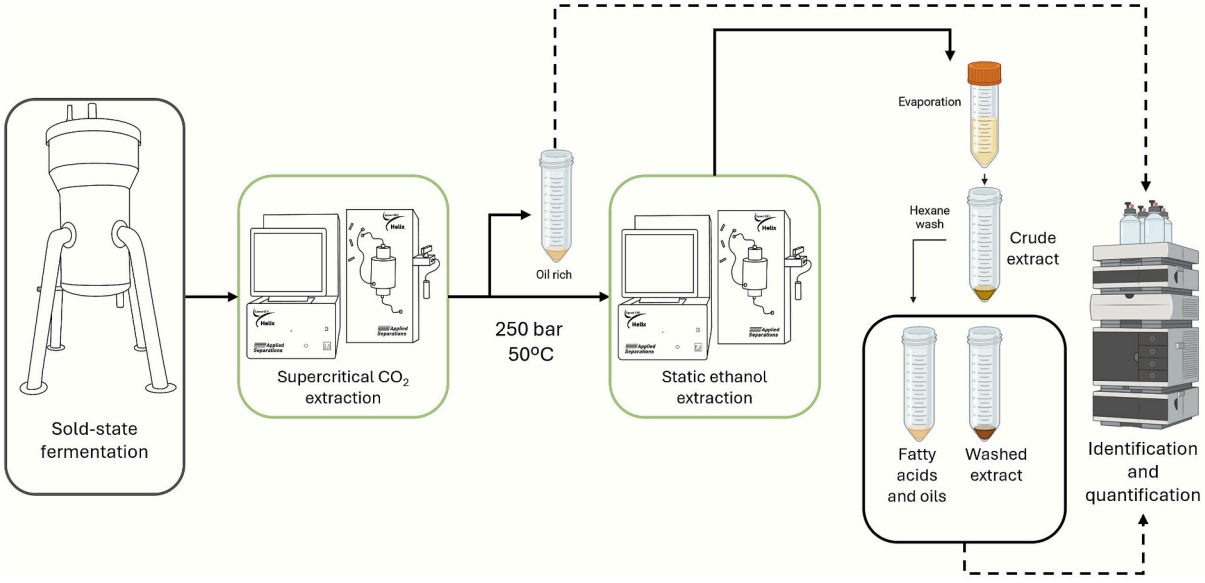

Fig. 5. Scheme of proposed downstream processing for SFE-produced SL, consisting of a SFE-CO_2 followed by pressurized ethanol extraction in a one vessel system.

the exhausted fermented solid can be used in composting or in anaerobic digestion processes. Therefore, this biorefinery can be conceptualized as a zero-waste process.

Further studies should be focused on optimizing and scaling up this technology and with that estimating the economic and environmental benefits of this alternative in comparison with traditional steps, as to clarify if this new processes better yields warrant the use of more complex equipment and energy compared to higher volumes of toxic solvents.

3.7. Environmental performance of alternative extraction strategies for biosurfactants produced by solid-state fermentation from organic waste

Assessing how "green" the developed extraction processes are is essential to ensure their alignment with sustainability goals and the principles of circular bioeconomy. While the most comprehensive approach for evaluating environmental performance is Life Cycle Assessment (LCA), its application at laboratory scale can be challenging due to data limitations and complexity. For this reason, several simplified metrics and tools have been proposed to estimate the environmental impact of chemical and bioprocesses, as compiled in resources such as Chemistry for Sustainability (https://chemistryforsustainability.org/tools). In this study, we selected Path2green (de Souza Mesquita et al., 2024) because it is specifically designed for unit operations for biomass valorization and biorefinery processes and is grounded in the Twelve Principles of Green Chemistry [3]. This tool enables a preliminary yet systematic comparison of extraction strategies, providing an accessible benchmark for environmental performance and guiding future optimization toward greener and more sustainable processes. The main things evaluated were the different solvents used, energy consumption, scaling and purification. The evaluation is carried out following 12 principles, a table with all the selected values is available in the supplementary information:

Principle 1 (biomass): Is related to the selection of biomass used in the process. Since the fermentation process is performed using wastes from different origins, a value of 1 was selected.

Principle 2 (transportation): since all waste were locally sourced and are commonly, a value of 50 km was assigned.

Principle 3 (Pre-treatment): In all cases samples were dried and crushed. Following the documentation these both fall under physical treatments.

Principle 4 (solvent): This refers to the solvent used in the extraction step, ethanol is considered a recommended solvent, while ethyl acetate is problematic. In the case of SFE, ethanol was used in the second extraction, so that was selected as the value.

Principle 5 (scaling): All systems except the last one are based in batch steps, where first the extraction is performed, the solvent is evaporated and then the washing is performed. However, SFE extraction can be performed in a "in flow" mode, where when the CO_2 extraction finishes, the ethanol can directly be pumped into the vessel without having to depressurize and repressurize the system.

Principle 6 (purification): All systems except SFE need the use of n-hexane to remove the impurities of the extracts, for this reason, it was considered based on hazard solvents. Meanwhile, SFE if completely optimized, the majority of residual fatty acids could be extracted before the SL extraction thus removing the need for solvent purification.

Principle 7 (yield): Since SFE allowed the recovery not only of the SL fraction but also the fatty acids, this principle was set as complete valorization, while in the other systems it was deemed exhaustive extraction.

Principle 8 (post-treatment): In all cases it was left to combining up to two recommended solvents, due to the possibility of incorporating post-treatment practices such as chromatography independently of the extraction evaluated in this work.

Principle 9 (Energy): In the scope of a biorefinery we imagine these systems using a mixture of renewable energy, biogas being one of the possibilities as explained previously. Taking this into account, maceration was deemed low-dependent renewable, while all the others were high-dependent renewable.

Principle 10 (application): Since SFE allowed us to obtain a fatty acid fraction free of n-hexane and the SL fractions have high purity, we deemed they decided that this higher flexibility would be reflected in the application to up to five domains, while in other cases it would be down to two domains. This is especially since cosmetic and pharmaceutical applications have higher purity requirements.

Principle 11 (repurposing): Due to the scale of the experiments conducted in this work, the solvent recovery rates could not be evaluated. However, at industrial scale both the recovery of organic solvents and CO_2 is always implemented [1].

Principle 12 (waste): this principle was set to 0 in all cases as more optimizations would be needed to truly understand the potential of each technology.

With these considerations, SFE emerges as the clear greener option, with a score of 0.482 compared to 0.1 of traditional solvent extraction using ethyl acetate (Table S1). This difference comes mostly from the change of ethyl acetate to ethanol and the substitution of hexane for CO_2, while archiving higher purity levels of SL and providing a side stream of fatty acids. On top of this, higher yields were obtained, signaling this as a viable strategy for SL recovery in SSF processes.

4. Conclusions

This study addresses a critical gap in product recovery from SSF by comparing different extraction technologies, namely PLE, UAE, solvent extraction, and SFE. Although previous studies have employed these technologies in the context of SSF, this is the first study to utilize these technologies for biosurfactant recovery and to compare the resulting yields. We demonstrated that PLE achieved higher crude yields (0.443 g/g DM) with ethyl acetate and the highest washed SL extract with ethanol (0.039 g/g DM). Solvent selection was demonstrated to have a significant effect on the selectivity of SL extraction. This resulted in higher SL content compared to the traditional ethyl acetate. The use of SFE-CO_2, a greener alternative to hexane, as a pretreatment provided a selective removal of fatty acids. SFE-CO_2 also enabled the recovery of a clean fatty acid extract, opening an opportunity for its revalorization within a biorefinery concept. SFE-CO_2 followed by static ethanol extraction within a single reactor, resulted in a one vessel system, which achieved 181 % washed extract, and 308 % SL yields when compared to traditional solvent extraction with a higher SL content in the final extract. This new system also proved to score better in the Path2green assessment (0.482 compared to 0.1).

Future works regarding SL extraction could explore the further optimization of SFE-CO_2 parameters to reduce to the minimum the fatty acids carried on to subsequent extraction. This work serves as an initial screening of possible extraction methodologies not traditionally used in SSF processes and further optimizes the reduction in organic solvent use for obtaining a high purity SL extract. The advantages of these news systems have to be assessed both environmentally and economically in future work.

CRediT authorship contribution statement

Nicolás Oiza: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Javier Moral-Vico:** Writing – review & editing. **Jose A. Mendiola:** Writing – review & editing, Funding acquisition, Conceptualization. **Elena Ibañez:** Writing – review & editing, Funding acquisition, Conceptualization. **Antoni Sánchez:** Writing – review & editing, Funding acquisition. **Teresa Gea:** Writing – review & editing, Validation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2025.136501.

Data availability

Data will be made available on request.

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