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Second-generation feedstocks for sustainable sophorolipids production: submerged and solid-state fermentation approaches

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Second-generation feedstocks for sustainable sophorolipids production: submerged and solid-state fermentation approaches.

PhD Thesis

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Teresa Gea Leiva, professora titular i **Xavier Font Segura**, catedràtic laboral del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

Certifiquem:

Que la graduada en Biotecnologia y màster en Microbiologia Avançada **Estefanía Nathaly Eras Muñoz** ha realitzat sota la nostra direcció, i als laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, el treball amb el títol "*Second-generation feedstocks for sustainable sophorolipids production: submerged and solid-state fermentation approaches*", que es presenta en aquesta memòria i que constitueix la seva tesi per optar al Grau de Doctor en Biotecnologia per la Universitat Autònoma de Barcelona.

I, per a què se'n prengui coneixement i consti als efectes oportuns, presentem a l'Escola de Postgrau de la Universitat Autònoma de Barcelona l'esmentada tesi, signant el present certificat

Dra. Teresa Gea Leiva

Dr. Xavier Font Segura

Bellaterra, setembre de 2024.

A familia por ser mi motor de vida.

A Marina, Victoria, Rosa y Fernando en el cielo.

"You cannot get through a single day without having an impact on the world around you. What you do makes a difference, and you have to decide what kind of difference you want to make." - Jane Goodall.

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Summary

Sustainable processes centered on residues valorization and circular bioeconomy have driven the scientific community to develop new value chains for producing marketable products. Within this context, sophorolipids (SLs), a class of microbial surfactants produced mainly by *Starmerella bombicola*, exhibit environmentally friendly properties, making them potential substitutes for conventional chemical surfactants.

This research contributes to the "Surfing Waste" project (*Biosurfactantes glicolipidos a partir de residuos, una alternativa a los surfactantes quimicos en la bioeconomia circular, Ministerio de Ciencia e Innovación,* PID2020-114087RB-I00) and extends ongoing research on the production of SLs from waste through solid-state fermentation (SSF). The primary focus of this thesis was SSF, with an additional evaluation of the process in submerged fermentation (SmF) conducted in collaboration with Dr. James Winterburn at The University of Manchester, thereby contributing this knowledge to the group.

The initial phase of this research (Chapter 4) involved the optimization of hydrophilic carbon sources (glucose) and nitrogen sources (urea) using a Box-Behnken experimental design within SSF at the Erlenmeyer scale. A subsequent comparison was made between the optimized ratio and those reported in the literature, using 0.5 L reactors. Following the optimization, pure substrates (glucose, urea) were substituted with industrial and municipal waste.

Beyond the described nutrient sources, the process requires a hydrophobic carbon source. Consequently, in the second phase (Chapter 5), the scope of hydrophobic residues in SSF was broadened by replacing sunflower winterization

oil cake (employed by the group as the model hydrophobic residue) by different oilseed cakes (a byproduct of the oil industry). These experiments were conducted using 0.5 L reactors, with a scale-up to 22 L reactors, where corn oilseed cake and rapeseed oilseed cake yielded promising results. Additionally, the study highlighted the correlation between the free fatty acid content in the hydrophobic residue and the final composition of the SLs crude extract.

The third phase (Chapter 6) was conducted as part of an international research collaboration, where commercial protease from *Aspergillus niger* was employed to evaluate the use of hydrolysates derived from agro-industrial residues as nitrogen sources in SmF. Several nitrogen concentrations were evaluated and the best outcome was scaled from the shake flask (250 mL) to a 2 L reactor, obtaining promising results with wheat hydrolysate.

Throughout this thesis, significant efforts were dedicated to the identification and quantification of SLs using chromatographic techniques (HPLC-UV/HPLC-MS) to enhance the precision of results, which have commonly been based on SLs crude extract.

Finally, Chapter 7 presents a global discussion on the application of second-generation feedstock in the sustainable production of SLs, proposing selection criteria for their use. Additionally, a novel approach is introduced, advocating for the integration of SSF and SmF as complementary strategies with the same goal: the absolute utilization of a residue within the framework of circular bioeconomy.

Resumen

La búsqueda de procesos sostenibles enfocados en la valorización de residuos y la bioeconomía circular por parte de la comunidad científica ha promovido el desarrollo de nuevas cadenas de valor para la generación de productos comercializables. Dentro de este marco, los soforolípidos (SLs), un tipo de tensoactivo microbiano producido principalmente por *Starmerella bombicola*, presentan características amigables con el ambiente, lo que los convierte en posibles sustitutos de los surfactantes químicos.

El presente trabajo está enmarcado dentro del proyecto *Surfing Waste* cuyo propósito es la producción de biosurfactantes glicolípidos a partir de residuos, una alternativa a los surfactantes químicos en la bioeconomía circular, Ministerio de Ciencia e Innovación, (PID2020-114087RB-I00) y representa la continuación de la línea de investigación para la producción de SLs mediante fermentación en estado sólido (SSF). Esta tesis se enfocó principalmente en la SSF y, mediante colaboración internacional con el Dr. James Winterburn *The University of Manchester*, también se evaluó el proceso en fermentación sumergida (SmF), aportando con este conocimiento al grupo.

El primer bloque (Capítulo 4) de esta investigación se centró en optimizar las fuentes de carbono hidrofílico (glucosa) y la fuente de nitrógeno (urea) mediante un diseño experimental tipo Box-Behnken en SSF a escala Erlenmeyer. Posteriormente, se realizó una comparativa entre el ratio optimizado y el ratio reportado en la literatura en reactores de 0.5 L. Finalmente, a partir de los valores optimizados, se sustituyeron los sustratos puros (glucosa, urea) por residuos industriales y municipales.

Además de las fuentes de nutrientes descritas, una fuente de carbono hidrofóbico es esencial en el proceso, por ello en el segundo bloque (Capítulo 5), se amplió el rango de residuos hidrofóbicos utilizados hasta el momento en SSF sustituyendo el residuo de winterización de aceite de girasol (utilizado por el grupo como residuo modelo) por diferentes tortas de prensado de semillas (subproducto de la industria del aceite). Los experimentos se realizaron en reactores de 0.5 L y se escaló el proceso a 22 L, donde las torta de prensado de germen de maíz y colza mostraron resultados prometedores. A su vez, se destacó la relación entre el contenido de ácidos grasos libres en el residuo hidrofóbico y la composición del extracto crudo de SLs.

El tercer bloque (Capítulo 6) se realizó como parte de una estancia internacional, donde se utilizó la proteasa comercial de *Aspergillus niger* para evaluar el uso de hidrolizados de residuos agroindustriales como fuentes de nitrógeno en SmF. Donde se evaluaron distintas concentraciones de nitrógeno y se escaló la mejor combinación de Erlenmeyer (250 mL) a biorreactor de 2 L, obteniendo resultados prometedores con el hidrolizado de trigo.

Además de ello, a lo largo de la tesis se trabajó constantemente en la identificación y cuantificación de SLs mediante técnicas cromatográficas (HPLC-UV/HPLC-MS), con el fin de aportar precisión a los resultados reportados hasta el momento, los cuales se basan en el extracto crudo de SLs.

Finalmente, en el Capítulo 7 se plantea una discusión global sobre la aplicación de residuos para la producción sostenible de SLs, estableciendo un criterio de selección para su uso ya su vez, se presenta un enfoque novedoso donde se plantea el uso de SSF y SmF como estrategias complementarias con un mismo fin: el aprovechamiento absoluto del residuo y la bioeconomía circular.

Resum

La recerca de processos sostenibles enfocats a la valorització de residus i la bioeconomia circular per part de la comunitat científica ha promogut el desenvolupament de noves cadenes de valor per a la generació de productes comercialitzables. Dins aquest marc, els soforolípids (SLs), un tipus de tensioactiu microbià produït principalment per *Starmerella bombicola,* entre altres llevats, presenten característiques respectuoses amb el medi ambient, fet que els converteix en possibles substituts dels surfactants químics. El present treball s'emmarca dins del projecte *Surfing Waste* el propòsit del qual és la producció de biosurfactants glicolips a partir de residus, una alternativa als surfactants químics en la bioeconomia circular, Ministeri de Ciència i Innovació, (PID2020-114087RB-I00) i representa la continuació de la línia d'investigació per a la producció de SLs mitjançant fermentació en estat sòlid (SSF). Aquesta recerca es va centrar principalment en la SSF i, mitjançant col·laboració internacional amb el Dr. James Winterburn *The University of Manchester*, es va avaluar també el procés en fermentació submergida (SmF), aportant aquest coneixement al grup.

El primer bloc (Capítol 4) d'aquesta investigació es va centrar en optimitzar les fonts de carboni hidrofílic (glucosa) i la font de nitrogen (urea) mitjançant un disseny experimental de tipus Box-Behnken en SSF a escala Erlenmeyer. Posteriorment, es va fer una comparació entre el ràtio optimitzat i el ràtio reportat a la literatura utilitzant reactors de 0,5 L. A partir dels valors optimitzats, es van substituir els substrats purs (glucosa, urea) per residus industrials i municipals.

A més de les fonts de nutrients descrites, és essencial una font de carboni hidrofòbic en el procés, per la qual cosa, en el segon bloc (Capítol 5), es va ampliar el rang de residus hidrofòbics utilitzats en la SSF fins al moment en el procés mitjançant la substitució del residu de winterització d'oli de gira-sol (utilitzat pel grup com a residu hidrofòbic model) per tortes de premsat de llavors (subproducte de la indústria de l'oli). Aquests experiments es van realitzar utilitzant reactors de 0,5 L i escalant el procés fins a 22 L, on la torta de premsat de germen de blat de moro i la torta de premsat de colza van mostrar resultats prometedors. A més, es va destacar la relació entre el contingut d'àcids grassos lliures en el residu hidrofòbic i la composició final de l'extracte cru de SLs.

El tercer bloc (Capítol 6) es va dur a terme com a part d'una estada internacional, on es va utilitzar la proteasa comercial d'*Aspergillus niger* per avaluar l'ús d'hidrolitzats de residus agroindustrials com a fonts de nitrogen en SmF. On es van avaluar diferents concentracions de nitrogen i es va escalar la millor combinació d'Erlenmeyer (250 mL) a bioreactor de 2 L, obtenint resultats prometedors amb l'hidrolitzat de blat.

A més, al llarg de la tesi es va treballar constantment en la identificació i quantificació de SLs mitjançant tècniques cromatogràfiques (HPLC-UV/HPLC-MS) per aportar precisió als resultats reportats fins al moment, que es basen en l'extracte cru de SLs.

Finalment, en el Capítol 7 es planteja una discussió global sobre l'aplicació de residus per a la producció sostenible de SLs, establint un criteri de selecció per al seu ús. A més, es presenta un enfocament innovador on es planteja l'ús de SSF i SmF com a estratègies complementàries amb un mateix objectiu: l'aprofitament absolut del residu i la bioeconomia circular.

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CFU	Colony forming unit	Total CFU
		CFU g^{-1} DM_i
CMC	Critical micellar concentration	
CNC	Corn oilseed cake	
COC	Cumulative oxygen consumption	mgO_2 g^{-1} DM
CSL	Corn steep liquor	
CW	Coconut waste hydrolysate	
db	dry basis	
DM	Dry matter	%
DM_i	Initial dry matter	
DoE	Design of experiments	
E168	Emulsion index at 168 h	
E24	Emulsion index at 24 h	
HPLC-MS	High-Performance Liquid Chromatography with Mass Spectrometry	
HPLC-UV	High-Performance Liquid Chromatography with Ultraviolet Detection	
LC18:1, 2ac	Diacetylated lactonic C18:1	
LCFA	Long-chain fatty acids	
MC	Moisture content	%
MELs	Mannosylerythritol lipids	
OFMSW	Organic fraction of municipal solid waste	
OM	Organic matter	%

PW	Palm waste hydrolysate	
RAC	Clean house products sludge	
RHP	Cosmetic industry sludge	
RM	Rapeseed meal hydrolysate	
ROF	OFMSW hydrolysate	
RPC	Rapeseed oilseed cake	
RSC	Sugar candy wastewater	
RSO	Sugar candy wastewater and OFMSW hydrolysate	
SBC	Soybean oilseed cake	
SFC	Sunflower oilseed cake	
SL	Sophorolipid	
SLs	Sophorolipids	
SmF	Submerged fermentation	
sOur	Specific oxygen uptake rate	$mgO_2\ g^{-1}$ DM h^{-1}
SSF	Solid-state fermentation	
TC	Total carbon	g L^{-1}
		g kg^{-1}
TN	Total nitrogen	g L^{-1}
		g kg^{-1}
WCNC	Artificial winterization corn oil cake	
WF	Wheet feed hydrolysate	
WOC	Winterization sunflower oil cake	
WOC-O	Nutrients optimal ratio	
WOC-R	Nutrients reference ratio	

Chapter 1

Introduction

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Microbial biosurfactants: a review of recent environmental applications
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Aplicaciones ambientales de los biosurfactantes.
Eras-Muñoz, E., Sánchez, A., Font, X., Moral, J., Barrena, R and Gea, T. (2022).

1.1 Microbial surface-active compounds

The exploitation of natural resources and the rise in industrial activities have resulted in serious environmental problems, highlighting the need for increased efforts to address these issues (Pontevedra-Pombal et al., 2013). In the 1990s, the concept of the circular bioeconomy emerged as a strategy centered on environmental responsibility and as an alternative to the traditional linear economy, which relies on a model of extraction, use and disposal. This new approach advocates for a closed-loop system where biological resources are utilized efficiently; waste is minimized and value is maximized at every stage. The circular bioeconomy promotes resource use and eco-efficiency while aiming to reduce the carbon footprint and dependence on fossil carbon (Gutiérrez-Soto et al., 2024; Stegmann et al., 2020). However, despite these advances, petroleum remains the most widely used non-renewable resource, particularly as a source of energy and for the production of related products such as chemical surfactants (Singh and Rathore, 2019).

Chemical surfactants are tensioactive agents used to reduce surface tension between two immiscible phases and promote their solubility by the formation of micelles. However, they present high environmental toxicity and are not biodegradable (Otzen, 2017). Consequently, it is necessary to search for natural substitutes that can be used in an eco-friendly way. In this sense, microbial surface-active compounds (MSAC) have risen as a novel alternative.

MSAC are amphiphilic molecules that contain hydrophobic and hydrophilic parts that promote the presence of interfaces between fluids with different polarities. The hydrophilic part generally consists of one of the following

structures: amino acids, anionic or cationic peptides and carbohydrates; while the hydrophobic tail is generally constituted by peptides, proteins, or fatty acids that can be saturated or unsaturated (Sarubbo et al., 2015b; Farias et al., 2021).

The capacity of MSAC to reduce the interphase tension varies according to its chemical structure. MSAC can be classified into two principal groups based on their molecular weight: low molecular weight (glycolipids and lipopeptides) and high molecular weight (polysaccharides, lipopolysaccharides, proteins and lipoproteins) (Figure 1.1). Based on their properties, low-molecular-weight MSACs are more effective in reducing surface tension and are therefore known as biosurfactants, while high-molecular-weight is better for stabilizing oil-water emulsions, making them known as bioemulsifiers (Otzen, 2017; Karlapudi et al., 2018). Indeed, the chemical differences in the molecular structure are directly related to their biological activities and applications (Morita et al., 2015).

1.1.1 *Biosurfactants: Glycolipids*

Biosurfactants are secondary metabolites that can be produced from renewable sources by different kinds of bacteria, yeast, fungi and archaea. Previous findings reported that a microorganism is considered a biosurfactant producer if it can reduce the surface tension under values of 40 mN m^{-1} which allows the research of new producer microorganisms (Ayed et al., 2015).

Besides, biosurfactants present antibacterial, antifungal and antiviral properties (Mnif et al., 2017; Naughton et al., 2019) that may broaden its applications. They present low toxicity, high biodegradability and are functional in wide ranges of conditions of temperature, pH and salinity (Karlapudi et al., 2018; Zang et al., 2021). Glycolipids are a group of biosurfactants, which are conformed

by mono-, di-, tri- and tetra-saccharides in combination with one or more chains of aliphatic acids or hydroxy aliphatic acids. They are classified as trehalolipids, mannosylerythritol lipids, rhamnolipids and sophorolipids (SLs) (Malkapuram et al., 2021).

Trehalolipids are constituted by trehalose disaccharides in which each glucose is linked to a fatty acid (mainly mycolic acids) by an α-1-glycosidic bond and its production is associated with species of *Mycobacterium*, *Corynebacterium* and *Nocardia* (Sen, 2010; Varjani and Upasani, 2016). In the case of mannosylerythritol lipids, they are better known as MELs and are produced by microorganisms of the genera *Pseudozyma* and *Ustilaginaceae* (Beck and Zibek, 2020). Rhamnolipids were the first biosurfactants described and are mainly produced by *Pseudomonas aeruginosa* (Edwards and Hayashi, 1965). Rhamnolipids are the most researched biosurfactants and they are formed by mono- and di-saccharides of rhamnose linked by a glycolic bond to a β-hydroxy fatty acid molecule (Patowary et al., 2016). Finally, SLs are extracellular biosurfactants that are mainly produced by the non-pathogenic and bumblebee honey-isolated *Starmerella bombicola* (De Graeve et al., 2018).

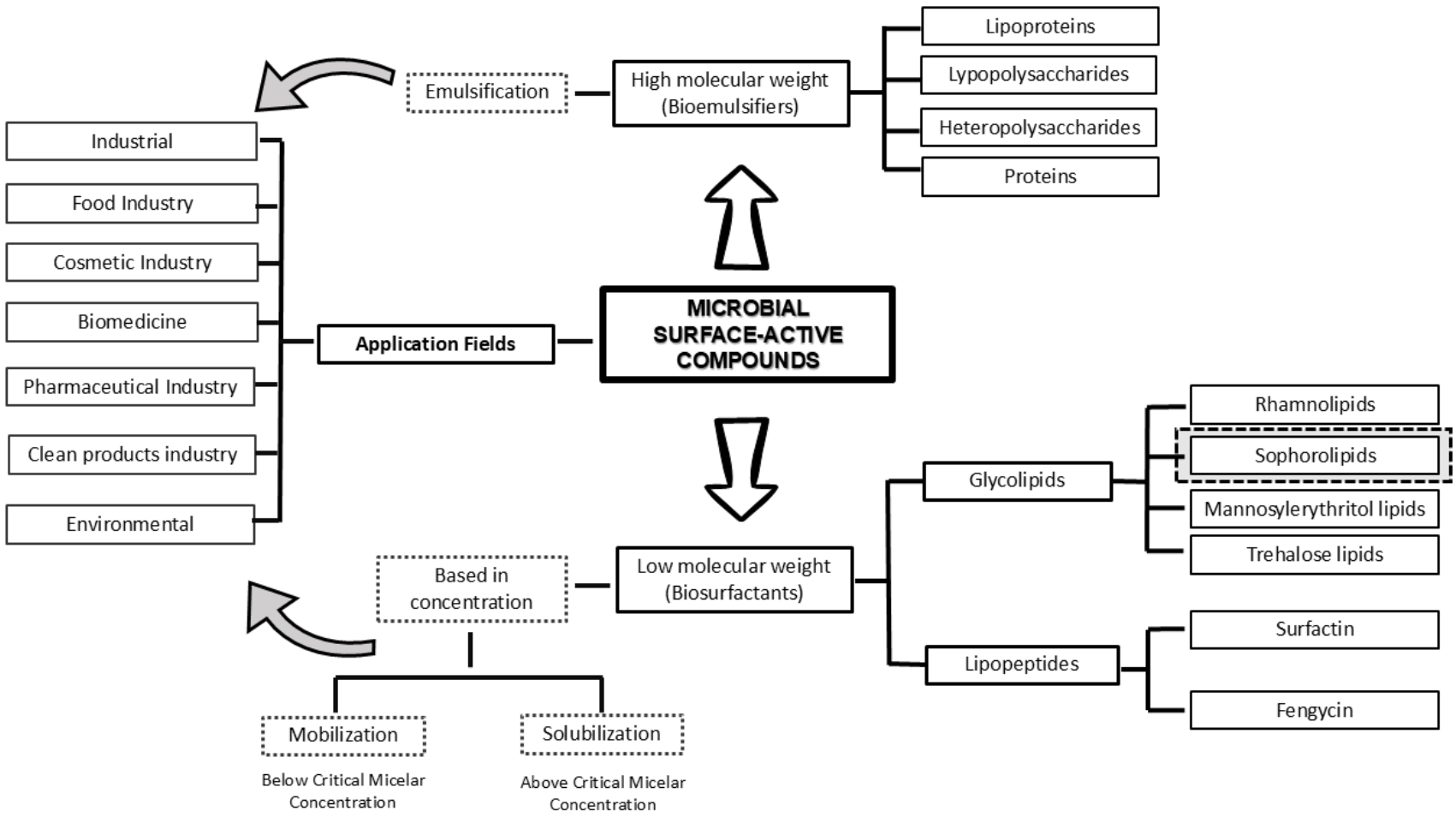

Figure 1.1 Microbial surface-active compounds classification and applications.

1.2 Biosurfactants Production Overview

According to the literature, different types of microorganisms belonging to the genera *Pseudomonas*, *Bacillus*, *Candida*, *Rhodococcus* and *Corynebacterium* are used to produce biosurfactants (Martins and Martins, 2018). These microorganisms can use several compounds as carbon sources for their growth. The most common carbon sources are glucose and glycerol. Occasionally, biosurfactant production media must be supplemented with yeast extract or macronutrients in the form of chlorine, sulfate, or phosphate salts of different metals (Na, Mg, K, Ca, or Fe, among others). Frequently, the main fermentation media use the mineral fermentation medium, which may slightly vary in composition for each biosurfactant production case (Table 1.1). Because the use of pure substrates and media supplementation has an economic impact (Ejike Ogbonna et al., 2021), the use of low-cost byproducts and waste as feedstock as well as fermentation strategies will be discussed later (section 1.8 and 1.6, respectively).

The recovery of the final product (downstream process) represents around 60% of the production costs and makes the process expensive in comparison with chemical surfactant production (Sarubbo et al., 2015a; Teixeira et al., 2018). Nevertheless, the downstream process is vaguely commented in literature and techniques such as precipitation, extraction and crystallization are described for purification purposes (Venkataraman et al., 2021). Biosurfactants extraction using organic solvents is the most widely employed method in both SmF and SSF. For instance, mixtures of 2:1 (v v^{-1}) methanol: chloroform, 3:1:1 (v v^{-1}) methanol: n-hexane: water, or 1:4 (w v^{-1}) ethyl acetate: n-hexane are applied,

with the latter being successfully used for SSF recovery (Jiménez-Peñalver et al., 2016; Shen et al., 2019). Interestingly, Sahebnazar et al. (2018), have reported the use of zero-valent iron nanoparticles with purification purposes. Moreover, another novel method for biosurfactant recovery involves the implementation of integrated gravity separation which has been applied for sophorolipid recovery at industrial scale (Holiferm, UK; Dolman et al., 2019). Regarding the purification methods and structural characterization, the main analysis described are thin layer chromatography with silica gel plates (TLC), liquid chromatography coupled with mass spectrometry (HPLC-MS) and Fourier-transformed infrared spectrophotometry (FTIR) (Jiménez-Peñalver et al., 2020). However, the choice of method will depend on the specific application and the required purity level.

Biosurfactant-specific properties can serve as an indirect measure for their further application. Commonly, biosurfactants are mainly characterized by assays such as collapse test, oil drop, surface tension, emulsification index, foaming index and critical micellar concentration (CMC) (Malkapuram et al., 2021). It is important to note that when a biosurfactant is added to an aqueous phase at a concentration above the CMC, which is the threshold needed for maximum surface tension reduction, it can self-assemble into micelles (clusters of biosurfactant monomers). Therefore, CMC is a crucial parameter to consider, as it significantly affects the behavior, interactions and surface properties of biosurfactants (Santos et al., 2024). In environmental applications such as bioremediation, biosurfactants are preferred over chemical surfactants because low concentrations can effectively combat pollutants without negative consequences, promoting their use in this field (Ayed et al., 2015).

Table 1.1 Examples for glycolipids biosurfactants production, microorganisms, media composition and fermentation conditions.

Biosurfactant	Producer Microorganism	Operating Conditions								Reference
		Carbon Source	Medium and Supplement	Volume (L)	T (°C)	pH	Time (h)	Speed (rpm)	Yield (g L^{-1})	
Rhamnolipid	*Pseudomonas aeruginosa* TGC01	Glycerol 4% (w/v)	Mineral salt medium and (g L^{-1}): 4 $NaNO_3$	0.5	30	-	96	150	11.0	Bezerra et al., 2019
Rhamnolipid	*Lysinibacillus sphaericus* IITR51	Glycerol 1.5% (w/v)	Basal salt medium	0.008	30	7	72	160	1.6	Gaur et al., 2019b
Rhamnolipid	*Pseudomonas aeruginosa* DR1	Mango oil and glucose 1% (w/v)	Mineral salt medium	0.25	30	-	96	-	2.8	Reddy et al., 2016
Rhamnolipid	*Pseudomonas aeruginosa*	Glucose 6% (w/v)	Mineral salt medium	0.25	37	-	168	330	3.8	Sahebnazar et al., 2018
Rhamnolipid	*Pseudomonas aeruginosa* SR17	Glucose 2% (w/v) and paneer whey waste	Mineral salt medium	0.5	35	7	48	150	4.8	Patowary et al., 2016
Rhamnolipid	*Pseudomonas aeruginosa*	Glycerol, glucose, mannitol, molasses and n-hexadecane at 2% (w/v)	Mineral salt medium	-	35	-	120	150	0.84	Patowary et al., 2018

Rhamnolipid	*Pseudomonas aeruginosa* PBS	Glucose 5% (w/v) and kerosene 3% (w/v)	($g\ L^{-1}$): 5 KNO_3, 1 $KH_2PO_4.2H_2O$ 1 $K_2HPO_4.2H_2O$ 0.2 $MgSO_4 \cdot 7H_2O$ 0.02 $CaCl_2 \cdot 2H_2O$	0.1	37	7	72	150	2.65	Sharma et al., 2018
Rhamnolipid	*Pseudomonas aeruginosa* PA1	Glycerol 2% (w/v)	Nutrient Broth	-	28	-	288	180	-	Tomar et al., 2019
Mono- and di-rhamnolipid	*Bacillus algicola* 003-Phe1, *Rhodococcus soli* 102-Na5, *Isoptericola chiayiensis* 103-Na4 and *Pseudoalteromonas agarivorans* SDRB-Py1	Mannitol 2%, glucose, glycerol, starch and crude oil 1% (w/v)	Mineral salt medium and ($g\ L^{-1}$): 10 $(NH_4)_2SO_4$ 0.5 yeast extract	0.1	28	7	72 168	180	2.0 strain 003-Phe1 and 1.2 SDRB-Py1	Lee et al., 2018
Rhamnolipid	*Pseudomonas aeruginosa*	Glycerol 2% (v/v)	Mineral salt medium	0.25	30	7	192	180	5.07	Huang et al., 2020
Rhamnolipid	*Pseudomonas aeruginosa* UCP 0992	Corn steep liquor 0.5% and vegetable oil residue 4% (v/v)	-	1.2	28	7	120	225	26.0	Silva et al., 2018
Rhamnolipid	*Pseudomonas aeruginosa*	Glucose 1% (w/v)	Mineral salt medium	0.25	30	7	168	180	1.8	Sun et al., 2019

Sophorolipid	*Candida albicans* and *Candida glabrata*	Glucose 2% (w/v)	Synthetic defined medium with yeast nitrogen base 0.67%	0.2	30	-	72	150	1.32 *C. albicans* and 1.6 *C. Glabrata*	Gaur et al., 2019a
Sophorolipid	*Starmerella bombicola* ATCC 22214	Winterization oil cake (WOC) and molasses	-	0.5 22 100	30	5.6	168	-	0.16 0.19 0.14 (gSL gDM^{-1})	Rodríguez et al., 2021
Sophorolipid	*Candida sphaerica* UCP 0995	Corn steep liquor 9% and ground-nut oil refinery residue 9% (v/v)	Basal medium	0.25 50	27	-	144	150	8.0 21.0	Luna et al., 2015
Sophorolipid	*Candida tropicalis*	Corn liqueur 4%, molasses 2.5% and canola frying oil 2.5% (v/v)	-	0.5 3 50	28	6	144	200	9.5 20.0 27.0	Da Rocha Junior et al., 2019
MELs	*Pseudozyma* sp. NII 08165	Soybean oil, diesel, kerosene and petroleum	Bushnell Hass broth	-	30	7	216	200	-	Sajna et al., 2015

1.3 Biosurfactants applications

According to the literature, biosurfactants present several applications (Farias et al., 2021; Mulligan, 2021; Soberón-Chávez et al., 2021) including already commercialized products (Miao et al., 2024). In the industrial field, biosurfactants have been used as eco-friendly ingredients in products including their use in detergents, cleaning products, food packaging among others (Roelants et al., 2019; Hausmann et al., 2024; Bueno-Mancebo et al., 2024). Figure 1.2 illustrates some commercial products containing biosurfactants. In biomedicine, their use is focused on their biocidal activity, for example, some authors have reported that SLs have antiviral activity against RNA viruses, suggesting their possible use against SARS-CoV-2 (Daverey et al.,2021).

Figure 1.2 Commercial products containing biosurfactant. Modified from Miao et al. (2024)

Apart from the above mentioned applications, biosurfactants are extensively applied in the environmental field based on two principal interaction mechanisms: on one hand, the presence of biosurfactant increases the pollutant's bioavailability and, on the other hand, it promotes the interaction with the cell surface by increasing its hydrophobicity, thus allowing hydrophobic pollutant to interact with bacterial cells (Pacwa-Płociniczak et al., 2011; Chamy and Rosenkranz, 2013; Ayed et al., 2015). As well known, the biodegradation of hydrophobic organic contaminants is typically limited by their solubility. Therefore, the use of biosurfactants is a well-explored technique, as they can mobilize, emulsify and solubilize these compounds thus improving biodegradation processes in soil bioremediation (Zouari et al., 2019) and wastewater treatment (Malkapuram et al., 2021). Reducing interfacial tension property enhances the interaction between the water-oil-solid matrix to decrease the capillary forces that avoid the migration of pollutants such as PAHs, metals, petroleum, chlorinated pesticides, among others (Chaprão et al., 2015; Geetha et al., 2018).

Additionally, potential biosurfactant application areas are being explored in agriculture and phytoremediation processes. Kumar et al. (2021a) concluded that biosurfactants promote plant growth through phytohormone production, microbial stimulation and an increase in nutrient availability in soil. Furthermore, the addition of biosurfactants improves plant resistance to biotic and abiotic stresses by acting as biocontrol agents. In comparison with chemical surfactants, biosurfactants' nature, properties and versatility make them a good choice for sustainable applications.

1.4 Biosurfactants Market

In 2022, the microbial biosurfactants global market was valued at USD 1.2 billion and is expected to reach USD 1.9 billion by 2027 with an annual growth rate of 11.2%. Notably, in 2021, SLs emerged as the largest subtype in terms of value within the biosurfactants market (Markets and Markets, 2022). As a result, some companies (Table 1.2) have implemented SLs production for further commercial applications.

Table 1.2 Sophorolipids producing companies and their associated commercial application. Adapted from Santos et al. (2024).

Company	Location	Commercial application
SyntheZyme LLC. http://www.synthezyme.com	USA	Cleaning products, food products, cosmetics
Saraya Co. Ltd. http://worldwide.saraya.com	Japan	Cleaning products, cosmetics, hygiene products
Allied Carbon Solutions (ACS) Ltd. https://www.allied-c-s.co.jp	Japan	Hair care, anti-acne, antimicrobials
Urumqi Unite Bio-Technology Co.Ltd. https://www.tradeindia.com	China	Cleaning and oil recovery
Ecover https://www.ecover.com	Belgium	Cleaning products, bioremediation, pest control, pharmaceuticals
Givaudan https://www.givaudan.com	Switzerland	Cosmetics (anti-acne)
Holifem https://www.holiferm.com	UK	Wholesale
Amphistar https://amphistar.com	Belgium	Wholesale

However, despite increasing demand, economical large-scale biosurfactants production remains a challenge when compared on a manufacturing cost basis to chemical surfactants approximately 34-40 USD per kilogram for biosurfactants and 1-4 USD per kilogram for chemical surfactants (Ahalliya et al., 2023; Nagtode et al., 2023). These challenges can be attributed to several factors, including the requirement for pure substrates, low final product concentrations and the formation of product mixtures which contribute to high downstream processing costs (Roelants et al., 2018). According to previous studies, highly productive strains, optimized fermentation conditions and the use of cheaper substrates are essential for affordable costs and for expanding the industrial production and applications of biosurfactants (Silva et al., 2018; Durval et al., 2020). Moreover, considering the current environmental situation, the higher production cost of biosurfactants should not be considered as the sole factor. Environmental costs associated with product use should also be internalized and in this context, biosurfactants may offer an advantage over chemical surfactants.

1.5 Sophorolipids: a promising substitute of chemical surfactants

SLs are a class of glycolipids that were first reported in the early 1960s. They exhibit significant potential due to the high productivity levels of the wild-type producer *S. bombicola* ATCC 22214 (~200 g L^{-1}) and the possibility of producing target congeners using engineered strains (Wang et al., 2019; Van Bogaert et al., 2016; Dierickx et al., 2022). Moreover, literature reported that strains from the genera *Candida* such as *Candida apicola*, *Candida floricola* and *Candida rugosa* as well as *Wickerhamiella domercqiae*, *Rhodotorula*

muciliginosa and *Lachancea thermotolerans* are considered SLs producer microorganisms (Pal et al., 2023)

SLs are extracellular metabolites and their structure relies on a sophorose disaccharide (di-glucose with a β-1,2 bond) and a long fatty acid chain as the hydrophilic and hydrophobic moiety, respectively (Ingham and Winterburn, 2022). SLs are mainly produced as a mix of congeners in acid or lactonic form (acyclic and cyclic, respectively) (Figure 1.3).

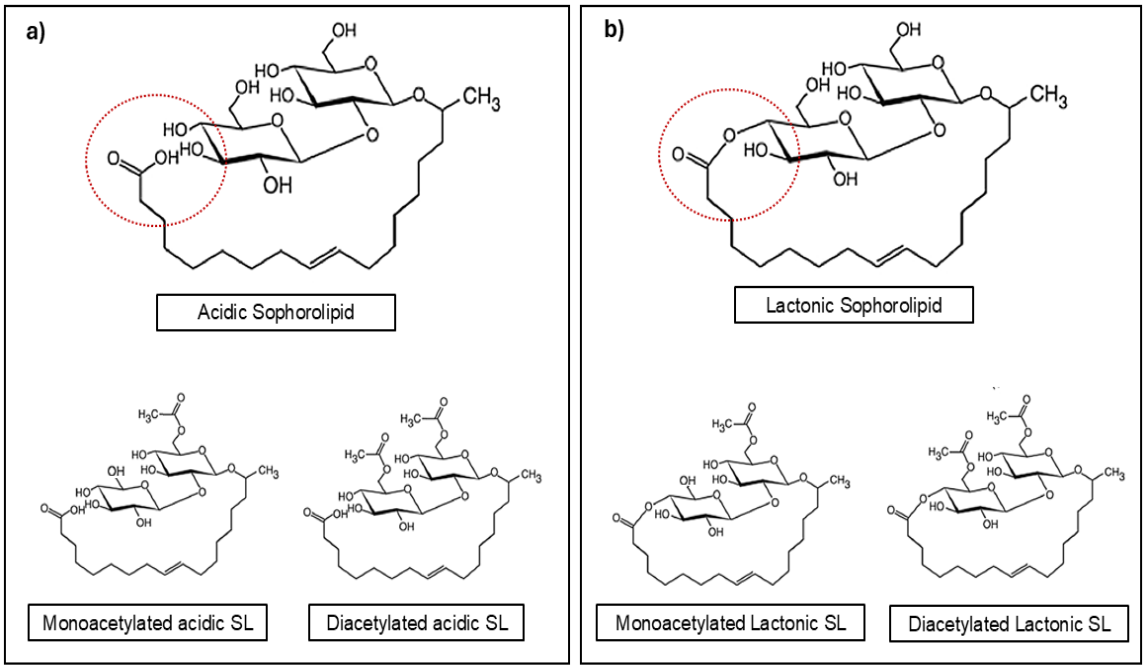

Figure 1.3 SLs structures. a) Acids SLs (acyclic form)and b) lactonic SLs (cyclic form). Note that non-, mono- and di-acetylated forms are represented in both cases. Independently from the acetylation and lactonization other fatty acids can constitute the long fatty acids chain with different saturation levels.

SLs main structural variations rely on the sophorose structure that can be acetylated or lactonized. The acetylation may occur at the 6’ or 6” positions of the sugar. Moreover, the hydrophobic tail could be free (acid form) or lactonized presenting an internal esterification between the carboxylic end of the fatty acid and the 4” position of the sophorose (lactonic form). In addition, the hydrophobic

tail could present variations such as fatty acid length (16 or 18 carbon atoms) and different degrees of saturation (Van Bogaert et al., 2011). Due to the reduction in rotational freedom caused by lactonization, lactonic SLs often form crystals rather than the more common yellow-viscous oil (Figure 1.4). Indeed, diacetylated lactonic C18:1 is identified as the predominant congener produced by wild-type yeast (Van Bogaert et al., 2016).

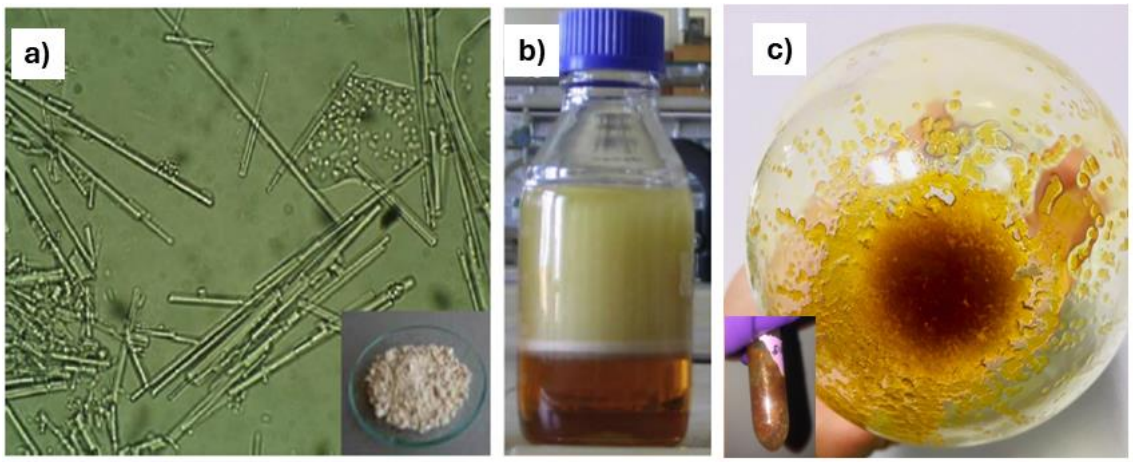

Figure 1.4 SLs appearance. a) SLs crystals at light microscope (100x), b) SLs crude extract obtained by submerged fermentation and c) SLs crude extract obtained by solid-state fermentation. Modified from Van Bogaert et al. (2011).

Literature indicates that the biological function of SLs and the reason microorganisms produce them are linked to their role as a carbon reservoir, supporting growth and cellular development in nutrient-limited and unfavorable environments. Additionally, SLs help microorganisms survive in high-sugar conditions and improve the solubilization of hydrophobic substrates, such as oils and fats, making these compounds more accessible to yeast (Garcia-Ochoa and Casas, 1996; Pal et al., 2023).

1.6 Fermentation process for sophorolipids production

Solid-state fermentation (SSF) and Submerged fermentation (SmF) are successfully applied for SLs production. These fermentation strategies are widely employed to enhance SLs yields, with a focus on optimizing nutrient concentrations (Shah et al., 2017; Jadhav et al., 2019; Van Renterghem et al., 2019). From a production standpoint, SLs maximum production by SSF is reported at a pilot scale (100 L) (Rodriguez et al. 2021a) while SmF offers advantages over SSF as it can be implemented at an industrial scale (~600 L) due to the reached yields and the final SLs crude extract purity (Holiferm, UK).

1.6.1 Solid-state fermentation

Solid-state fermentation (SSF) is defined as a heterogeneous process developed in the absence or limitation of free water, that guarantees microorganism growth and metabolic activity. Yields are expressed as grams of bioproduct per gram of initial dry matter in the solid substrate (Sánchez et al., 2015). SSF is constituted by solid, liquid and gaseous phases as shown in Figure 1.5 (Pandey, 2003). The solid phase could function either as a nutrient source for the microorganism or as an inert support that is not biodegraded. The liquid phase consists mainly of water or nutrient solutions contained within the solid matrix, while the gaseous phase is primarily composed of air that continuously circulates through the pores of the solid providing oxygen for the aerobic metabolism (Chen and Xu 2004).

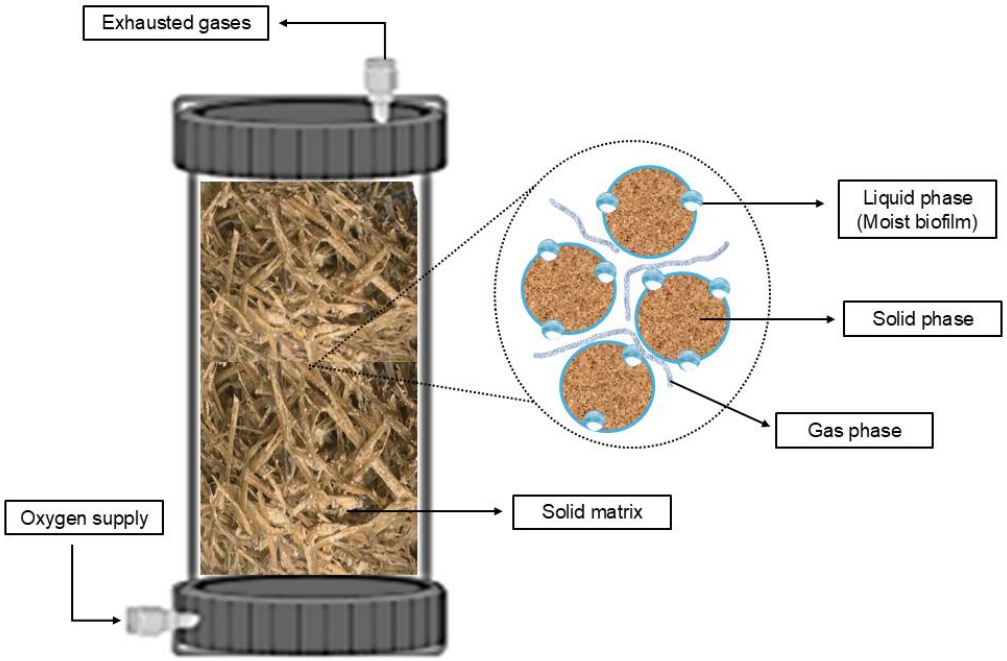

Figure 1.5 Scheme of the phases present in an SSF process.

From an environmental point of view, SSF seems to be a promising technology to increase efforts toward a circular economy due to this fermentation strategy allows the sustainable conversion of organic insoluble solid waste into marketable products (Jiménez-Peñalver et al., 2016; Hu et al., 2021). Moreover, SSF offers advantages as allowing the use of solid industrial residues and agro-industrial waste, low energy and water consumption, among others that make the processes rentable (Sánchez et al., 2015).

However, the main challenges around SSF involve sample heterogenicity and mass and heat transfer, which are intrinsic characteristics of solid matrices (Kumar et al., 2021b; Oiza et al., 2022). Temperature and composition gradients are often reported when scaling up SSF systems, pointing to the co-existence of different metabolic states for cells growing in the solid matrix. Even though efforts have been developed to decrease and remove heat during the fermentation such as aeration (Rodriguez et al., 2021a), the conventional methods for its control

applied in SmF are not easily adaptable to SSF, being temperature a critical parameter in the process (Chen and Xu, 2004). Moreover, some drawbacks are also associated with difficult downstream processing which limits its industrial application (Kumar et al., 2021b).

Nevertheless, the production of SLs by SSF has been already proven feasible at different operation scales as reported by the two previous theses on the topic (Jimenez-Peñalver, 2017; Rodríguez, 2020). Successfully, SSF has been reported for SLs production, achieving yields of around 0.20 g SLs per g of initial dry mass (DM_i) when using residues such as winterization sunflower oil cake as a hydrophobic carbon source and molasses as hydrophilic carbon source (Rodríguez et al., 2021a).

1.6.2 *Submerged fermentation*

Generally, both the research and industrial production of SLs are carried out through SmF processes. In this case, hydrolyzed waste or liquid/solubilized substrates have been used as a source of carbon or nitrogen by the yeast (Wongsirichot et al., 2021). Although, the production yields are higher than those achieved by SSF, this technology has drawbacks related to foam formation due to the surface-active nature of SLs during its production. Indeed, this issue affects oxygen transfer and requires process adaptations to mitigate it, such as sparging air, using foam fractionation columns, gravity separation, among others. These measures lead to increased production costs, making their commercialization more expensive compared to chemical surfactants (Dolman et al., 2019; Vučurović et al., 2024).

1.7 Nutrient sources for sophorolipids production

When producing SLs, several authors reported that the use of both hydrophilic and hydrophobic carbon sources increases their production by 85% compared to using only one carbon source (Amiri and Habibi, 2024; Ma et al., 2020). The suggested SLs pathway is illustrated in Figure 1.6. The first step involves the hydroxylation of fatty acids, followed by the production of the glucolipid through the action of glycosyltransferase I (gene UGTA1). After that, glucosyltransferase II (gene UGTB1) produces the acidic non-acetylated SL, which can either be acetylated by the action of acetyltransferase (gene AT) or lactonized by lactone esterase (gene SBLE), allowing the formation of lactonic SLs (Van Bogaert et al., 2011).

It should be noted that if only the hydrophilic carbon source (mainly glucose) is provided, it might not be directly incorporated into the SLs metabolic pathway for sophorose moiety, because it is redirected to *de novo* fatty acid synthesis by glycolysis and TCA cycle. On the other hand, if the hydrophobic carbon source (fatty acids) is only provided, it is metabolized by the yeast through β-oxidation and can also be used in other pathways to generate acetyl-CoA units, which are utilized for the synthesis of sophorose through the action of enzymes such as UDP-glucose synthase and UDP-glucose glucosyltransferase (Wongsirichot et al., 2021; Yang et al., 2019). Subsequently, sophorose is conjugated with the fatty acid chains derived from the hydrophobic carbon source by sophorolipid synthase enzymes (Wang et al., 2019). Recently, Ingham et al. (2022) highlighted that fatty acid supply is the most important component for SL synthesis, as it can co-supply glycerol to drive the hydrophilic carbon requirements during synthesis.

Literature reported that, an effective media combination that favors SLs synthesis and is widely reported for their industrial production, is constituted by glucose often combined with a hydrophobic carbon source rich in oleic acid (Van Bogaert et al., 2016; Wongsirichot et al., 2021). When the hydrophobic carbon source is a triglyceride, it is first converted into fatty acids by enzymes such as aldehyde dehydrogenase or long-chain alcohol oxidase and then, they are used for SL biosynthesis (Intasit and Soontorngun, 2023). Van Bogaert et al. (2011) observed that when the hydrophobic carbon source is constituted by shorter fatty acids, a toxic effect is observed. Specifically, the shorter the fatty acid (C12:0 < C10:0 < C8:0), the greater the toxicity to *S. bombicola*. This toxicity is likely caused by the interference of these fatty acids with the yeast's membrane.

In addition to the carbon sources, a nitrogen source is also required for yeast growth and SLs production. SLs are produced under nitrogen limitation when the microorganism reaches the stationary growth phase. They are produced by the yeast as a defense and nutrient acquisition mechanism and as energy storage (Roelants et al., 2019; Wang et al., 2019). Moreover, when there is an excess of a nitrogen source in the media, nutrients are used for microorganism growth and maintenance; consequently, SLs production decreases (Ingham and Winterburn, 2022). It is well known that yeast growth is nitrogen-dependent since it affects the formation of biomass, which in turn affects the duration and kinetics of the fermentation process (Ma et al., 2011; Christofi et al., 2022). Indeed, nitrogen is an essential source that needs to be well-balanced to allow growth and reach the stationary growth phase for an optimal process (Albrecht et al., 1996; Wongsirichot et al., 2022a).

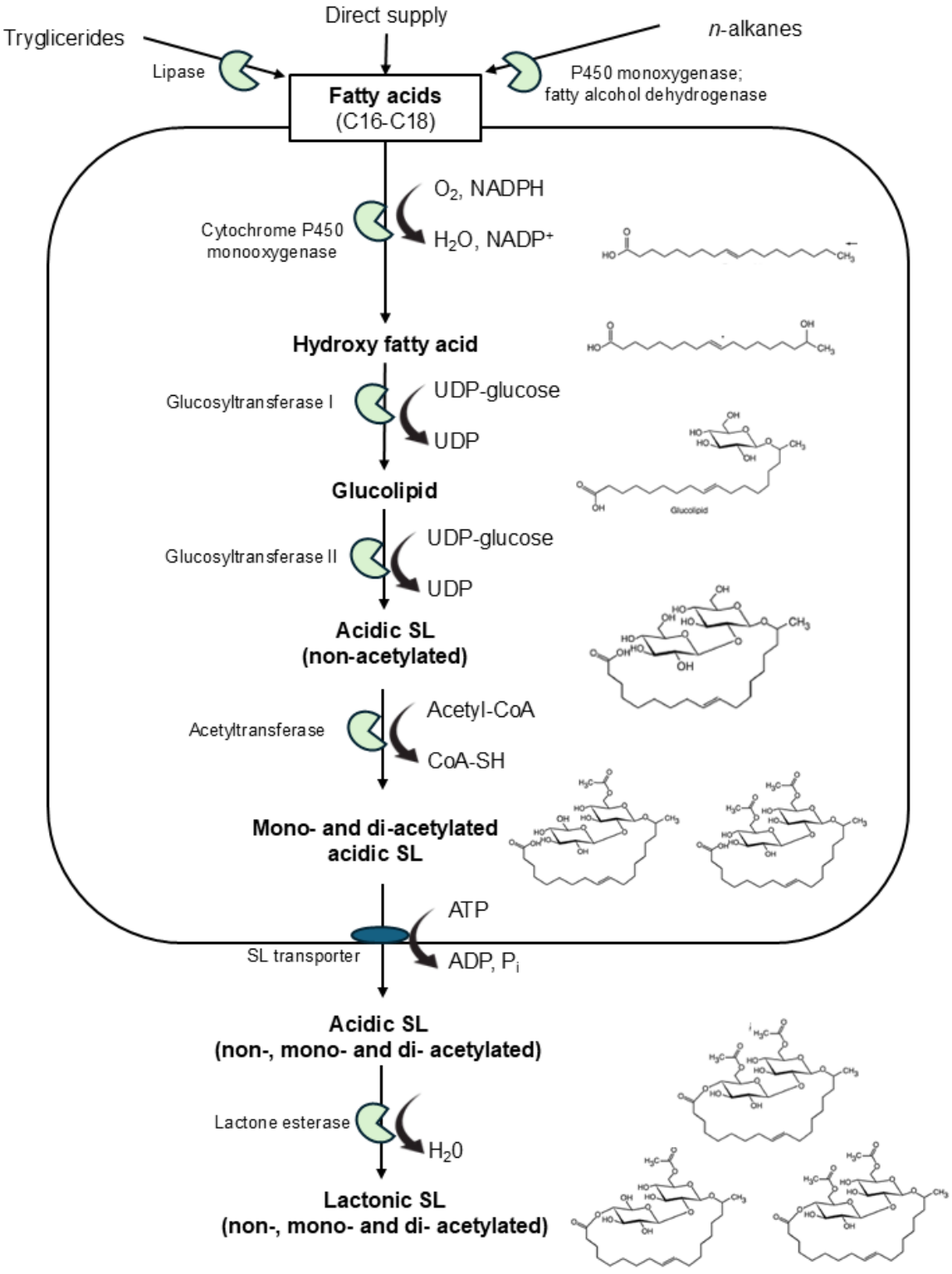

Figure 1.6 Summarized SLs biosynthesis pathway. Acidic SLs forms are transported to the extracellular space where lactonization occurs. Modified from Van Bogaert et al., 2011.

Because of SLs high market demand, biotechnology tools are being used to identify biosurfactant production pathways and to obtain hyperproducing strains or recombinant mutants. For example, a *S. bombicola* mutant (ΔatΔsble) produces a variety of SLs called bolaform (Soetaert et al., 2013). This molecule structure consists of two sophorose units located at each side of the hydrophobic tail and presents more stability at higher pH conditions, which impulses their application in a wide range of sectors such as biomedicine for drug delivery (Van Renterghem et al., 2019)

Several studies have shown that SLs can be produced from water-insoluble substrates, making them attractive for industrial applications based on a circular economy strategy (Luna et al., 2015; Gaur et al., 2019b; Rodríguez et al., 2021b). From a circular economy perspective, the successful production of SLs relies on the implementation of cost-effective processes and the utilization of low-cost substrates as raw materials to achieve zero-waste generation. Therefore, research efforts have focused on a circular bioeconomy context, promoting the use of alternative substrates that have been successfully used for SLs production (Jiménez-Peñalver et al., 2019; Wongsirichot et al., 2021).

1.8 Sophorolipids production using alternative substrates

In recent times, efforts have been made to reduce production costs, enhance the economic competitiveness of SLs, design more sustainable processes and encourage circular bioeconomy. The use of pure substrates such as glucose and pure oils (e.g. oleic acid) has been reported as the main carbon source for SLs production increasing production and scaling costs (Ma et al., 2020; Wongsirichot et al., 2021). Indeed, purified substrates constitute around

10-30 % of the total production costs (Gudiña et al., 2015), thus an encouraging approach to foster a sustainable process involves the utilization of second-generation substrates. Additionally, Baccile et al. (2017) determined that glucose and rapeseed oil together accounted for 87% of the total environmental impact in an SLs production process.

Second-generation substrates such as byproduct streams, obtained from industrial, agricultural and food waste seem to be a promising alternative to pure substrates because they do not compete with food crops, mitigating ethical and resource allocation concerns (Raza et al., 2021; Sundaram et al., 2024). In 2023, the amount of agricultural biomass generated by the European Union (EU) was 500 million tonnes of dry matter (Mtdm) (European Commission, 2024). Despite agricultural biomass is used for food supply, globally agricultural waste burning represents around 25% of total agricultural biomass production (Babu et al., 2022; Venkatramanan et al., 2021). Moreover, bio-waste such as food waste generated in the EU in 2018 amounted to 147 Mtdm, of which 133 Mtdm was recovered either as recycled new material or for energy recovery while the remaining bio-waste that is not recovered is either landfilled or incinerated which impact negatively in the environment.

In addition, oil residues such as soap stocks and oilseed cakes are composed mainly of triglycerides, diglycerides as well as monoglycerides and free fatty acids (Martino et al., 2014; Domínguez et al., 2019). Additionally, oils and their derivates such as vegetable oils and waste cooking oil (WCO) are a major source of water and soil contamination due to the low degradability of lipid compounds (Dumont and Narine, 2007). In EU countries, the WCO refinery sector has a capacity of over 21 million tons per year however only 11.6 million

tons per year of WCO was recovered and converted into biodiesel. This suggests that a significant amount of WCO is still being disposed of annually (Foo et al., 2022).

Agricultural waste is primarily composed of cellulose, hemicellulose and lignin (Khare and Arora, 2021). Oil residues are rich in fatty acids and food waste contains high levels of available carbon, nitrogen and phosphorus (Wongsirichot et al., 2024). Due to their rich nutrient composition, these kinds of residues have been widely reported for SLs production, often achieving competitive yields. In this regard, their utilization provides a dual benefit by reducing residues and creating marketable bioproducts, thereby aligning the process with the principles of the circular bioeconomy (Domínguez Rivera et al., 2019; Dierickx et al., 2022).

Literature reported the use of several alternative substrates for SLs production as shown in Table 1.2. Some examples are animal fat supplemented with a variety of sugars (Minucelli et al., 2017), vegetable oils (Konishi et al., 2015), molasses and winterization oil cakes (Jiménez-Peñalver et al., 2020), sugar cane molasses (Daverey and Pakshirajan, 2010), among others. Based on the literature, Kaur et al. (2019) achieved the highest production using food waste as a hydrophilic carbon source supplemented with oleic acid, resulting in a yield of 115.2 g L^{-1}. Additionally, Kim et al. (2021) reported a yield of 315.6 g L^{-1} using WCO as a hydrophobic carbon source in a fed-batch reactor. Regarding alternative nitrogen sources, limited information is available, as yeast extract and urea are typically preferred for their production. However, some studies, such as those by Imura et al. (2013) and To et al. (2022), have explored the use of wheat bran and rice bran hydrolysates, reaching 100 g L^{-1} and 96.4 g L^{-1}, respectively.

As reported, the utilization of solid second-generation substrates is mainly applied in SmF. Consequently, their application in this process involves biomass pre-treatments to increase the availability of nutrients (Kaur et al., 2019; Wongsirichot et al., 2022a; Eras-Muñoz et al., 2024b). These pre-treatments include the use of acid, alkali, heat, or enzymes among others (Nayak and Bhushan, 2019). Enzymatic hydrolysis is an intermediate step in the biomass conversion process that yields a high-value hydrolysate, typically rich in sugars, which must also be capable of supporting fermentative organisms in the subsequent production stages (Modenbach and Nokes, 2013).

Within the range of pre-treatment approaches employed thus far, enzymatic hydrolysis using amylases, cellulases, glucoamylases and enzymatic cocktails has exhibited the most promising efficacy in achieving competitive yields for biosurfactants production (Domínguez Rivera et al., 2019; Zhang et al., 2021). Indeed, enzymatic hydrolysis has been applied successfully for SLs production by authors such as Kaur et al. (2019) and Wongsirichot et al. (2022a) using food waste and agricultural residues in SmF, respectively.

Hu et al. (2021) highlight through their dynamic Life Cycle Assessment that enzymatic hydrolysis presented a lower environmental impact compared to other unit steps during SLs crystal production. However, the primary challenge of this pretreatment remains centered around production costs. Wang et al. (2020) conducted a techno-economic evaluation and calculated an annual enzyme cost, including protease, lipase and glucoamylase, amounting to US$ 209,032.00 for an annual production of 1,400 MT of SL crystals or 1,793 MT SL syrup which represents the 1 % of the total raw material cost. Conversely, Molina-Peñate et

al. (2024) reported that commercial enzymes represent up to 91 % of the variable cost for marketable bioproducts production.

For that reason, the implementation of SSF emerges as a potential alternative, enabling the utilization of cost-effective solid substrates like oil cakes and avoiding the challenges related to foaming during SLs production (Jiménez-Peñalver et al., 2016). Although second-generation substrates are widely reported in the literature as low-cost alternatives, the main challenge associated with these substrates is selecting suitable residues with the optimal nutrient balance to enable cell growth and product accumulation (Makkar et al., 2011; Nitschke et al., 2005). Notably, among the reviewed studies, few have been developed using alternative sources as the sole nutrient feedstock, indicating a potential area for future research.

In addition, literature in this field focuses on exploring alternative substrates that can serve as hydrophilic and/or hydrophobic carbon sources, particularly in SmF, creating a gap in terms of alternative nitrogen sources. On the other hand, in SSF, the implementation of alternative substrates has focused on the use of molasses and winterization oil cake as well as evaluating several agro-waste and inert materials that can serve as support in the process (Rodriguez et al., 2021b). This has created a gap in the exploration of other types of alternative substrates that could be utilized, leading to a lack of understanding of their influence and behavior in the SSF process. In this context, this thesis aims to address these gaps and highlight the challenges associated with their use.

Although alternative substrates present a promising option for green biosurfactant production, it is important to note that complex substrates can

introduce impurities into the final fermented extract (Chapter 7). However, from an industrial perspective, SLs can be used in three main forms: as SLs crude extract, as purified SL congeners, or as partially purified SLs with promising achievements in all cases (Pal et al., 2023; El-Shahed et al., 2022; Oiza et al., 2024).

Table 1.2 Alternative substrates used for SLs production by SmF and SSF.

Hydrophilic source	Hydrophobic source	Nitrogen source	Micoorganism	Fermentation strategy	Reactor scale (L)	SLs production	Time (h)	Reference
Glucose	Glycerol	Yeast extract	*Candida antarctica*	SmF	0.5	2.2 g L^{-1}	168	Accorsini et al. (2012)
Corn steep liquor	Soybean oil waste	-	*Cunninghamella echinulata*	SmF	0.3	4 g L^{-1}	96	Andrade Silva et al. (2014)
-	Glycerol - oleic acid	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.5	12.7 g L^{-1}	168	Ashby and Solaiman (2010)
Glucose	Rapeseed oil	Corn steep liquor	*S. bombicola ATCC 22214*	SmF	2.5 and 5	226.7 g L^{-1}	119	Chen et al. (2019)
Deproteinized whey concentrate	Rapeseed oil	Yeast extract	*S. bombicola ATCC 22214*	SmF	30	280 g L^{-1}	n.d	Daniel et al. (1998)
Sugarcane molasses	Olive oil	-	*S. bombicola ATCC 22214*	SmF	0.25	18.96 g L^{-1}	120	Daverey and Pakshirajan (2009)
Sugarcane molasses	Sunflower oil	-	*S. bombicola ATCC 22214*	SmF	0.25	17.46 g L^{-1}	120	Daverey and Pakshirajan (2009a)
Deproteinized whey concentrate	Oleic acid	Yeast extract	*S. bombicola ATCC 22214*	SmF	0.3	23.29 g L^{-1}	34	Daverey and Pakshirajan (2010)

Sugarcane molasses	Dairy wastewater composed of fat and oil	Yeast extract	*S. bombicola ATCC 22214*	SmF	3	1-2 g L^{-1}	40	Daverey and Pakshirajan (2015)
Glucose	White choice frease	Corn steep liquor and urea	*S. bombicola ATCC 22214*	SmF	10 and 15	97 g L^{-1}	72	Deshpande and Daniels (1995)
Glucose	Coconut fatty acids	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.5	40 g L^{-1}	240	Felse et al. (2007)
Glucose	Tallow fatty acids	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.5	120 g L^{-1}	240	Felse et al. (2007)
Corn steep liquor	Rpeseed oil	Ammonium sulphate	*S. bombicola ATCC 22214*	SmF	0.3	39.5 g L^{-1}	168	Ingham et al. (2023)
Glucose	Jatopha oil	Wheat bran	*S. bombicola ATCC 22214*	SmF	5	100 g L^{-1}	216	Imura et al. (2013)
Glucose	Sunflower oil waste	Yeast extract and urea	*S. bombicola MTCC 1910*	SmF	2 and 5	51.5 g L^{-1}	192	Jadhav et al. (2019)
Glucose	Jatropha oil	Yeast extract and peptone	*S. bombicola ATCC 22214*	SmF	0.45	15.25 g L^{-1}	96 120	Joshi-Navare et al. (2013)
Food waste	Oleic acid	-	*S. bombicola ATCC 22214*	SmF	1.15 and 2	115.2 g L^{-1}	92	Kaur et al. (2019)
Food waste	-	-	*S. bombicola ATCC 22215*	SmF	1.15 and 2	28.15 g L^{-1}	72	Kaur et al. (2019)
Glucose	waste cooking oil	Yeast extract	*S. bombicola ATCC 22214*	SmF	2	315.6 g L^{-1}	288	Kim et al. (2021)

Waste glycerol	Palm oil	Yeast extract and urea	*Candida floricola ZM1502*	SmF	0.030 and 0.3	42.7 g L^{-1}	168	Konish et al. (2018)
Corncob	Olive oil	Ammonium nitrate	*S. bombicola ATCC 22214*	SmF	0.5 and 1	49.2 g L^{-1}	96	Konishi et al. (2015)
Waste glycerol	Palm oil	Yeast extract and urea	*Candida floricola ZM1502*	SmF	0.03 and 0.3	42.7 g L^{-1}	168	Konishi et al. (2018)
Pretreated Rice straw	Oleic acid	Yeast extract	*Wickerhamiella domercqiae CGMCC 1576a*	SmF	0.5 and 0.3	53.7 g L^{-1}	168	Liu et al. (2016)
Delignined corncob residue	Oleic acid	Yeast extract	*S. bombicola CGMCC 1576*	SmF	0.05 and 0.3	38.29 g L^{-1}	168	Ma et al. (2014)
Detoxified delignined corncob residue	Oleic acid	Yeast extract	*S. bombicola CGMCC 1576*	SmF	0.05 and 0.3	50.2 g L^{-1}	168	Ma et al. (2014)
Glucose	Waste cooking oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	2.5 and 2.25	51 g L^{-1}	240	Maddikeri et al. (2015)
Glucose	Horse oil	Yeast extract	*Candida bombicola*	SmF	5	71.7 g L^{-1}	168	Maeng et al. (2018)
Sugarcane molasses	chicken fat	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.025 and 0.125	9.78 g L^{-1}	120	Minucelli et al. (2017)
Glucose	Catfish fat	Yeast extract	*S. bombicola ATCC 22214*	SmF	0.1 and 0.25	21.8 g L^{-1}	168	Nguyen et al. (2017)

Glucose	Corn oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	3 and 5	400 g L^{-1}	432	Pekin et al. (2005)
Glucose	Palm Oil	Yeast extract	*Cyberlindnera samutprakarnensis JP52T*	SmF	n.d	n.d.	168	Poomtien et al. (2013)
Rice bran	Rapeseed oil	-	*S. bombicola Y-6419*	SmF	0.2 and 2	51 g L^{-1}	168	Rocha et al. (2023)
Corn fiber	Soybean oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.1	15.6 g L^{-1}	240	Samad et al. (2015)
Sweet sorghum bagasse	Soybean oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.5	84.6 g L^{-1}	240	Samad et al. (2015)
Corn stover	Yellow grase	-	*S. bombicola ATCC 22214*	SmF	0.71 and 3	52.1 g L^{-1}	168	Samad et al. (2017)
Glucose	Waste cooking oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	3.3	34 g L^{-1}	n.d	Shah et al. (2007)
Glucose	Waste frying	Yeast extract	*S. bombicola ATCC 22214*	SmF	0.025 and 0.125	22 g L^{-1}	n.d	Shah et al. (2007)
Soy molasses	Oleic acid	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	12	21 g L^{-1}	120	Solaiman et al. (2004)
Glucose	Bakery waste oil	rice bran hydrolysate	*S. bombicola ATCC 22214*	SmF	1.5	96.4 g L^{-1}	144	To et al. (2022)
Glucose	Waste frying sunflower oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.05 and 0.25	4.26 g L^{-1}	200	Wadekar et al. (2012a)

Glucose	Karanja oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.25	7.7 g L^{-1}	200	Wadekar et al. (2012b)
Glucose	Jatropha oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.25	6 g L^{-1}	200	Wadekar et al. (2012b)
Glucose	Neem oil	Yeast extract and urea	*S. bombicola ATCC 22214*	SmF	0.25	2.63 g L^{-1}	200	Wadekar et al. (2012b)
Food waste	Oleic acid	-	*S. bombicola ATCC 22215*	SmF	2.0	36.5 g L^{-1}	72	Wang et al. (2019)
Potato processing scraps	Rapeseed oil	-	*S. bombicola ATCC 22214*	SmF	0.2 and 2	60.5 g L^{-1}	168	Wongshirichot et al. (2022a)
Sugar beet pulps	Rapeseed oil	-	*S. bombicola ATCC 22214*	SmF	0.2 and 2	25.6 g L^{-1}	168	Wongshirichot et al. (2022a)
Detoxified corn straw	Oleic acid	Ammonium nitrate	*S. bombicola ATCC 22214*	SmF	0.5	27.45 g L^{-1}	168	Yu et al. (2021)
Molasses	Winterization oil cake	-	*S. bombicola ATCC 22214*	SSF	0.5	0.18 g $g^{-1}DM_i$	240	Jiménez-Peñalver (2016)
Molasses	Winterization oil cake	-	*S. bombicola ATCC 22214*	SSF	22	0.19 g $g^{-1}DM_i$	168	Rodriguez et al. (2021a)

Abbreviation: n.d, not determined; SmF, submerged fermentation; SSF, solid-state fermentation, DM_i, initial dry matter

1.9 Surfing Waste project

New global trends emphasize the use of sustainable products and the enhancement of circularity in production processes. In this context, Circular Economy and Bioeconomy initiatives are being actively promoted by many governments. Spain has developed its "*Estrategia Española de Economía Circular. España Circular 2030*" and *"Estrategia Española de Bioeconomía. Horizonte 2030*." These two strategies intersect in a circular bioeconomy approach, where products are either bio-based or produced from second-generation feedstocks. Based on the biosurfactants market and their capacity to substitute chemical surfactants the project hypothesis is based on the development of a sustainable and reproducible process for glycolipids biosurfactants focusing on SLs and MELs production from wastes and side-streams that can be competitive at commercial scale which contribute to develop novel value chains in the framework of circular bioeconomy. Also, an assessment of the techno-economic and environmental feasibility of the proposed bioprocesses will be required, to ensure the sustainability of the use of organic wastes and side streams to produce marketable bioproducts (Figure 1.7).

Based on the extensive experience of the research group working with SSF, this project contributes to understanding the general challenges that SSF presents for biosurfactant production, specifically for SLs. This has been explored in two previous theses by Jiménez-Peñalver (2017) and Rodríguez (2020). The first thesis laid the groundwork for the process and introduced initial approaches for SLs production via SSF, while the second thesis focused on screening new agro-waste and inert materials as supports for the process, as well as scaling-up

and exploring potential strategies for product recovery. In this context, this thesis contributes by expanding the range of second-generation feedstocks used for SLs production, evaluating their impact on the process and by bringing valuable know-how from the established SmF process to the group. For instance, this is the first thesis of the research group where both SSF and SmF are applied with the same goal, making SLs production a sustainable process.

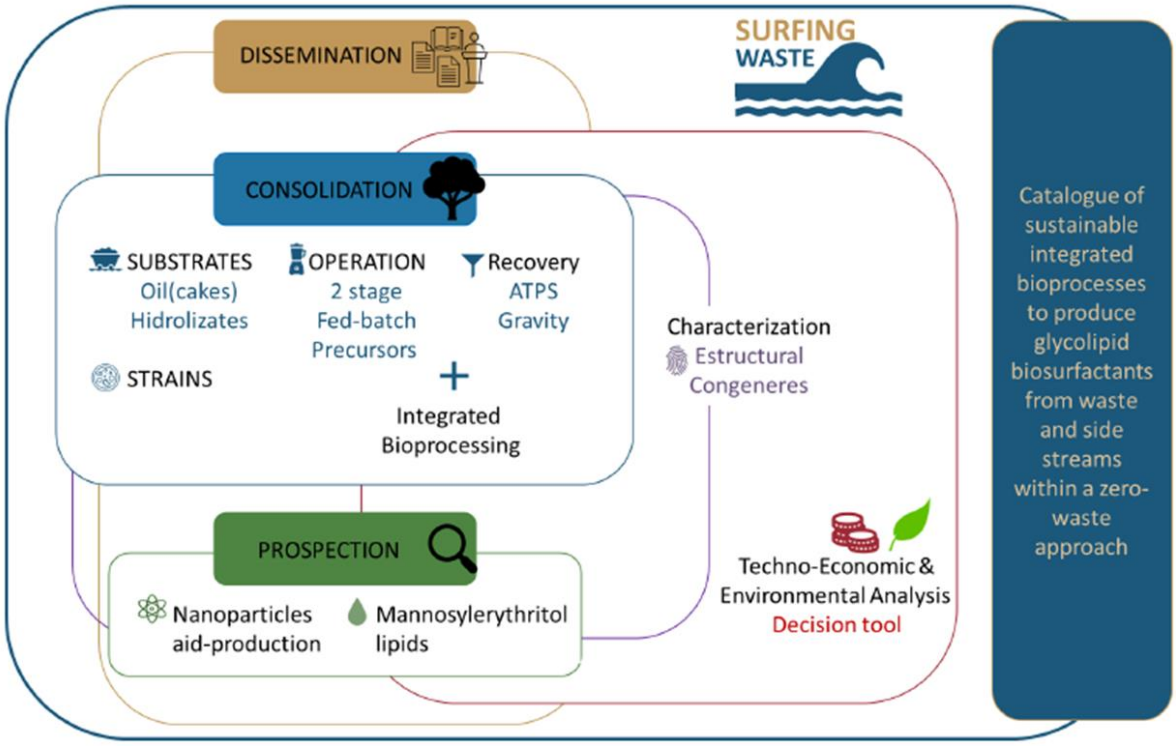

Figure 1.7 Surfing Waste project scheme

Chapter 2

Objectives

This PhD thesis is part of the research line in solid-state fermentation of the research group GICOM at UAB. In this field, the group is developing the Surfing Waste Project (PID2020-114087RB-I00), within which the objectives of this thesis are aligned. The background of the research group, which has worked with the valorization of organic (urban, agricultural and industrial) waste and has experience in sophorolipids production via solid-state fermentation laid the groundwork for this study.

The project includes several activities (**Table 2.1**) framed in creating a catalogue of sustainable integrated bioprocesses to produce glycolipid biosurfactants from waste and side streams, within a zero-waste approach. This thesis contribution relies on activities 1, 3, 7 and 9.

Table 2.1 List of Surfing waste project activities

Task	Description
Activity 1	**Screening of potential substrates (waste and side streams) for sophorolipids' production.**
Activity 2	Gaining knowledge on the process basis: metabolism and heat generation.
Activity 3	**Optimization of growth and synthesis of sophorolipids.**
Activity 4	Sophorolipid recovery through an integrated bioprocessing approach.
Activity 5	Zero waste approach.
Activity 6	Extrapolation of the optimized system to the production of mannosylerythritol lipids.
Transversal activities	
Activity 7	**Product characterization.**
Activity 8	Life Cycle and Techno-economic assessments.
Activity 9	**Project management and dissemination.**

Therefore, the main goal of this PhD thesis is to assess the technical feasibility of utilizing second-generation feedstocks as an alternative to edible pure substrates and food ingredients to produce sophorolipids using the wild-type *Starmerella bombicola* ATCC 22214. To go deeper, the following specific objectives were established and are presented below:

I. To optimize the ratio of hydrophilic carbon and nitrogen sources to increase sophorolipids production in solid-state fermentation by using pure substrates.
II. To evaluate the performance of different hydrophilic carbon, nitrogen and hydrophobic carbon sources derived from second-generation feedstocks on sophorolipids production by solid-state fermentation and its influence on the congeners profile.
III. To develop a reproducible down-scale method for solid-state fermentation that allows the simultaneous handling of multiple samples.
IV. To evaluate the scale-up viability for sophorolipids production by solid-state fermentation using second-generation feedstocks, going from down-scale (Erlenmeyer 0.5 L) to laboratory scale bioreactor (0.5 L) and bench bioreactor scale (22 L).
V. To investigate the influence of alternative nitrogen sources as feedstock for sophorolipids production using agricultural byproducts hydrolysates in submerged fermentation.
VI. To evidence the influence of nitrogen concentration on sophorolipids production and yeast growth in submerged fermentation.
VII. To scale up the best hydrolysate and nitrogen concentration for submerged fermentation from Erlenmeyer (0.5 L) to bioreactor scale (2 L)

VIII. To contribute fermentation process data for the creation of a database that supports initial inventories for Life Cycle Assessment, Economic and Technical Analysis and model development using second-generation feedstocks.

Chapter 3

Materials and Methods

Summary

This chapter provides a comprehensive overview of the methodology utilized in this research. It covers the preparation of yeast cultures, the use of both pure and alternative substrates and the general analytical methods employed. Additionally, it details the setup of fermentation systems, including both solid-state fermentation (SSF) and submerged fermentation (SmF).

3.1 General materials

3.1.1 *Microorganism*

The yeast *Starmerella bombicola* ATCC 22214 was purchased from the American Type Culture Collection (Manassas, USA) and cryopreserved at -80°C with glycerol 10% (v v^{-1}) following the methodology by Simione (2009).

3.1.2 *Pure substrates*

Analytical grade substrates such as glucose, urea, yeast extract, soy peptone, malt extract, bacteriological agar, ammonium sulfate, sodium sulfate Ringer® solution, corn steep liquor (CSL), ethanol, ethyl acetate and n-hexane were provided by Sigma-Aldrich (St. Louis, MO, USA). Moreover, commercial rapeseed oil (Crisp ŃDry, UK) was used due to its high content of oleic acid in Chapter 6.

3.2 Solid-State Fermentation: materials

3.2.1 *Inoculum preparation*

S. bombicola ATCC 22214 was grown for 48 h at 30 °C on agar plates containing 10 g L^{-1} of glucose, 5 g L^{-1} of peptone, 3 g L^{-1} of malt extract, 3 g L^{-1}

of yeast extract and 20 g L^{-1} of bacteriological agar (Figure 3.1a). Then, it was transferred to 100 mL broth in a 500 mL Erlenmeyer flask with the same medium composition described above but without bacterial agar (Figure 3.1b). After incubation time two tasks were performed routinely to confirm inoculum quality. First, optical microscopy was carried out to control culture morphology and any possible contamination (Figure 3.1c). Then optical density (OD) at 600nm was measured in a spectrophotometer (Cary 50 Bio, UV-Visible Spectrophotometer) and calculated using Equation 1. For this procedure, samples were diluted (x50 times) with the inoculation culture media that was used as well as a blank. To get reproducible results between different experimental batches the absorbance readings range was 0.1-0.3 with a target OD_{600} of 12–15 units, approximately 10^9 CFU mL^{-1} (Figure 3.1d).

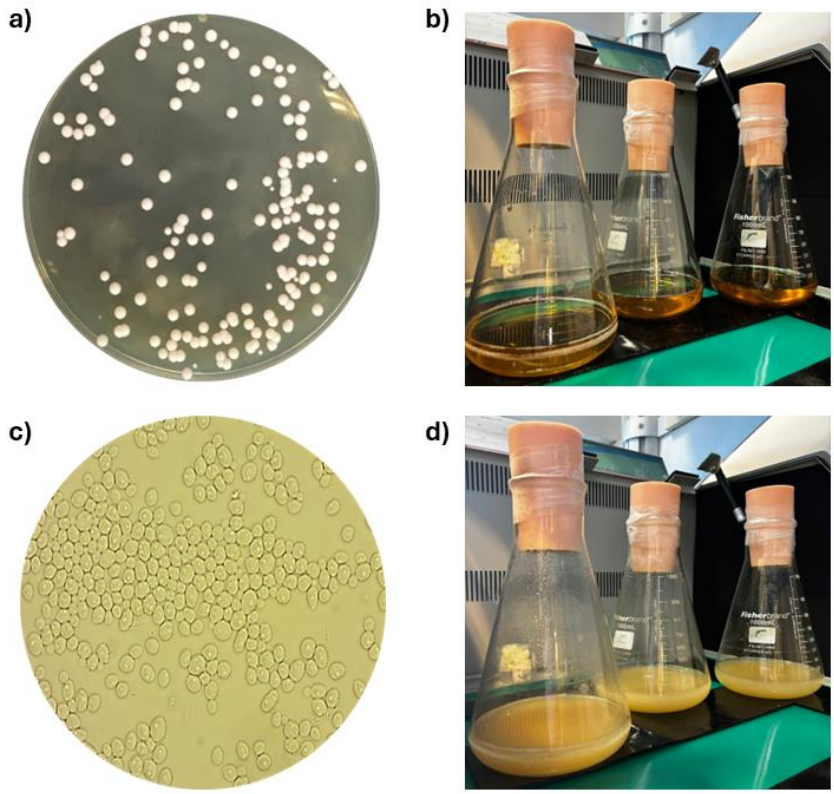

Figure 3.1 *Starmerella bombicola* growth. a) Yeast colonies in agar plates, b) Initial liquid media appearance, c) Cells in an optical microscope (x100 augments) and d) Broth after 48 h of growth at 30ºC and 180 rpm.

$$OD = Abs_{600}\ x\ N$$

(Eq.1)

Where: OD, optical density; Abs_{600}, absorbance values at 600 nm; N, inoculum dilution.

3.2.2 *Second-generation feedstocks used in SSF*

Different organic industrial waste and agricultural oilseed cakes were used as feedstock in SSF (Figure 3.2). Sugar candy wastewater (RSC) was provided by *Chupa Chups S.A.U*. (Barcelona, Spain). The organic fraction of municipal solid waste (OFMSW) was kindly provided by *Mancomunitat La Plana* (Malla, Barcelona) and the hydrolysate (ROF) was prepared as described by Molina-Peñate et al. (2022). Nitrogenous sludges (RHP and RAC) were provided by two local industries. The RHP sludge was provided by *HIPERTIN S.A* (Barberà del Vallès, Barcelona) and comes from the cleaning of the reactors used in the production of cosmetics, hair treatments and body creams, among others. RAC was provided by *AC Marca, S.L* (L'Hospitalet de Llobregat, Barcelona) and comes from the cleaning of the reactors employed to produce household cleaning products.

Winterization oil cake (WOC) obtained from sunflower oil refining was used as the model hydrophobic substrate, with an oil content ranging from 44% to 80%, composed mainly of 84% of C18:1 fatty acid, as described in previous studies (Jiménez-Peñalver et al., 2016; Rodríguez et al., 2021) and was provided by *Lípidos Santiga S.A.* (Santa Perpètua de Mogoda, Barcelona). This oil cake is obtained after subjecting the oil to low temperatures (5 ºC) to precipitate waxes, followed by filtration with perlite. In addition, corn (CNC), sunflower (SFC),

soybean (SBC) and rapeseed (RPC) oilseed cakes were obtained after seed pressing for vegetable oil extraction and were provided by *Aceites de Semillas S.A* (Caldes de Montbui, Barcelona). In all SSF assays, the organic support used for SSF was wheat straw provided by the *Facultat de Veterinària UAB*, which was autoclaved twice before use. Before its use, all substrates were characterized and kept at −20 °C until their use.

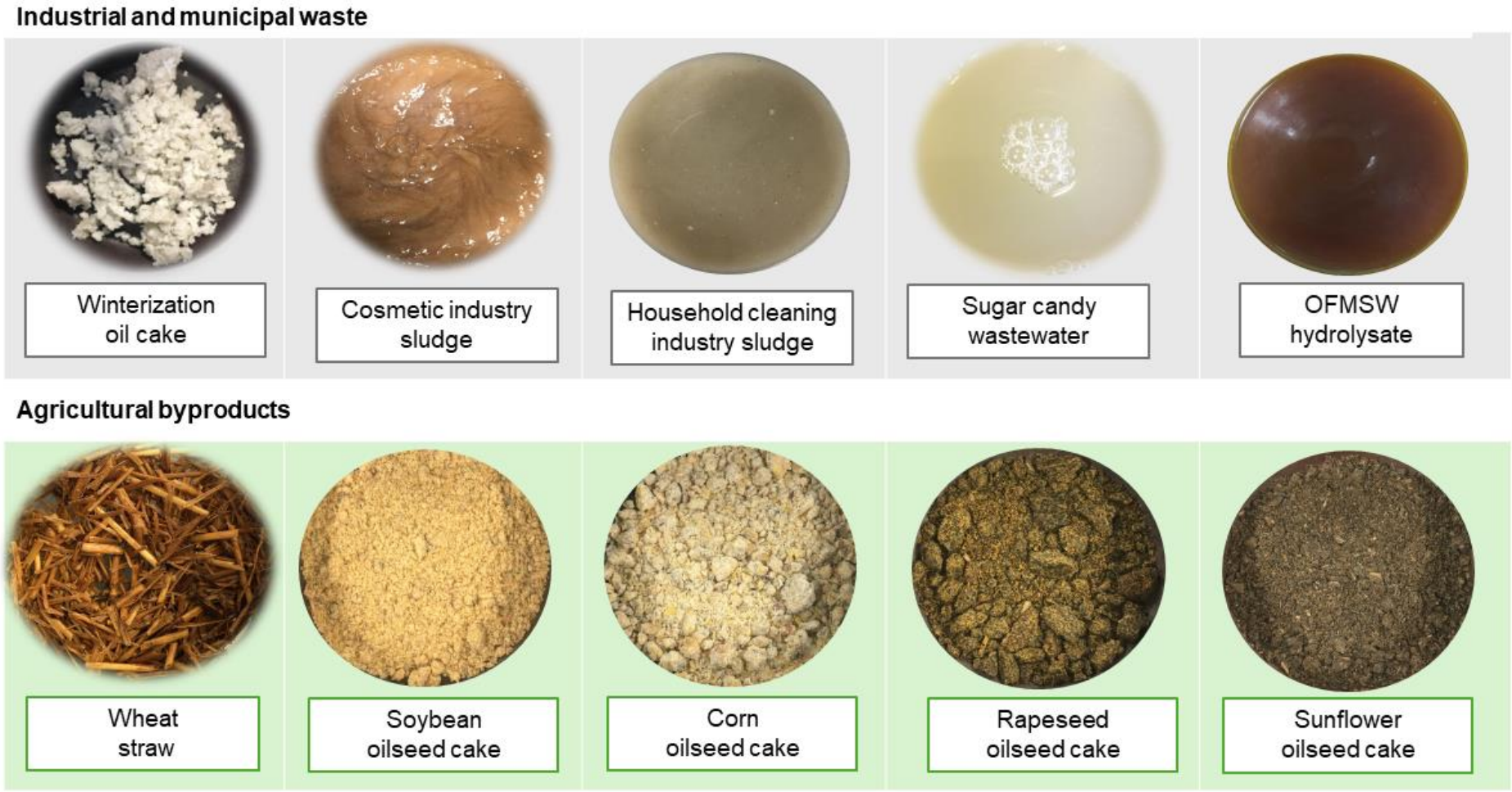

Figure 3.2 Second-generation feedstocks used in solid-state fermentation. Up: industrial and municipal organic waste, down: agricultural byproducts.

3.3 Solid-State Fermentation: set-up

In this work, SSF experiments were conducted at different scales, including 0.5 L scale-down system, as well as 0.5 L and 22 L bioreactors, with packed-bed forced-aerated systems used in the latter two. The scale-down system was developed as part of the thesis due to it was adapted based on the 0.5 L bioreactor scale used as the standard method by the group.

3.3.1 *SSF: 0.5 L scale-down system*

Due to equipment availability limitations, a scale-down adaptation was developed within this thesis to allow the simultaneous fermentation of several replicates. The experiments were carried out using 0.5 L Erlenmeyer flasks, with a total working volume of 0.18 L. The system was modified by placing the Erlenmeyer flasks inside a plastic box with water, which was then placed inside an incubator (Memmert GmbH, IN750) under temperature control at 30 ºC (Figure 3.3). It is important to note that the water was not in contact with the Erlenmeyer flasks, it was used just to humidify the air and prevent the drying of the fermented solid. The replicability of this system and its analogy with the reference packed bed system were verified.

The total solid substrate weight was approximately 20.3 ± 0.6 g with a dry matter of 53.5% (10.84 g). Each sample was loaded with a solid matrix made up of 3.8 g of wheat straw as the support, working at 75% water-holding capacity and 6.3 g of WOC. The final production mixture was 43.1% aqueous phase composed of 7 mL of a nutrient dilution mix (glucose, yeast extract and urea) and 1.7 mL of *S. bombicola* inoculum. This mixture was based on previous works using 0.1 g glucose g^{-1} dry matter as an initial stage for SLs production (Jiménez-

Peñalver et al., 2016; Rodríguez et al., 2020). Moreover, nutrients were added following reference glucose:yeast extract:urea ratio of 100:10:01 (w w^{-1} based on the initial mixture dry weight), as extensively reported for SmF. This ratio was later modified according to the experimental design (Chapter 4). Fermentations were carried out under sterile conditions, for which wheat straw was autoclaved (121°C, 30 min) twice before the preparation of the solid matrix and then, the total mixture was autoclaved (the same conditions) before assembly.

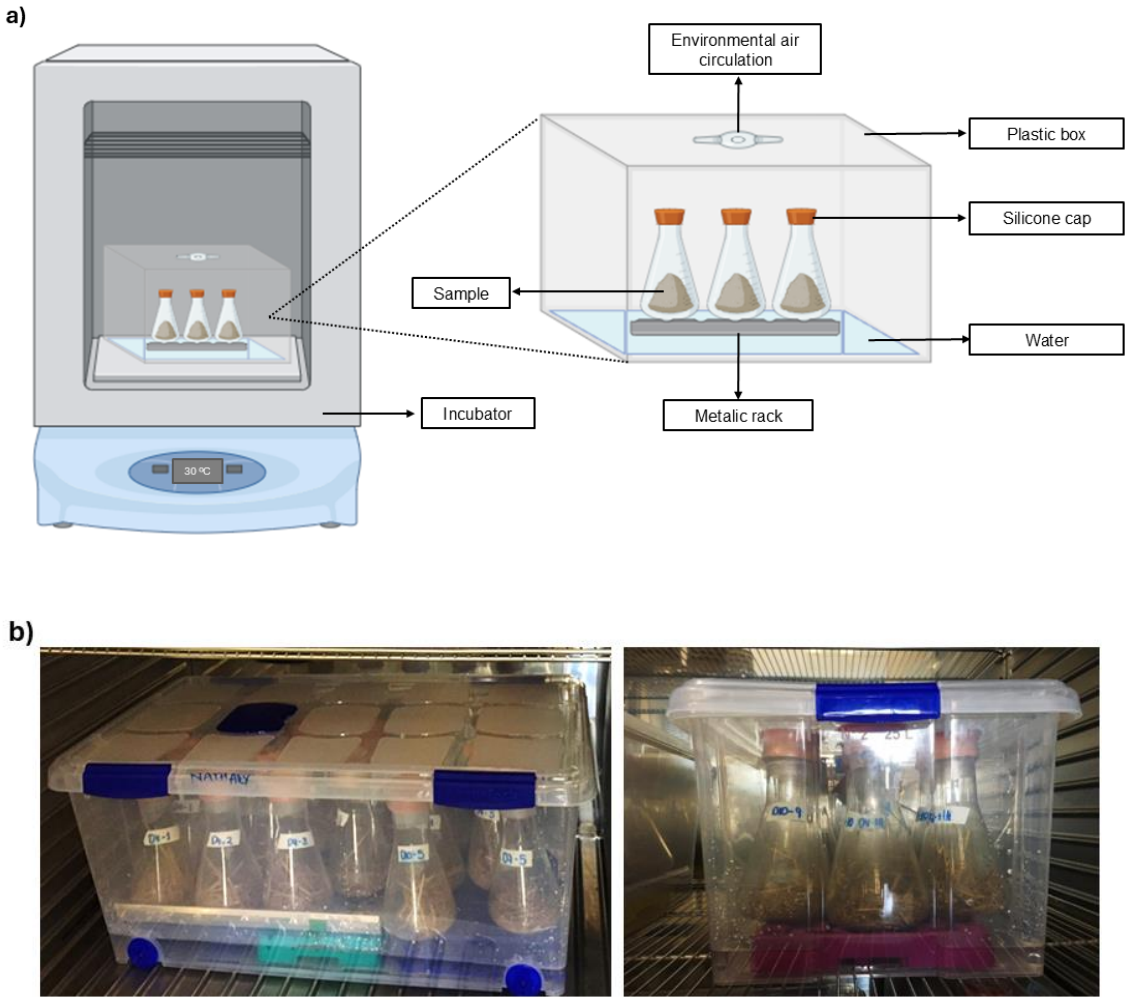

Figure 3.3 Custom-designed downscale SSF system. a) System set-up to keep sample humidity and b) Erlenmeyer and sample appearance.

3.3.2 *SSF: 0.5 L bioreactor system*

The base of this system was designed and built by the GICOM group and has been thoroughly described in several previous studies. It is used as the reference scale by the group, the experimental setup is shown in Figure 3.4. Briefly, experiments were conducted under aseptic conditions using cylindrical 0.5 L PVC reactors (13 cm height × 7 cm diameter), with a working volume capacity of 75% corresponding to 0.38 L. Each reactor was filled with a solid matrix that included 14 g of wheat straw (water-holding capacity 75%), along with 23.20 g of hydrophobic substrate (WOC or oilseed cakes). Furthermore, the fermented solid consisted of a 45.1% aqueous phase, comprising 25.86 mL of a nutrient solution (glucose, yeast extract and urea) and 6.4 mL of the *S. bombicola* inoculum. Subsequently, the total weight of the solid substrate reached 77.42 g, with a dry matter content.

The aseptically filled reactors were placed at 30 ºC in a controlled water bath. Then following Jiménez-Peñalver et al. (2016) optimized rates for SLs production, humidified air at 30 mL min^{-1} was continuously provided to the reactors controlled with a mass flow meter (Bronkhorst, Netherlands). Exhaust gases exited from the top of each reactor to an O_2-A_2 oxygen sensor (Alphasense, UK) connected to a custom acquisition system (Arduino®). Finally, the bioreactors were sacrificed for analysis at as described in each chapter

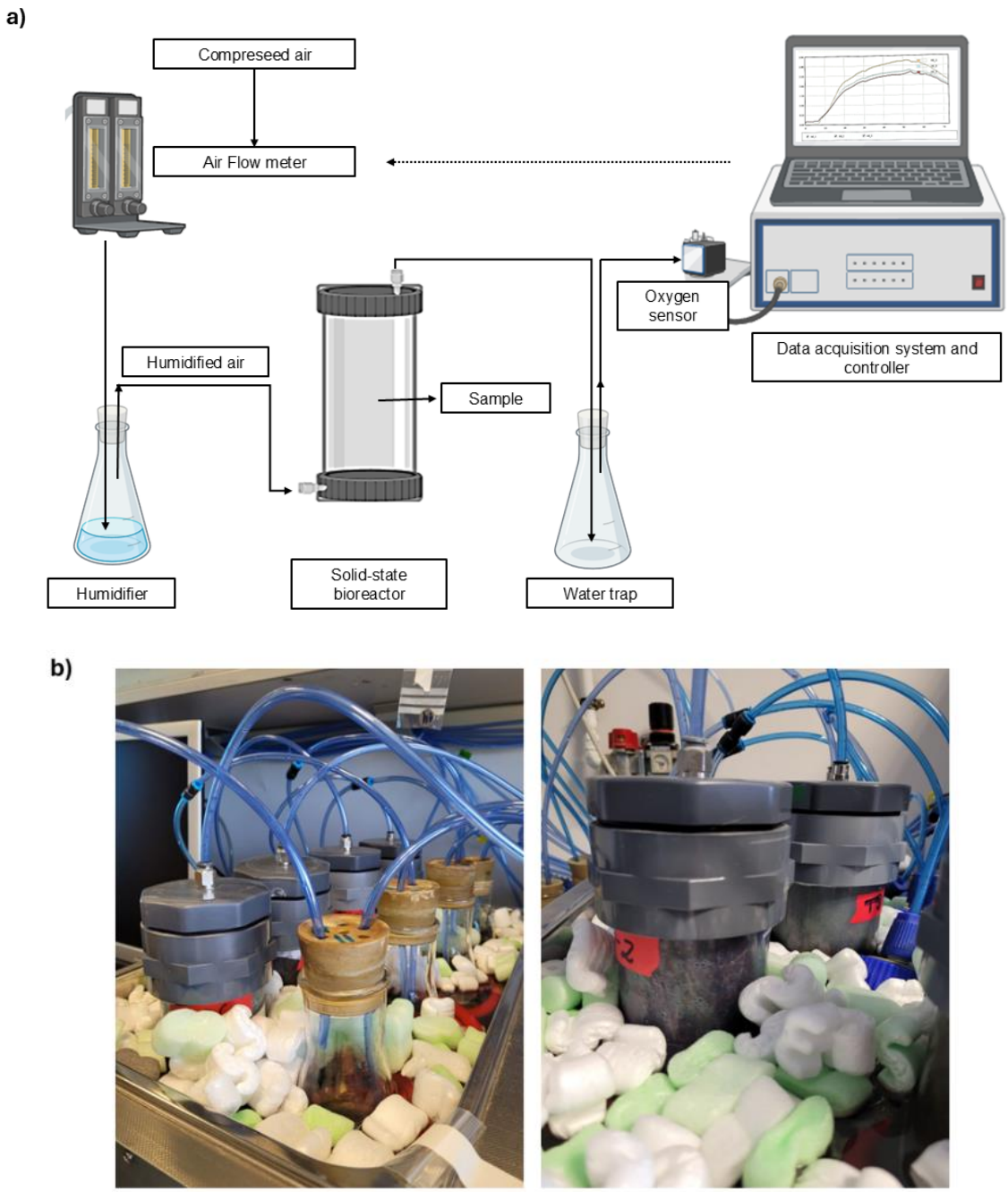

Figure 3.4 Solid-state fermentation set-up, air flow and oxygen monitoring. a) SSF system and b) 0.5 L bioreactor appearance.

3.3.3 *SSF: 22 L bioreactor system*

A 22 L bioreactor was used consisting of a cylindrical stainless-steel fermenter (70 cm height × 30 cm diameter) with a removable inner basket (48 cm height × 24.5 cm diameter). The basket contains a perforated dish (2 mm holes) which separates the solid matrix chamber from the air distribution chamber

(Figure 3.5). Before and after each set-up, the reactor was first cleaned with a water-bleach solution (30% v v^{-1}), then with a commercial disinfectant (Vilafant PP) that was left for 24 h before being removed. Moreover, the removal basket was autoclaved twice at 121 ºC for 30 minutes before the setup, while the fermentation solid was prepared under a flow hood to maintain as sterile conditions as possible.

The aeration system for the large scale, is analogue to the process previously described for the 0.5 L bioreactors. Working conditions were set as described by Rodríguez et al. (2021a), operating at a bioreactor capacity of 75%. The reactor was filled with 3.4 kg of wet mass, composed of 1.01 kg of hydrophobic substrate, 0.61 kg of wheat straw, 1.2 L of a nutrient solution (including 0.33, 0.003 and 0.02 kg of glucose, urea and yeast extract, respectively) and 0.28 L of *S. bombicola* inoculum. During the first 24 hours, aeration was maintained at 1237 mL min^{-1} and after this period, the airflow was increased to 2000 mL min^{-1} to assist with heat removal. Additionally, temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S) were placed at different reactor heights (top, middle and bottom) to monitor solid media temperature Finally, samples were analyzed at 96 h by duplicates at different heights.

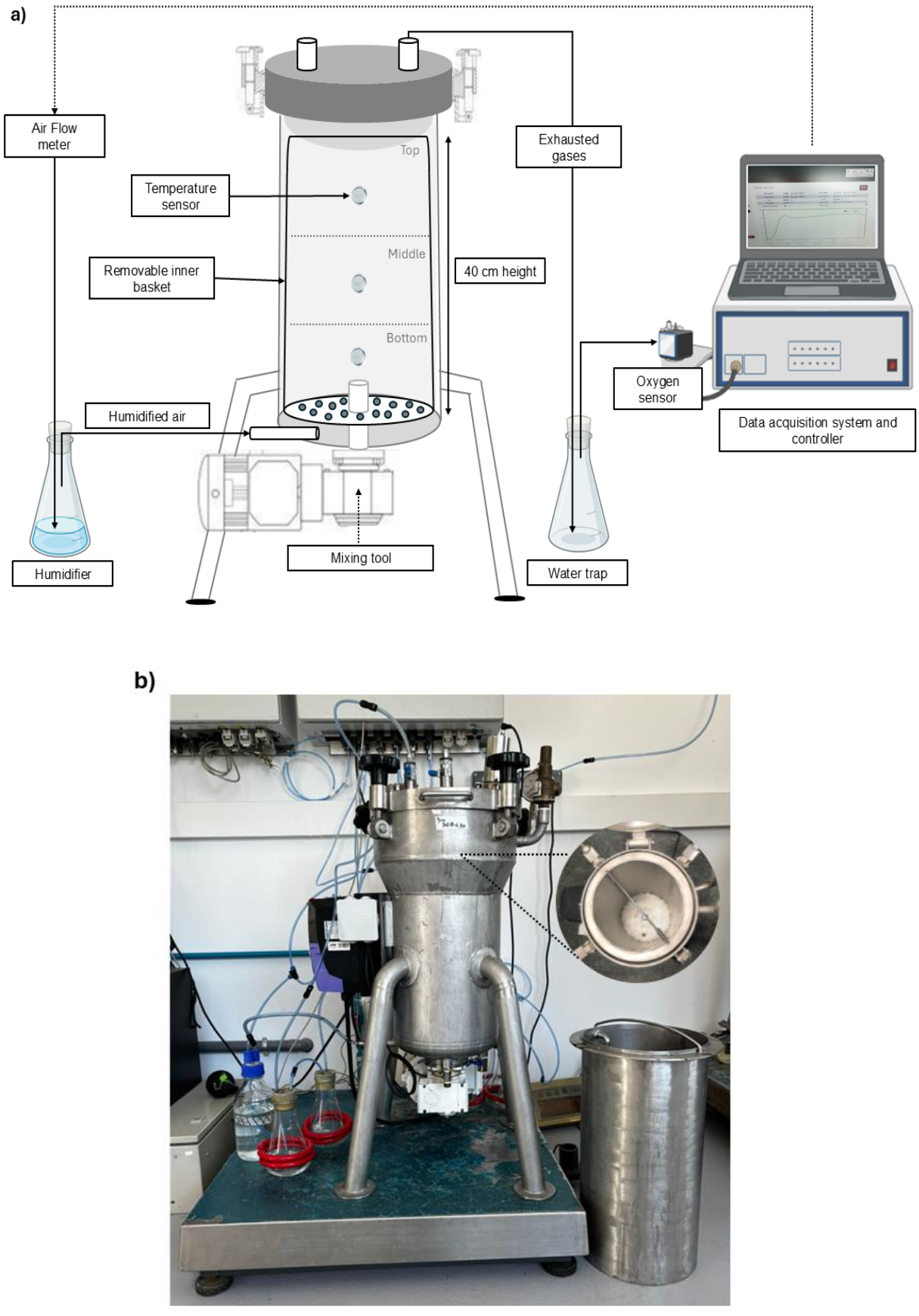

Figure 3.5 22 L bioreactor set-up. a) Bioreactor system and b) Bioreactor appearance. Modified from Sala, 2022.

3.4 Solid-State Fermentation: monitoring methods

3.4.1 *Yeast growth estimation*

- *Biological activity calculation*

The recorded oxygen data allow for the calculation of the specific oxygen uptake rate (sOUR, Eq.2) and the cumulative oxygen consumption (COC, Eq.3) as an indirect measure of biological activity (Ponsá et al., 2010).

$$SOUR = \frac{\Delta O_2 \; x \; F \; x \; 31.98 \; x \; 60 \; x \; 1000^{a}}{1000^{b} \; x \; 22.4 \; x \; DM}$$

(Eq. 2)

Where: $sOUR$, represents the specific oxygen uptake rate at a given time (mgO_2 g^{-1} DM h^{-1}); ΔO_2, oxygen variation between airflow in and the reactor exhaust gases (volumetric); F aeration rate (mL min^{-1}); 31.98, oxygen molecular weight (g mol^{-1}); 60, time conversion factor (min h^{-1}); 1000^{a}, weight conversion factor (mg g^{-1}); 1000^{b}, volume conversion factor (mL L^{-1}); 22.4, volume (L) occupied at normal conditions by one mol of ideal gas (273 K and 1 atm) and DM, dry matter of the solid sample.

$$COC = \int_{0}^{t} sOUR \; x \; dt$$

(Eq. 3)

Where: COC, cumulative oxygen consumption (mgO_2 g^{-1} DM); $sOUR$, Oxygen consumption rate (mgO_2 g^{-1} DM h^{-1}) and t final time of solid-state fermentation (h).

- *Viable cell numbers*

Viable cell numbers were quantified by counting colony-forming units (CFUs), as described by Rodríguez et al. (2020). First, under sterile conditions, 10 g of fermented solid was mixed with 90 mL of Ringer® sterile saline solution for eukaryotic cells (1:10, w v^{-1}). Then, the mixture was shaken in an orbital incubator at 200 rpm, 25°C for 20 min. After that, serial dilutions were carried out (1:10, v v^{-1}) in 15 mL sterile falcon tubs by adding 1 mL of extract in 9 mL of Ringer solution. Later, 100 µL of each dilution was inoculated on agar plates in triplicate and incubated at 30°C for 48 h (Figure 3.6). After incubation, the formed colonies were counted using the Schuett counter (Göttingen, Germany) and calculations were developed as detailed in Equation 4 and Equation 5.

$$Total\ CFU = \frac{Nº\ CFU\ x\ DF\ x\ EF}{0.1}\ x\ W_{FS} \quad \text{(Eq. 4)}$$

Where: $Total\ CFU$, total colony forming unit; $Nº\ CFU$, number of colonies counted by petri dish (in the range of 25-250); DF, dilution factor; EF, extraction factor (9 mL g^{-1}); 0.1 mL of diluted extract plated and W_{FS}, fermented solid weight (g).

$$CFUg^{-1}DM_i = \frac{Total\ CFU}{DM_i} \quad \text{(Eq. 5)}$$

Where: $CFUg^{-1}DM_i$, colony forming unit per initial dry matter; $Total\ CFU$ (Eq.4) and DM_i, initial dry matter weight.

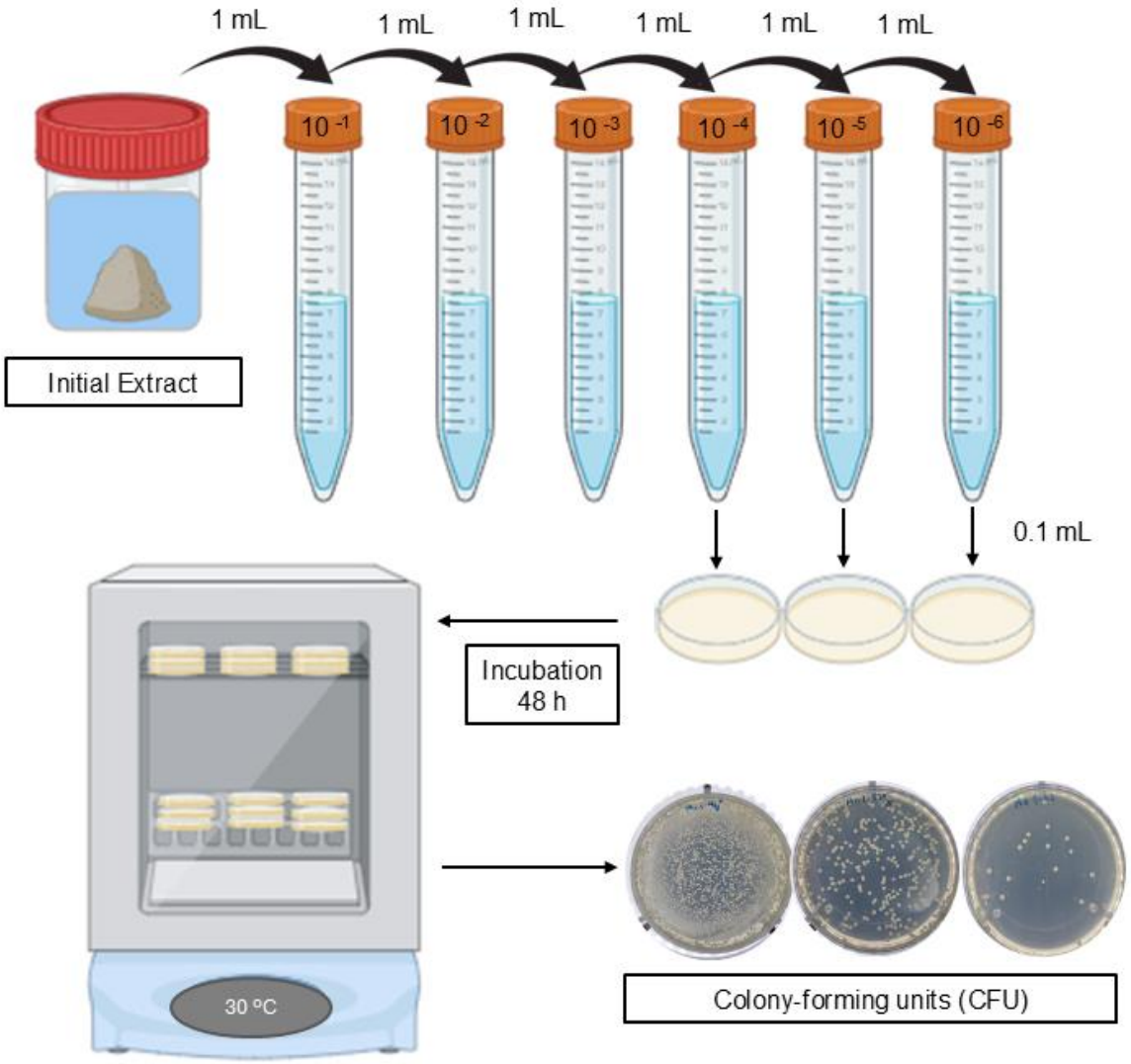

Figure 3.6 Colony-forming units serial dilution protocol.

3.4.2 *Sophorolipids crude extraction from a solid matrix*

Sophorolipids crude extract was obtained using solvents as described by Jiménez-Peñalver et al. (2018). First, 10 g of the ground fermented solid underwent two rounds of ethyl acetate extraction (1:10, w v^{-1}) using an orbital shaker at 200 rpm, 25 °C for 1 hour. After extraction, the solids were removed and the extracts were pooled together. To remove any remaining moisture, anhydrous Na_2SO_4 was added. Then the extract was filtered using Whatman filter paper No. 1 and vacuum-dried using a rotary evaporator at 40 °C for

approximately 20 min. Following this, the resulting SLs crude extract was cleansed of any oily residue by washing it with n-hexane (1:4 w v^{-1}) and leaving it to air-dry overnight (Figure 3.7). Finally, the SLs crude extract was determined gravimetrically by applying Equation 6 and 7 and stored at 4°C until further use. Moreover, using SLs crude extract, productivity values were calculated (Eq.8).

$$SLs\ crude\ extract\ (total\ g) = \frac{W_f - W_i}{Ws}\, x\, W_{FS}$$

(Eq.6)

Where: $SLs\ crude\ extract$ (total g); W_f, final round bottom flask weight after rotary evaporation (g); W_s, initial sample weight used for the extraction (g); W_{FS}, fermented solid weight (g).

$$SLs\ crude\ extract\ (g\ g^{-1} DM_i) = \frac{SLs\ crude\ extract\ (total\ g)}{DM_i}$$

(Eq.7)

Where: $SLs\ crude\ extract$ (g g^{-1} DM_i), grams of SLs crude extract per gram of initial dry matter $SLs\ crude\ extract$ (Eq.6) and DM_i, initial dry matter from the fermented sample (g).

$$Productivity\ (g\ L^{-1} h^{-1}) = \frac{SLs\ crude\ extract\ (total\ g)}{V_R\ x\ t}$$

(Eq.8)

Where: $Productivity$ (g L^{-1} h^{-1}), total grams of SLs crude extract per liter per hour. $SLs\ crude\ extract$ (Eq.6), V_R, reactor (0.38 L) or Erlenmeyer (0.18 L) working volume (L), t, fermentation time (h).

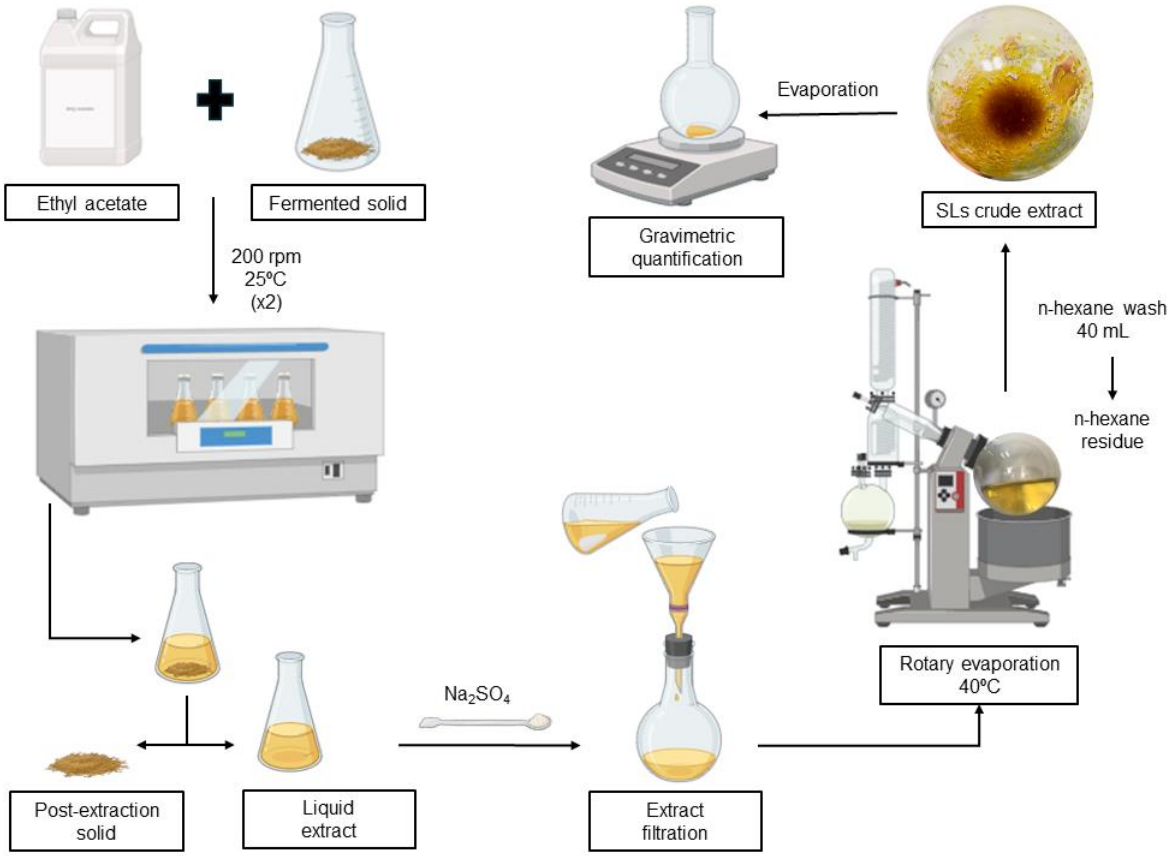

Figure 3.7 Scheme of the procedure for SLs crude extract obtained from a fermented solid.

3.4.3 *Fat content*

The fat content analysis was conducted using a standard Soxhlet method (Figure 3.8) as outlined by the U.S. Environmental Protection Agency (Method 9071B). Before extraction, glass flasks, cellulose cartridge and solid samples were dried at 105 ºC for 24 h. Then, 5 g of the crushed sample was loaded on a cellulose cartridge and placed on the Soxhlet-E816 equipment (Büchi Ibérica S.L.U, Spain.). After that, sequential n-hexane (≥95% of purity, Sigma-Aldrich) extractions were carried out for 4 h and the samples were allowed to dry overnight before gravimetric weighing.

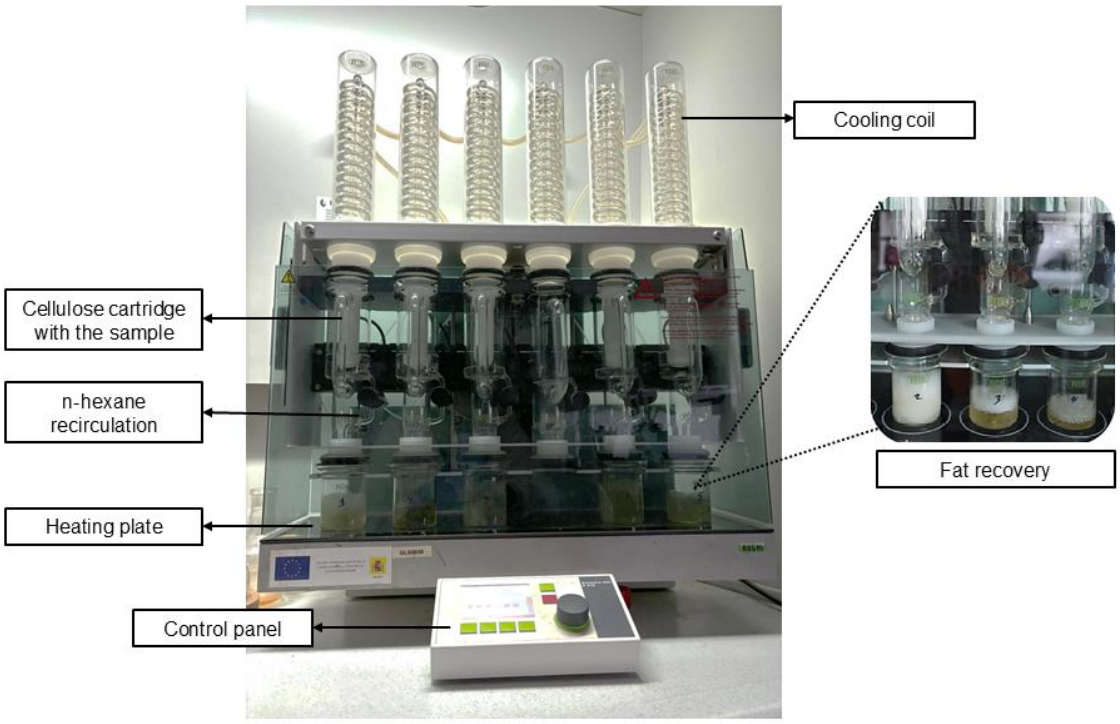

Figure 3.8 Soxhelet-E816 equipment for fat extraction.

Results were calculated as stated in Equation 9 and were presented as a percentage of fat content on a dry base. Moreover, using standard protocols, fatty acids were characterized via gas chromatography coupled with a flame ionization detector (GC-FID) by Eurofins Scientific (Barcelona, Internal Method C5127285) when required.

$$Fat\ content\ (\%, db) = \frac{W_{Gf} - W_{Gi}}{W_S}\ X\ 100\%$$

(Eq. 9)

Where: W_{Gf}, Glass flask final weigh after overnight drying (g); W_{Gi}, Glass flask initial weight (g); W_S, Initial solid sample weight (g)

3.5 Sophorolipids crude extract characterization

Emulsion and Oil displacement tests using SLs crude extract were prepared and assessed following Jiménez-Peñalver et al. (2020) and Pornsunthorntawee et al. (2008) with some modifications. Results were expressed as mean ± standard deviation of three replicates.

3.5.1 *Emulsion activity*

For the emulsification test, a mix of 3 mL of cooking oil and 2 mL of a water solution of the obtained SLs crude extract at 1 mg mL^{-1} and 0.1 mg mL^{-1} underwent high-speed vortexing for 2 min. Subsequently, the mixtures were allowed to stand at room temperature (25 ºC). Then, emulsion activity was assessed after 24 h and 168 h by calculating the emulsification index (E24 and E168). A higher emulsification index indicates greater surfactant emulsification activity. In this way, E24 is obtained in percentage by measuring the emulsion layer's height and dividing by the total mixture's height, then multiplied by 100 (Cooper and Goldenberg, 1987). Results were compared with the laboratory grade Triton™ X-100 (Merck Life Science S.L.U., Spain).

3.5.2 *Oil displacement*

The oil displacement test was done by adding 40 mL of distilled water to a petri dish (90 × 13 mm). After that, 10 µL of cooking waste oil was dropped onto the surface of the water, followed by the addition of 10 µL of ethanol-SLs crude extract solution of 10 mg mL^{-1}. Then, the diameter of the clear zone formed after dropping the surfactant-containing solution on the oil-water interface was measured. The diameters of the clear zones of triplicate experiments were

determined for an averaged value of the clear zone diameter (Rodrigues et al., 2006).

3.6 Submerged Fermentation: materials

3.6.1 *Microbial seed culture*

From the cryopreserved *S. bombicola* ATCC 22214 seed culture flasks were prepared using 10 % (v v^{-1}) of the cryopreserved stock in 50 mL growth media containing: 4 g L^{-1} $(NH_4)_2SO_4$, 5 g L^{-1} corn steep liquor, 100 g L^{-1} glucose and 100 g L^{-1} rapeseed oil. Growing conditions were set up at 30 ºC, 200 rpm at 24 h (Figure 3.9). Once the seed culture reached the exponential phase with an optical density (OD_{600}) of around 12–15 units it was inoculated into the fermentation media.

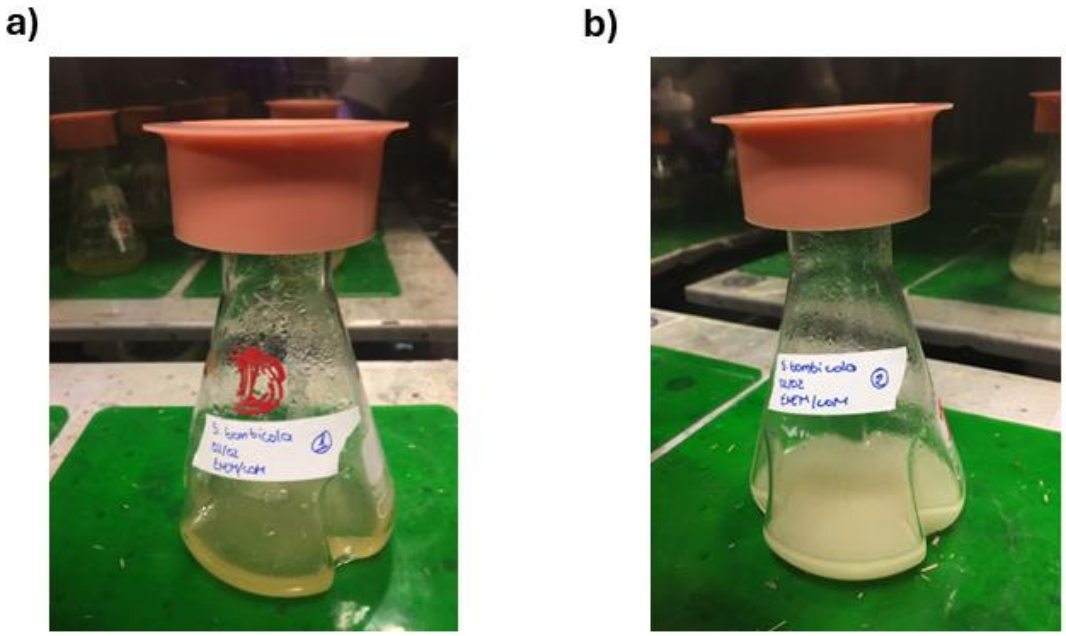

Figure 3.9 *S. bombicola* seed used in Submerged Fermentation experiments. a) Initial growing media and b) Microbial seed after 22-24 h of inoculation

3.6.2 *Alternative substrates used in SmF*

Four different agro-industrial wastes were used as nitrogen sources (Figure 3.10). Coconut waste (CW) and palm waste (PW) were acquired directly from local farmers in Thailand, the rapeseed meal (RM) was procured from Cargill (UK) and wheat feed (WF) was procured from the Nelstrops Albion Flour Mill (UK). The substrates were stored at -4 ºC until hydrolysis pre-treatment.

Figure 3.10 Alternative substrates used in submerged fermentation.

3.7 Agro-industrial biomass hydrolysis

The enzymatic hydrolysis process was conducted based on Wang et al. (2010) study with some modifications. The residues were autoclaved at 121 ºC for 30 min before the hydrolysis as well as the miliQ water used for the process. For 1 L hydrolysate, the solid loading was 1:10 (w v^{-1}) while the commercial protease from *Aspergillus oryzae* (Sigma-Aldrich, Merck, Germany) was used with a biomass:enzyme loading ratio of 20:1 (w v^{-1}). The operating conditions were 55 ºC, 24 h and 200 rpm (Figure 3.11a). Following enzymatic hydrolysis, the residual solids were separated through centrifugation at 7500 rpm for 20 min at 4 ºC (Figure 3.11b). The resulting supernatant was collected, characterized

(sections 3.10.3-3.10.6) and subsequently frozen at -20 ºC until it was utilized in the submerged fermentation (SmF) process.

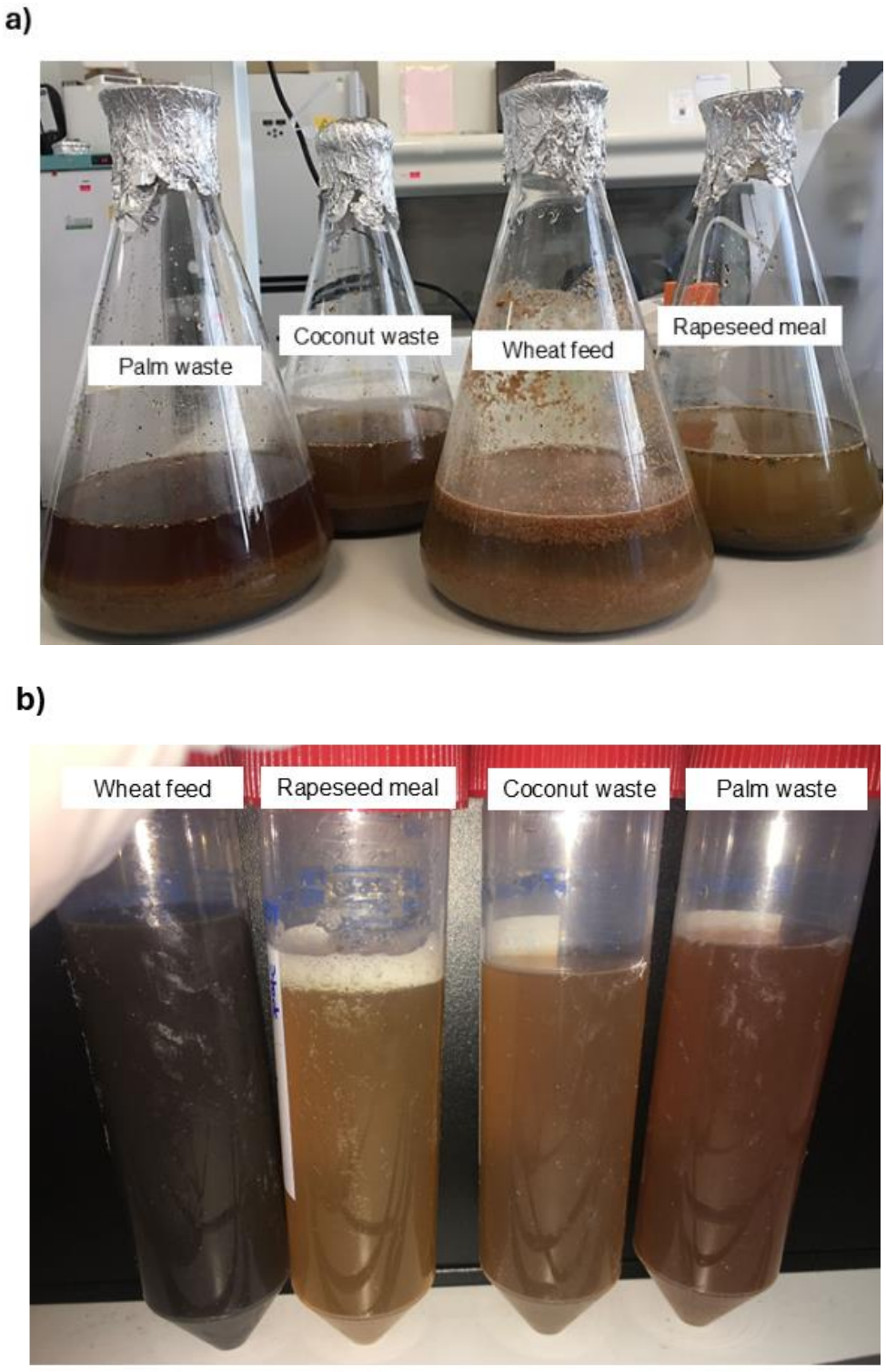

Figure 3.11 Agro-industrial residues hydrolysis. a) Initial samples and b) Hydrolysates appearance after 24 h and centrifugation.

3.8 Submerged Fermentation: set-up

3.8.1 *SmF: Erlenmeyer scale*

The experiments described in this section were carried out using 4-bottom baffled 250 mL Erlenmeyer flasks with a total working volume of 50 mL (Figure 3.12). Fermentation media composition was based on Ingham et al. (2022) as a starting point. Two control groups were set up with a composition of 100 g L^{-1} glucose as the hydrophilic carbon source, 100 g L^{-1} rapeseed oil as the hydrophobic carbon source and a target 0.15 g L^{-1} of total nitrogen (TN). Nutrient stock solutions were prepared and autoclaved separately to avoid inhibitor formation. The first control group (Control 1) contained $(NH_4)_2SO_4$ and CSL as nitrogen sources while the second (Control 2), contained $(NH_4)_2SO_4$ without CSL. Both control groups were set up for the experiments described in Chapter 6.

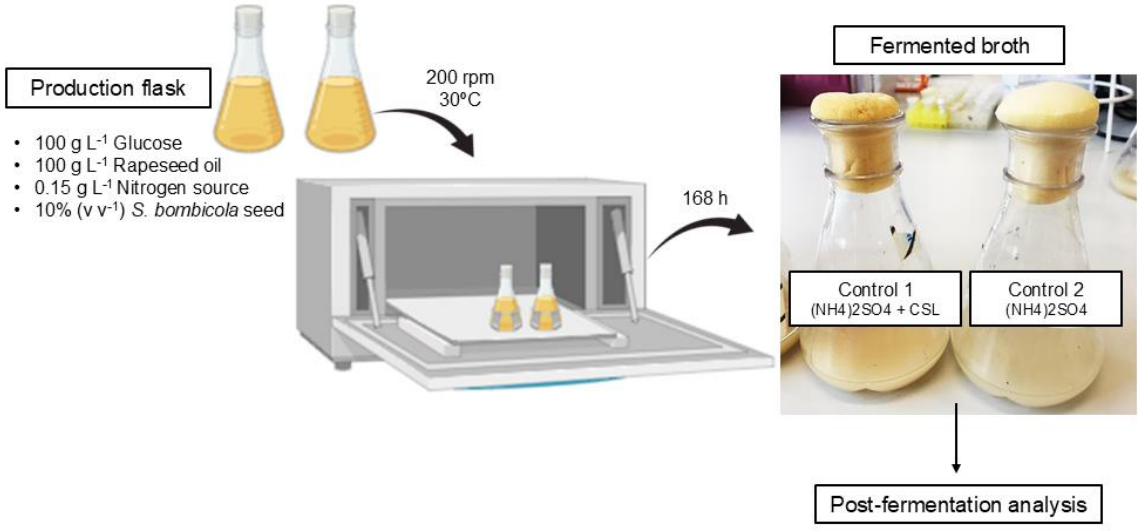

Figure 3.12 Fermentation process at Erlenmeyer flask scale.

Then, $(NH_4)_2SO_4$ and CSL were substituted by agro-industrial hydrolysates. Fermentation media combinations were sterilized via vacuum filtration using 0.45-µm aPES membrane filters (Nalgene, Thermo Scientific, UK). Following

filtration, sterile conditions were maintained as rapeseed oil and *S. bombicola* seed 10% (v v^{-1}) ratio were added. Finally, production flasks were incubated in an orbital shaker (Infors HT, UK) at 30 ºC, 168 h and 200 rpm as described by Wongsirichot et al., 2022a and Ingham and Winterburn, 2022 (Figure 3.13). After this time, samples were kept at -20 ºC for post-analysis process. Finally, production flasks were incubated in an orbital shaker (Infors HT, UK) at 30 ºC, 200 rpm for 168 h. After this time, samples were kept at -20 ºC for post-analysis processes.

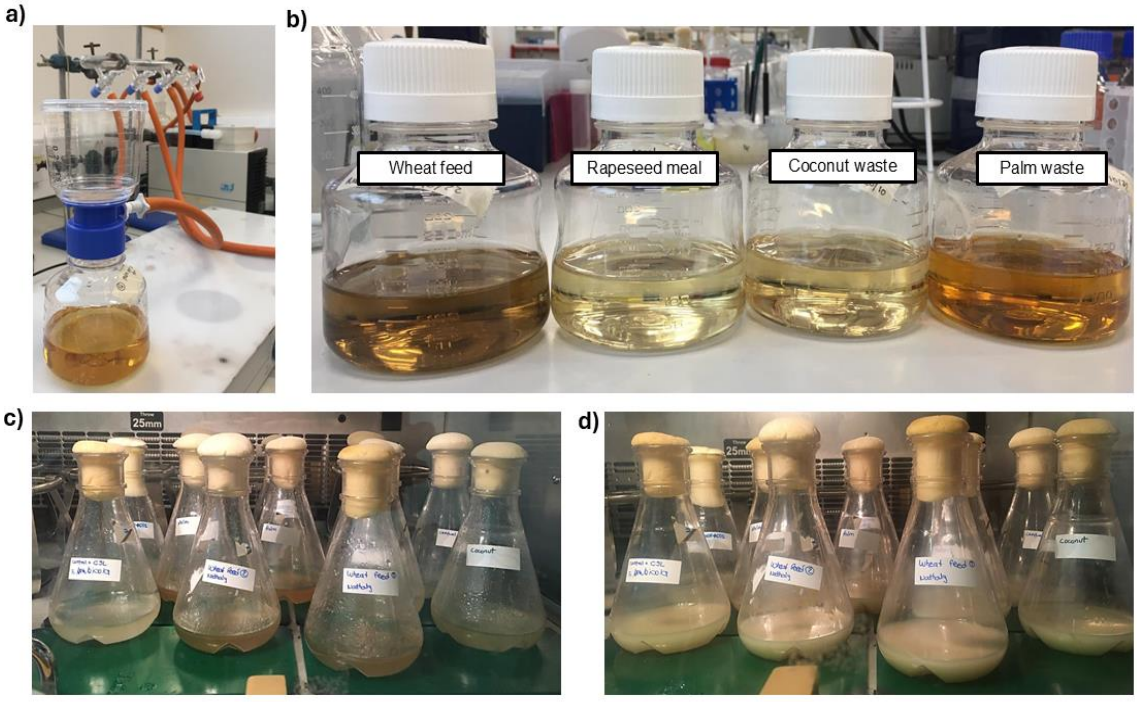

Figure 3.13 Screening of hydrolysates as nitrogen sources in SmF. a) Fermentation media vacuum filtration, b) Filtrated media appearance without rapeseed oil, c) Erlenmeyer set-up and d) Fermented broth appearance after 168 h.

3.8.2 *SmF:2 L bioreactor system*

Process scale-up was conducted using a 2 L bioreactor (BioStat® B, Sartorius, Germany) with a working volume of 1.8 L, in the facilities of the *Planta Pilot de Fermentació, Escola d'Enginyeria UAB*. The fermentation media

consisted of 100 g L^{-1} glucose and 100 g L^{-1} rapeseed oil as carbon sources while the nitrogen source was derived from a second batch of WF hydrolysate, providing a TN concentration of 0.31 g L^{-1} (Figure 3.14).

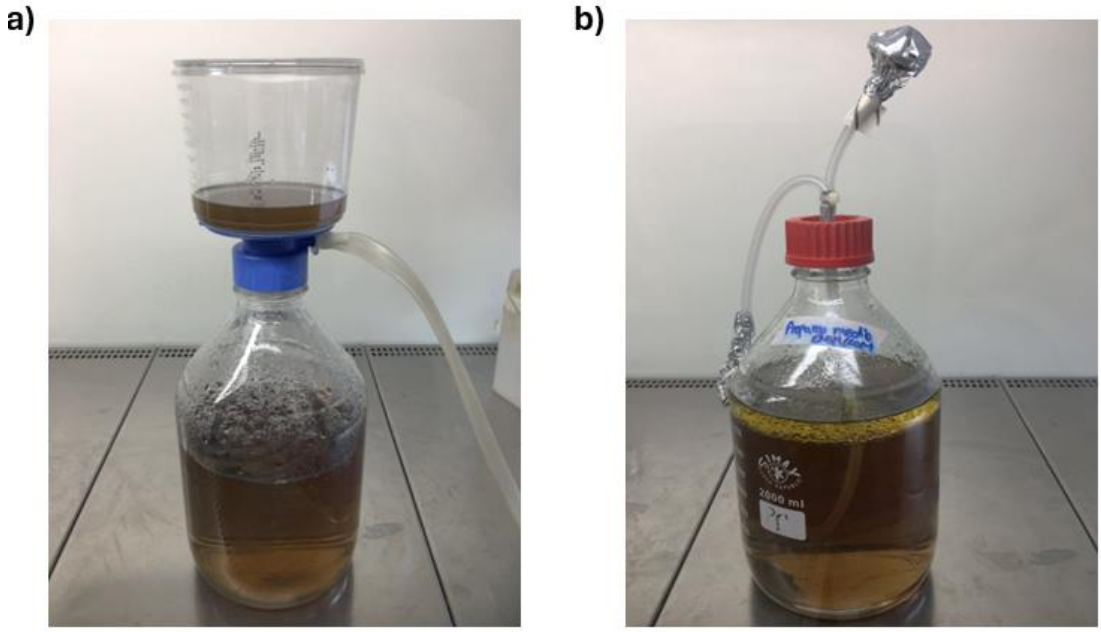

Figure 3.14 Wheat feed hydrolysate used for 2 L scale-up. a) Hydrolysate vacuum filtration and b) Fermentation media appearance.

The fermentation process was developed following Wongsirichot et al. (2022a) methodology with modifications. The operational parameters established were temperature 30 ºC, airflow rate 2 L min^{-1}, dissolved oxygen level was consistently maintained above 30% and regulated by adjusting the agitation speed within the range of 200 to 800 rpm. The pH was allowed to naturally decrease until reaching 3.5, after which it was regulated to the setpoint by the automatic addition of NaOH (35 % w v^{-1}) or H_2SO_4 (2 mM) as needed. An antifoam agent (Glanapon 2000 KONZ, Bussetti, Austria) was used when necessary to control foam formation (Figure 3.15). Finally, the fermentation time was 168 h and samples were aseptically withdrawn at regular 24 h intervals for subsequent post-fermentation analysis.

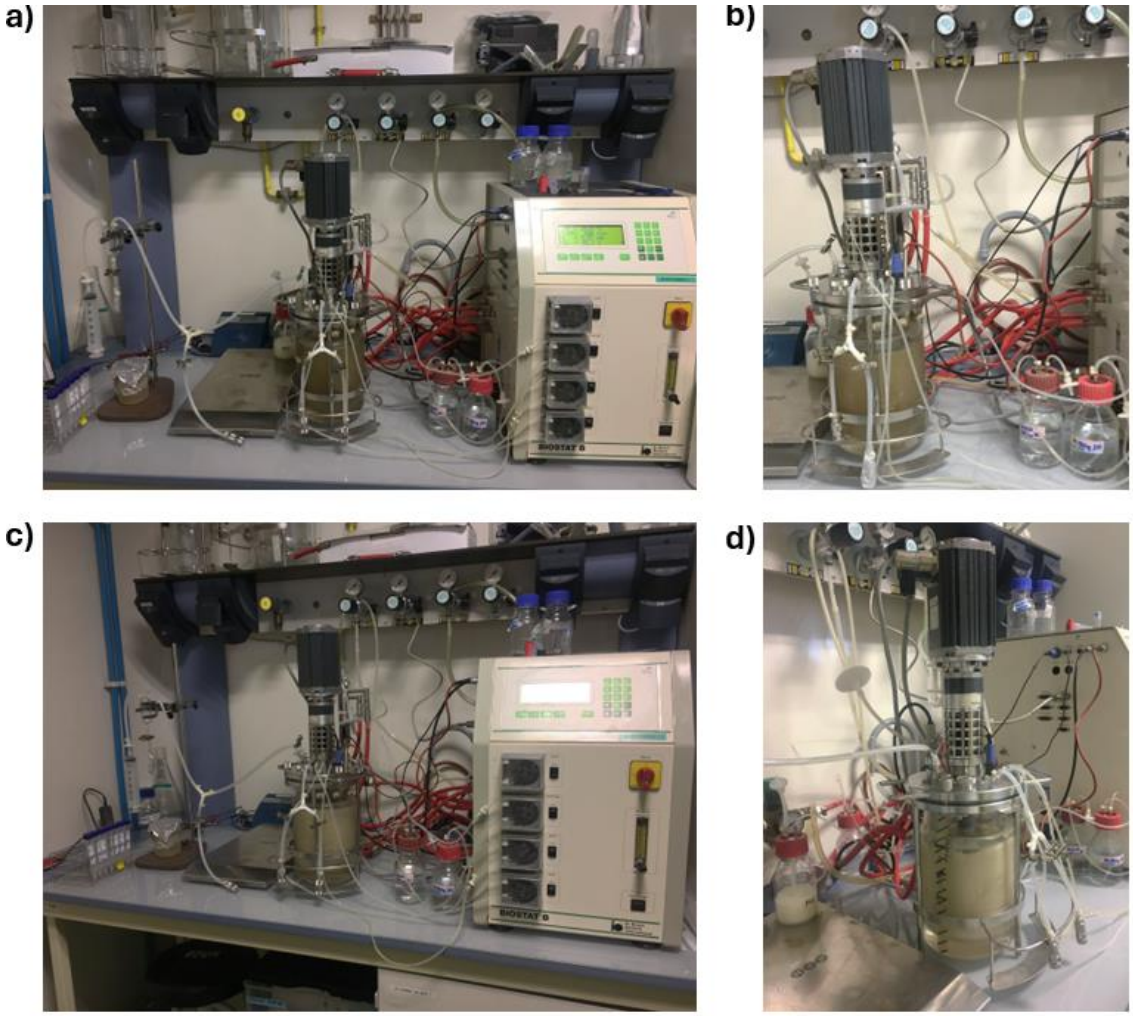

Figure 3.15 Batch bioreactor (2 L) used for SLs production using wheat feed hydrolysate as nitrogen source. a) Bioreactor set-up, b) Initial production media, c) Bioreactor after 168 h and d) Fermented broth appearance after 168 h.

3.9 Submerged Fermentation: monitoring methods

3.9.1 *Sophorolipids crude extraction and oil recovery from a fermented broth*

Oil recovery and SLs crude extract quantification were performed sequentially using n-hexane/ethyl acetate triple extractions following the method described by Wongsirichot et al. (2022a) with some modifications. The procedure combined equal volumes of fermented broth and the corresponding solvent (Figure 3.16). First, to quantify residual oil an equal volume of n-hexane (5 mL) was added to the sample (5 mL), followed by vigorous mixing through vortex

agitation at 2000 rpm for 1 min. Phase separation was facilitated by centrifuging the sample at 4000 rpm for 3 min, after which the top layer was carefully transferred into a pre-dried (105 ºC for 24 h) and labeled aluminum tray. The process was repeated two more times with a final organic phase volume of 15 mL in the aluminum tray. Once the n-hexane extraction was completed, the same procedure was repeated with the remaining sample using ethyl acetate for SLs quantification.

Subsequently, the organic phases on the aluminum trays (one for n-hexane and one for ethyl acetate) were air-dried overnight at room temperature. Finally, gravimetric measurements were then conducted to determine the concentrations of remaining oil and SLs crude extract in the hexane and ethyl acetate extractions, respectively. Calculations were developed as stated in Equation 10.

$$Oil\ residue\ or\ SLs\ crude\ extract\ (g\ L^{-1}) = \frac{W_{Tf} - W_{Ti}}{0.05} \quad \text{(Eq.10)}$$

Where: W_{Tf}, Final aluminum tray weight (g); W_{Ti}, Initial pre-dried aluminum tray weight (g); 0.05, fermented broth volume (L).

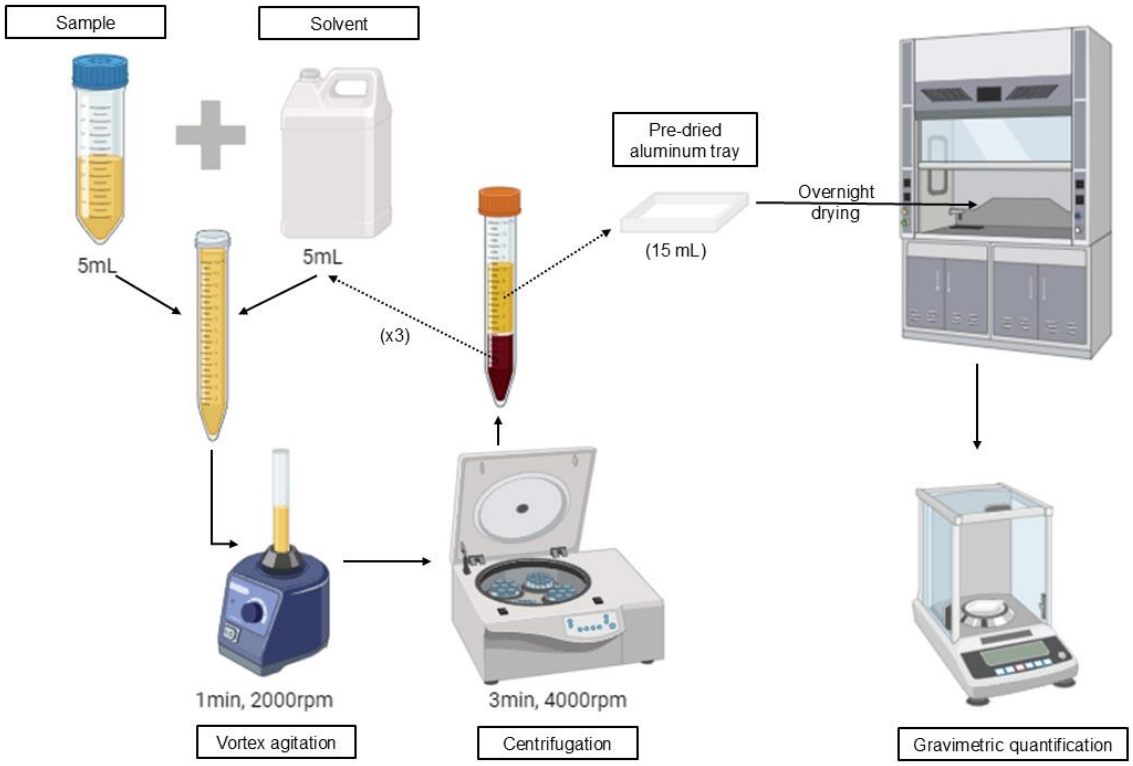

Figure 3.16 Solvent extraction scheme for oil and SLs crude extract obtention.

3.9.2 *Cell dry weight analysis*

After solvents extraction, the remaining aqueous phase was subjected to centrifugation at 7000 rpm for 15 min. Then the resulting pellet was washed twice with Ringer solution and centrifuged at the same conditions. Finally, the clean pellet was resuspended in 2 mL of miliQ water and placed on a pre-dried aluminum tray that was further dried at 105 ºC during 24 h for cell dry weight gravimetric analysis (Figure 3.17). Calculations were developed using Equation 11.

$$Cell\ dry\ weight\ (g\ L^{-1}) = \frac{W_{Tf} - W_{Ti}}{0.05} \quad \text{(Eq.11)}$$

Where: W_{Tf}, Final aluminum tray weight (g); W_{Ti}, Initial pre-dried aluminum tray weight (g); 0.05, fermented broth volume (L).

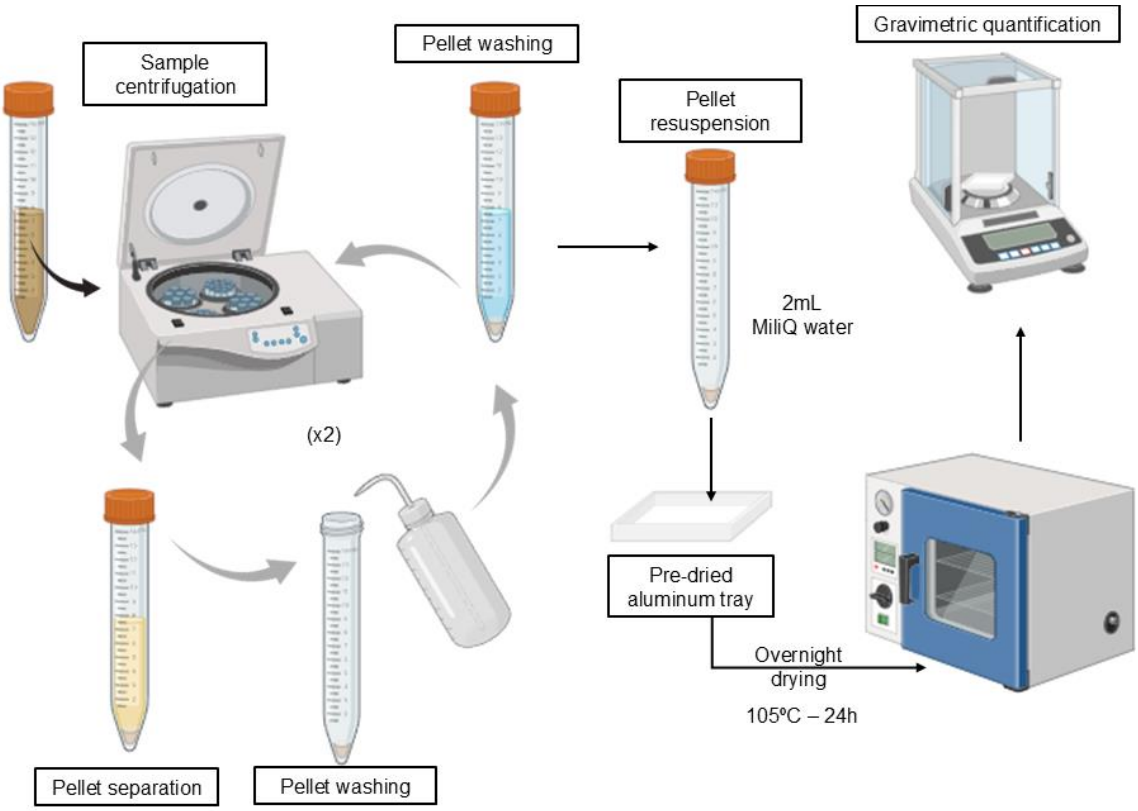

Figure 3.17 Cell dry weight process scheme.

3.10 General analytical methods

3.10.1 *High-performance chromatography for SLs quantification*

Based on SLs crude extract analysis, SLs mixture and diacetylated lactonic C18:1 (LC18:1, 2ac) quantification was developed following Ingham and Winterburn (2022) with some modifications. SLs molecules were separated in the HPLC UltiMate™ 3000 system (Thermo Fisher Scientific, Spain) using the Nucleosil™ 100 × 3 × 4.6 mm C18 EC column (Phenomenex, United States). The method conditions were a flow rate of 1.4 mL min^{-1}, column temperature of 45 °C and injection volume of 10 µL measured using a UV visible diode array detector at a spectrum of 198 nm. A solution of acetonitrile/water, both supplemented with 0.1% formic acid, was used as the mobile phase. The elution gradient was set at 70:30 for 10 min, followed by a linear gradient up to 10:90 in 50 min; this ratio

was maintained for an additional 10 min, after which it was set back to 70:30 for 15 min to restore initial conditions. Moreover, to identify the compounds by their mass/charge (m/z), samples after the HPLC-UV were ionized by electrospray (in the negative mode) and were analyzed using a MicroTOF-Q II mass spectrometer (Bruker, United States) coupled to the equipment. Finally, the calibration curve concentration (Figure 3.18) ranged from 2.5 to 20 g L^{-1} of the standard 1′,4″-sophorolactone 6′,6″-diacetate with a purification of ≥80% (Cayman Chemical, United States).

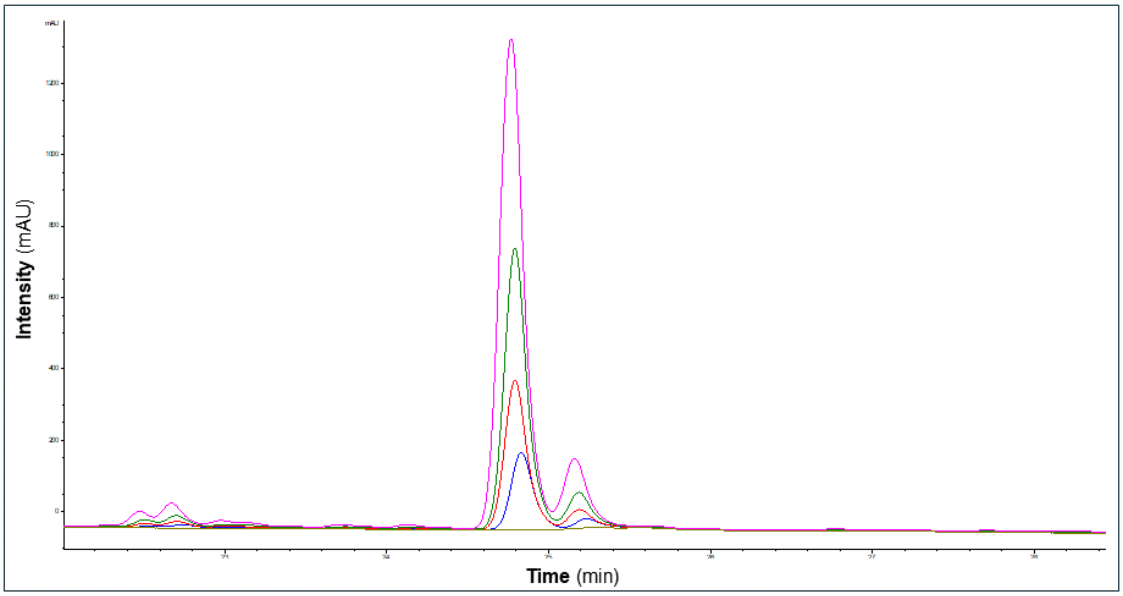

Figure 3.18 Diacetylated lactonic C18:1 calibration curve.

Besides diacetylated lactonic C18:1 quantification, SLs mixture present in the crude extract (g g^{-1}) is also reported in Chapter 4 and Chapter 5. This is done by summing the chromatogram areas obtained between 10 and 28 min of retention time and comparing them with the SLs area present in the commercial standard (Figure 3.19).

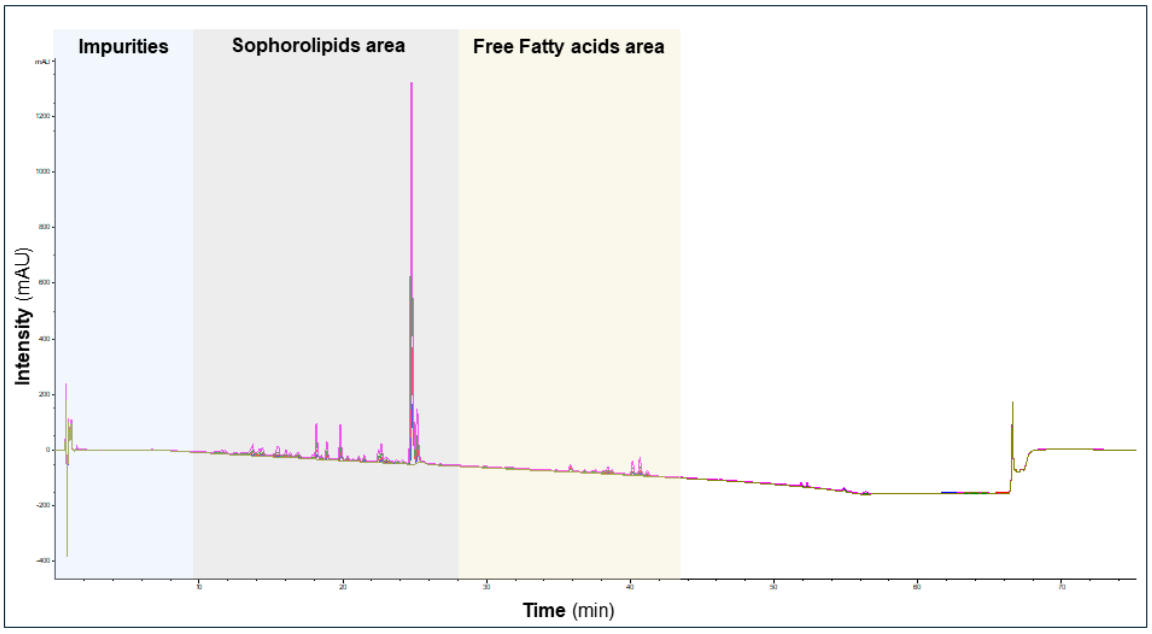

Figure 3.19 Chromatogram appearance of the standard. In blue, impurities area (0-9min); in grey, sophorolipids area (10-28 min) and in yellow, free fatty acids area (29-42 min).

For the analysis, samples were prepared and quantified according to their nature as follows:

- *Sample preparation from a fermented solid*

After gravimetric quantification, 20 mg of SLs crude extract were diluted in 1 mL of ethanol (10 g L^{-1}). After vortexing, the solution was heated at 60 °C for 15 min to dissolve the lactonic SLs. Then, samples were filtered in a glass vial using a 0.22-µm Nylon syringe filter. Diacetylated lactonic C18:1 quantification and productivity were calculated as started in equation 12 and 13.

$$Diacetylated\ lactonic\ C18{:}1\ (Total\ g\ or\ g\ g^{-1}DM_i) = A_q\ x\ 0.1\ x\ SLs\ crude\ extract \quad \text{(Eq.12)}$$

Where: $Diacetylated\ lactonic\ C18{:}1$ (total g); A_q, area quantification based in gram of LC18:,2ac per gram of SLs crude extract (g g^{-1}); 0.1, dilution factor (20

mg of crude extract in 2 mL ethanol); $SL\ crude\ extract$ (total g or g g^{-1} DM_i) as detail in Eq.6 and Eq.7.

$$Diacetylated\ lactonic\ C18{:}1\ productivity\ (g\ L^{-1}h^{-1}) = \frac{total\ g}{V_w\ x\ t}$$

(Eq.13)

Where: $Diacetylated\ lactonic\ C18{:}1\ productivity$ (g L^{-1} h^{-1}), total grams of diacetylated lactonic C18:1 per liter per hour, $Diacetylated\ lactonic\ C18{:}1$ (total g, Eq.12); V_w, working volume (L) for reactor (0.38 L) or Erlenmeyer (0.18 L); t, fermentation time (h).

- *Sample preparation from fermented broth*

Frozen samples were thawed at 60 ºC for 15 min to dissolve any SL crystallized during storage. Subsequently, the fermentation broths were subjected to high-speed vortexing at 2000 rpm for 30 s. To ensure a representative sample and prevent separation of the lipid/water-soluble phases, a 2.5 mL sample was collected immediately after vortexing whilst the broth was still agitated. Next, 6 mL of pure ethanol were added to the sample, followed by vortexing at the same conditions. After which the mixture was centrifuged at 7000 rpm for 10 min and the supernatant was carefully collected. Finally, it was filtered through a 0.22-µm Nylon syringe filter. Quantification and productivity were calculated as followed:

$$Diacetylated\ lactonic\ C18{:}1\ concentration\ (g\ L^{-1}) = A_q\ x\ 3.4$$

(Eq.14)

Where: $Diacetylated\ lactonic\ C18{:}1$ (g L^{-1}); A_q, area quantification based on LC18:1,2ac in the sample (g L^{-1}); 3.4, dilution factor (2.5 mL fermented broth in 6 mL of ethanol).

$$Diacetylated\ lactonic\ C18{:}1\ productivity\ (g\ L^{-1}h^{-1}) = \frac{C}{t}$$

(Eq.15)

Where: $Diacetylated\ lactonic\ C18{:}1\ productivity$ (g L^{-1} h^{-1}), grams per liter per hour; C, represents $diacetylated\ lactonic\ C18{:}1\ concentration$ (g L^{-1}, Eq.14); t, fermentation time (h).

3.10.2 *Solids characterization*

Substrate physiochemical characterization parameters such as moisture content (MC), dry matter (DM) and organic matter (OM) were measured according to standard methods (Thompson et al., 2001).

- *Moisture and Dry matter*

First, a specific amount of the solid sample (around 5-10 g) was placed in a pre-dried and weighed crucible. The filled crucible was then weighed and placed in an air oven for 24 h at 105 °C. After this time, the crucible containing the dry sample was weighed to calculate the quantity of water evaporated using Equation 16. Additionally, the DM content was calculated by subtracting the MC as stated in Equation 17.

$$Moisture\ (MC\%) = \frac{W_{Ci} - W_{Cf}}{W_{Ci} - W_{Ce}}\ x\ 100$$

(Eq. 16)

$$Dry\ matter\ (DM\%) = 100 - MC$$

(Eq. 17)

Where: W_{Ci} , crucible and wet sample initial weight (g); W_{Cf},crucible and dry sample final weight (g); W_{Ce}, empty crucible weight (g).

- *Organic matter*

For OM, the dried sample was weighed (2-3 g) in a pre-dried and weighed crucible. Then, the crucible with the sample was incinerated at 550 °C for 4 h. After this time, the crucible was allowed to cool and the crucible with the ashes was weighed to determine the organic matter (OM) using Equation 19.

$$Organic\ matter\ (OM\%) = \frac{W_{Cd} - W_{Ca}}{W_{Cd} - W_{Ce}} x\ 100$$

(Eq. 19)

Where: W_{Cd} , crucible and dry sample initial weight (g); W_{Ca},crucible and ashes final weight (g); W_{Ce}, empty crucible weight (g).

- *Elemental analysis*

A dry and ground sample was sent to the *Servei d'Anàlisi Química UAB* for elemental determination. Quantification was performed by gas chromatography using a CHNS elemental analyzer Flash 2000 (Thermo Scientific) after sample combustion at 1200 °C with an excess of air.

3.10.3 *pH*

For pH determination, liquid samples were analyzed directly, while solid samples underwent an aqueous extraction (1:5, w v^{-1}). Approximately 5-10 g of the solid sample was placed in a clean flask and mixed with Milli-Q water. The

mixture was then shaken at 25 °C and 200 rpm for 30 min to solubilize the salts present in the solid. Finally, the pH of the supernatant was measured using a pH meter (Crison®, micropH2001).

3.10.4 *Total carbon and total nitrogen*

The analysis of TC and TN was carried out using the multi-N/C 2100S analyzer (Analytik Jena, INYCOM, Instrumentación y Componentes, S.A, Spain) (Figure 3.20). The liquid sample was introduced into the analyzer, where it undergoes high-temperature combustion in the presence of a catalyst and oxygen to oxidize the carbon compounds and convert them into carbon dioxide (CO_2). For the measurement of inorganic carbon (TIC), the sample was acidified before combustion, releasing CO_2 from carbonates and bicarbonates. The CO_2 released from both organic (TOC) and inorganic (TIC) was then summed and reported as total carbon content (TC). Moreover, during the combustion, nitrogen compounds are also oxidized by converting them into nitrogen oxides (NOx), which were subsequently reduced to nitrogen gas (N_2) allowing total nitrogen (TN) quantification.

Samples were prepared as described below based on its nature:

- *For solid samples*

Solid-liquid water extraction was performed (1:10, w v^{-1}) at 200 rpm for 15 min. Then, the extracts were filtered using a 0.22-µm membrane filter. When it was required, previous filtration, samples were centrifuged at 7000 rpm for 15 min.

- *For liquid samples*

The liquid sample underwent centrifugation at 7000 rpm for 15 min. Then the pellet was discarded and 0.1 mL of the supernatant was diluted in 20 mL of miliQ water followed by membrane filtration using a 0.22-µm membrane filter.

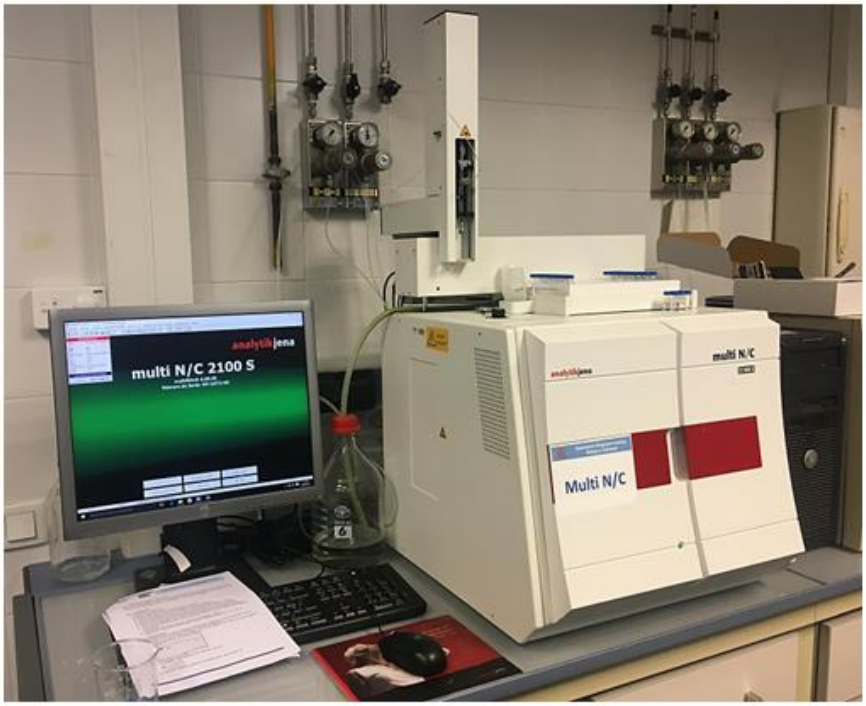

Figure 3.20 Multi N/C 2100S equipment for TC and TN quantification.

3.10.5 *Glucose content*

Glucose was quantified using YSI 2950D biochemistry analyzer (YSI Inc./Xylem Inc., United States). Sugar extracts were diluted to fit in the equipment calibration range (0.05-25 g L^{-1}). The extracts were obtained as described below:

- *For solid samples*

A solid-liquid extract (1:10, w v^{-1}) was prepared at 200 rpm, 50 ºC for 15 min and the supernatant was filtered using a 0.45-µm RC membrane filter.

- *For liquid samples*

The liquid sample underwent centrifugation at 7000 rpm for 15 min. Then the supernatant was filtered using a 0.45-µm RC membrane filter.

3.10.6 *Sugar profile (HPLC)*

The sugar profile was specifically used for hydrolysate characterization. The analysis was performed via HPLC (Ultimate 300, Thermo Fisher Scientific) using a CarboSep CHO 782 lead form 300 mm × 8 µm × 7.8 mm column (Concise Separations, USA), coupled to a refractive index detector (RI). A constant flow rate of HPLC grade water at 0.6 mL min^{-1} was used for 30 min with a column temperature maintained at 70 ºC. Subsequently, samples were compared with a calibration curve for nine different mono- and disaccharides (0-20 g L^{-1}).

3.11 Specific methodologies

3.11.1 *Experimental design*

The Design Expert 12® program (Stat-Ease, Inc, United States) was used to generate a DoE (Chapter 4). Glucose, urea and fermentation time were chosen as factors and were tested at three different levels low, medium and high. As outcomes, diacetylated lactonic C18:1 production (total g); diacetylated lactonic C18:1 productivity (g L^{-1} h^{-1}) reported by working volume (0.18 L) and yeast growth (total CFU) were evaluated using a BBD (Box and Behnken, 1960). The total set-up was developed at scale-down (section 3.3.1) and consisted of 33 runs setting a triplicate in the central point for each fermentation time for pure error estimation.

Equation 20 shows a second-order polynomial model that was fitted for each response result. The fit significance of the model equations was evaluated using the statistical analysis of variance (ANOVA) with a p-value below 0.05. The fit quality of the quadratic model was expressed by the coefficient of determination (R^2) and their prediction capability by the predicted R^2.

$$y = \beta o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j$$

(Eq. 20)

Where: y is the predicted response; βo is model constant and β_i, β_{ii}, β_{ij} are regression coefficients of linear, quadratic and cross-product terms; X_i and X_j are coded independent variables.

Optimum ratio predictions were generated focusing on maximizing diacetylated lactonic C18:1 production and productivity. The experimental and predicted response values were compared and the predictive capability of the model was assessed. Multiple regression analysis was applied to analyze the variables by obtaining a regression equation that could predict the response within the specified range. To verify the obtained models, the best ratio combination was assayed by triplicate using the same set-up system.

3.12 Statistical analysis and Software used throughout the thesis

One-way ANOVA and Tukey's tests were used to analyze the statistical differences between treatments (α = 0.05) while Pearson's correlation was used to investigate the relationship between the studied variables. Data analysis was performed using Minitab 17 statistical software (Minitab Inc.). Additionally, the Design Expert 12® program (Stat-Ease, Inc., United States) was used for

experimental design and JMP® 15 statistical software (JMP Statistical Discovery LLC, USA) was used to analyze SmF results. SigmaPlot 12.5 (Systat Inc., USA) and Biorender (https://www.biorender.com) were used throughout the thesis for self-made figures.

Chapter 4

Experimental Design and alternative hydrophilic and nitrogen sources in SSF

Part of this chapter has been presented as a poster at the *Biosurfactants International Conference*, Stuttgart-Germany.
Optimization of carbon and nitrogen sources for solid-state sophorolipid production.
Eras-Muñoz, E., Font, X., Barrena, R. and Gea,T. (2022)

Contents of this chapter were published in *Frontiers in Bioengineering and Biotechnology*, 11, 1252733.
Carbon and nitrogen optimization in solid-state fermentation for sustainable sophorolipid production using industrial waste.
Eras-Muñoz, E., Gea, T. and Font, X. (2024a).

Summary

The use of alternative feedstocks (to pure substrates) such as industrial or food waste is being explored for the sustainable production of SLs. Applying the advantages that SSF offers and with the aim of revalorizing industrial organic waste, the impact of carbon and nitrogen sources on the relationship between yeast growth and SL production is analyzed in this Chapter. The laboratory-scale system used winterization oil cake as the solid waste for a hydrophobic carbon source. Pure hydrophilic carbon (glucose) and nitrogen (urea) sources were used in a Box–Behnken statistical design of experiments at different ratios by applying the response surface methodology. Optimal conditions to maximize the production and productivity of diacetylated lactonic C18:1 were a glucose:nitrogen ratio of 181.75:1.43 (w w^{-1} based on the initial dry matter) at a fermentation time of 100 h, reaching 0.54 total gram of diacetylated lactonic C18:1 with a yield of 0.047 g per gram of initial dry mass. Moreover, fermentation time under optimized conditions increased the SLs crude extract and diacetylated lactonic C8:1 production by 22% and 30%, respectively, when compared to reference conditions. After optimization, industrial wastes were used to substitute pure substrates. Different industrial sludges, OFMSW hydrolysate and sugar candy industry wastewater provided nitrogen, hydrophilic carbon and micronutrients, respectively, allowing their use as alternative feedstocks. Sugar candy industry wastewater and cosmetic sludge are potential hydrophilic carbon and nitrogen sources, respectively, for sophorolipid production, achieving yields of approximately 70% when compared to the control group. Figure 4.1 illustrates the chapter scheme.

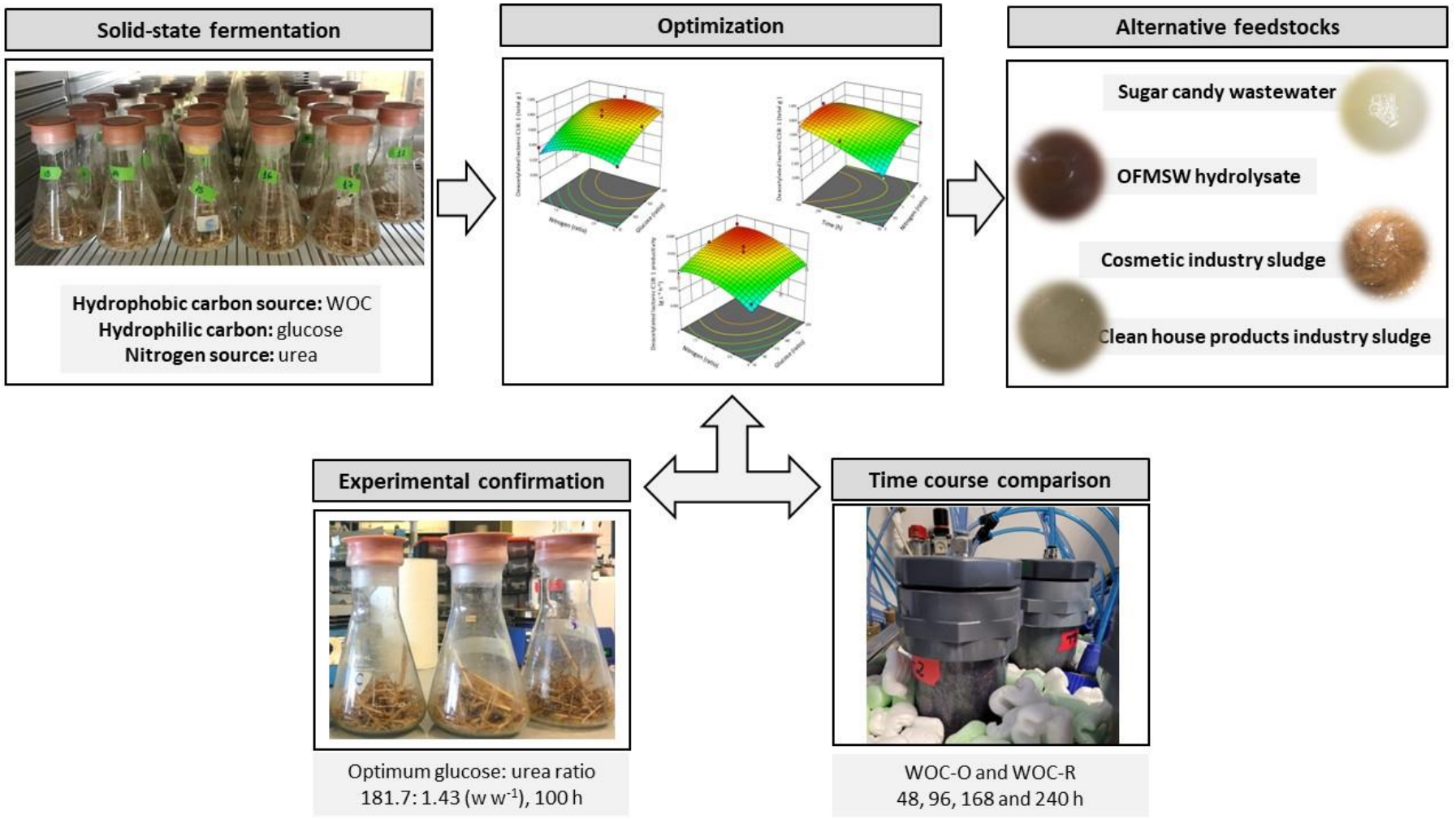

Figure 4.1 Schematic overview of the chapter.

4.1 Introduction

The application of statistical design of experiments (DoE) is widely used for biological process optimization and has already been used for biosurfactant production (Zhu et al., 2013; Minucelli et al., 2017). DoE provides an understanding of the interactions between factors (medium components) at different levels (concentration/ratio) and their effect on the evaluated output. The variables that are found significant and fit a statistical model (linear, quadratic and cubic curvature, among others) can be further optimized using the response surface methodology (RSM) (Rispoli et al., 2010). Since there are a large number of possible combinations to be tested when DoE is applied, the use of bioreactors is a limitation to the analysis being the shake flask scale the methodology reported in the literature (Ingham and Winterburn, 2022).

DoE was applied in this work to enhance our knowledge on how certain factors influence the production of SLs in SSF. It is crucial to evaluate the influence of nutrient sources on yeast growth, the production process and the final product composition. In this regard, this chapter aims to evaluate the influence of hydrophilic carbon (glucose) and nitrogen (urea) sources ratio on SLs crude extract and diacetylated lactonic C18:1 (LC18:1, 2ac SL) production. Our hypothesis is based on balancing nitrogen to ensure suitable cell growth levels and optimal SLs productivity. Then, to evidence the production increase, a time course comparison between the optimum ratio (WOC-O) and the established in previous works by the group (WOC-R) was developed at 0.5 L bioreactor. Finally, pure substrates were substituted by multiple industrial and municipal waste as

novel sources of hydrophobic carbon, hydrophilic carbon, nitrogen and micronutrients for SLs production.

4.2 Materials

As detailed in section 3.1.2, pure substrates such as glucose, urea and yeast extract were used. Then to substitute pure substrates, different organic industrial wastes were used as feedstock. Sugar candy industry wastewater (RSC), hydrolysate of organic fraction of municipal solid waste (ROF) and two nitrogenous sludges, one from the cosmetic industry (RHP) and one from the household cleaning production (RAC). Moreover, in all experiments wheat straw was used as support and winterization oil cake (WOC) as a hydrophobic carbon source. Residues were characterized as described in Chapter 3 (sections 3.10.2–3.10.5) and the obtained results are presented and discussed in section 4.4.7.

4.3 Experiments

Table 4.1 shows the factors and levels selected for the experimental design described in section 3.12. The levels were chosen due to reference conditions based on literature and previous works (Jimenez-Peñalver, 2017; Rodríguez, 2020).

Table 4.1 Selected factors and levels for the experimental design and the optimization process.

Parameter	Units	Level -1	Level 0	Level +1
Glucose	Ratio (w w^{-1})*	50	125	200
Urea	Ratio (w w^{-1})*	0	1	2
Time	Hour	96	168	240

*Ratio values were calculated based on the initial solid dry weight (10.8 ± 0.6 g).

Moreover, to compare the optimum glucose:nitrogen combination (WOC-O) and the reference ratio (100:1 w w^{-1}, WOC-R), the process was scaled up to 0.5 L packed bed bioreactors following section 3.3.2 with some modifications. The total weight of the solid substrate reached 77.42 g, with a dry matter content of 54.9% for WOC-O and 73.89 g with a dry matter content of 52.7% for the WOC-R. The bioreactors were sacrificed for analysis at 48, 96, 168 and 240 h and analyzed as described in section 3.4 and 3.10.1.

4.3.1 *Pure substrates substitution*

The second-generation feedstocks were dosed to achieve the optimal glucose and nitrogen levels dictated by the model obtained from the DoE. These experiments were designed based on the potential exhibited by alternative substrates such as carbon or nitrogen sources. To reach the optimal ratio conditions of the DoE, the solid matrix was supplemented with glucose and/or urea when necessary.

4.4 Results and Discussion

4.4.1 *Results from Experimental Design*

As mentioned earlier, the evaluation of the glucose as hydrophilic carbon source and urea as nitrogen source, was performed based on their weight ratio related to the initial total mixture dry weight. To screen potential alternative feedstocks for SLs production on SSF, a deep understanding of the media components, product range, as well as potential interactions is required. In this context, Table 4.2 shows the BBD matrix outcomes for the analyzed responses.

Table 4.2 Box-Behnken design matrix and observed responses: production and productivity of diacetylated lactonic C18:1 and yeast growth

Run	Combination	X_1: *Glucose*	X_2: *Urea*	X_3: *Time*	Diacetylated lactonic C18:1 production		Diacetylated lactonic C18:1 productivity	Yeast growth
		(ratio)	(ratio)	(h)	(total g)	(g g^{-1} DM_i)	(g L^{-1} h^{-1})	(total CFU)
1	+++	200	2	240	0.624	0.054	0.014	$4.57x10^{10}$
2	-++	50	2	240	0.408	0.040	0.009	$3.90x10^{10}$
3	-0-	50	1	96	0.396	0.039	0.023	$2.53\ x10^{10}$
4	+00	200	1	168	0.736	0.062	0.024	$3.36\ x10^{10}$
5	0--	125	0	96	0.242	0.022	0.014	$5.76\ x10^{10}$
6	+0+	200	1	240	0.866	0.073	0.02	$3.45\ x10^{10}$
7	0-+	125	0	240	0.735	0.067	0.017	$3.94\ x10^{10}$

8	--+	50	0	240	0.44	0.043	0.01	4.82 x10^{10}
9	000[a]	125	1	168	0.771	0.070	0.025	2.24 x10^{10}
10	0-0	125	0	168	0.45	0.041	0.015	3.22 x10^{10}
11	+0-	200	1	96	0.534	0.045	0.031	4.08 x10^{10}
12	+--	200	0	96	0.333	0.028	0.019	3.97 x10^{10}
13	++-	200	2	96	0.597	0.050	0.035	4.59 x10^{10}
14	-+0	50	2	168	0.409	0.040	0.014	2.08 x10^{10}
15	---	50	0	96	0.329	0.032	0.019	5.45 x10^{10}
16	-0+	50	1	240	0.438	0.043	0.010	2.32 x10^{10}
17	+-+	200	0	240	0.699	0.059	0.016	4.37 x10^{10}
18	000[b]	125	1	168	0.73	0.067	0.024	5.48 x10^{10}

19	000[c]	125	1	168	0.742	0.068	0.025	5.31×10^{10}
20	0+0	125	2	168	0.531	0.048	0.018	7.93×10^{10}
21	00+[a]	125	1	240	0.763	0.070	0.018	4.39×10^{10}
22	++0	200	2	168	0.626	0.053	0.021	3.78×10^{10}
23	-+-	50	2	96	0.311	0.030	0.018	3.07×10^{10}
24	--0	50	0	168	0.337	0.033	0.011	2.98×10^{10}
25	00-[a]	125	1	96	0.591	0.054	0.034	3.59×10^{10}
26	-00	50	1	168	0.338	0.033	0.011	2.50×10^{10}
27	00-[b]	125	1	96	0.587	0.053	0.034	2.78×10^{10}
28	00-[c]	125	1	96	0.537	0.035	0.031	4.99×10^{10}
29	0+-	125	2	96	0.529	0.048	0.031	2.58×10^{10}

30	+-0	200	0	168	0.685	0.058	0.023	6.53 x10^{10}
31	00+[b]	125	1	240	0.617	0.056	0.014	8.53 x10^{10}
32	00+[c]	125	1	240	0.838	0.076	0.019	5.70 x10^{10}
33	0++	125	2	240	0.528	0.048	0.012	4.57x10^{10}

Abbreviations: DM, initial dry matter; [a, b ,c] biological replicates at the central point for the corresponding fermentation time.

4.4.2 *Nutrients influence on sophorolipids production*

The applied levels of glucose, nitrogen and time yielded a range of diacetylated lactonic C18:1 quantity at harvest from 0.242 to 0.866 total gram, which corresponds to a yield from 0.022 to 0.073 g g^{-1} DM_i (Figure 4.2).

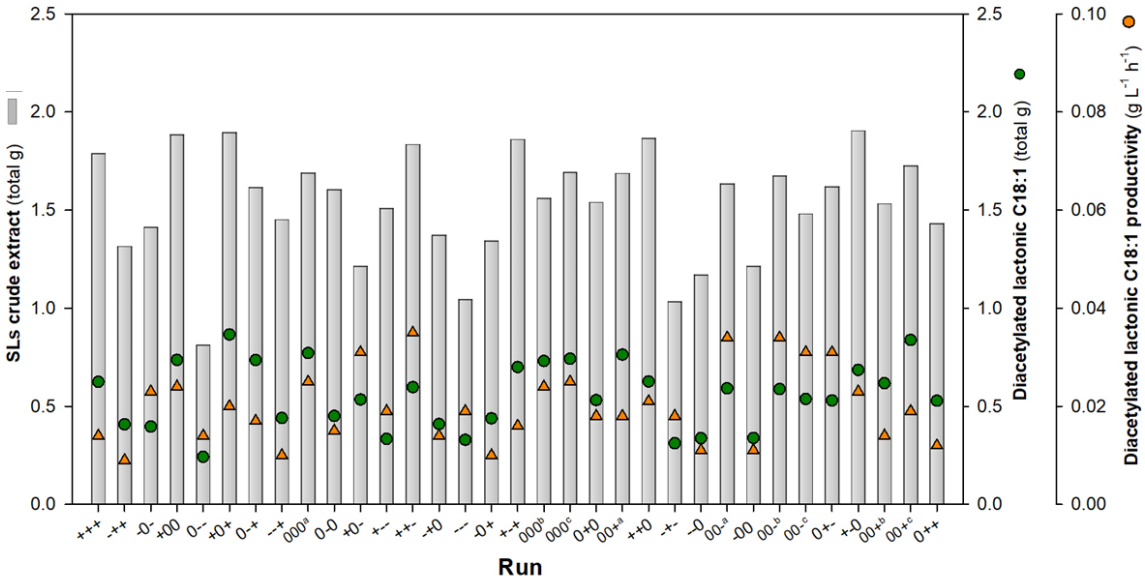

Figure 4.2 DoE outcomes based on glucose:nitrogen ratio and fermentation time. SLs crude extract and diacetylated lactonic C18:1 total gram were obtained from total initial solid wet weight (20.3 ± 0.63 g). Productivity values are expressed in working volume (0.18 L). [a, b, c] shows central point replicates.

The central point 000 (runs 9, 18 and 19) combined with a glucose:urea weight ratio of 125:1 at 168 h resulted in a mean of 0.748 ± 0.021 total grams of diacetylated lactonic C18:1, with a yield of 0.068 ± 0.002 g g^{-1} DM_i and a volumetric productivity of 0.025 ± 0.001 g L^{-1} h^{-1}. Moreover, the highest value of diacetylated lactonic C18:1 was achieved when a glucose:urea ratio of 200:1 was applied at 240 h (+0+). In contrast, the minimum value was achieved at 96 h, related to the combination of glucose:urea ratio of 125:0 (0--). Glucose would be depleted in the first 96 h in the combinations with a lower initial glucose ratio, showing a statistical decrease in SLs crude extract and diacetylated lactonic

C18:1 production, while high ratios increased productivity, which is aligned with the results obtained by Ingham and Winterburn (2022).

Considering the importance of time as a critical operation factor, the results were assessed at each fermentation time, recognizing that the duration required to reach the stationary phase is dependent on the medium concentration (Gao et al., 2013). The results revealed that runs 13 (++-, 0.597 total g), 9 (000, 0.771 total g) and 6 (+0+, 0.866 total g) exhibited the highest production levels at 96, 168 and 240 h, respectively. In addition, the highest productivity was achieved at 96 h by run 13 (++-), achieving 0.035 g L^{-1} h^{-1}. SLs crude extract production increased from 0.810 to 1.905 total g, with a yield range between 0.075 and 0.164 g g^{-1} DM_i. The literature shows that glucose is an important parameter for the SLs structure. When glucose is supplied, together with a hydrophobic carbon source, it is directly incorporated into the SL, although glucose is not taken from the fatty acid synthesis. In contrast, when the glucose concentration is low, part of the fatty acids will be used for cell maintenance rather than SLs synthesis being part of the fatty acids directed toward the β-oxidation (Hommel et al., 1994; Van Bogaert et al., 2007; Van Bogaert et al., 2016).

SLs production has been extensively reported based on crude extract both in SmF and SSF. Compared to the extraction of SLs from liquid matrices, downstream processing in SSF presents notable differences, primarily attributed to the unique characteristics of the involved solid fermented matrix which can decrease the efficiency of the n-hexane extraction process. Consequently, impurities can be present in the final crude product, affecting downstream process design and overall economic performance. Martínez et al., (2022)

described SLs recovery from the solid matrix as the major contributor to operating costs.

In this case, diacetylated lactonic C18:1 accounted for between 22-49% of total SLs crude extract. Literature reports that several enzymes are involved in the SLs metabolic pathway allowing the production of a mixture of more than 20 molecules. SLs can be classified into acidic and lactonic, being the diacetylated lactonic C18:1 the main molecule produced by *S. bombicola* when fed with a main hydrophobic carbon source rich in oleic acid (Van Bogaert et al., 2016; Roelants et al., 2019; Liu et al., 2020). Our findings are consistent with those reported by Jiménez Peñalver et al. (2020) in SSF, who documented a SLs crude extract containing a mixture of congeners primarily composed of diacetylated lactonic C18:1 when using WOC as hydrophobic carbon source. Thus, when considering the total equivalent area of SL congeners present in the crude extract, the total relative abundance of this congener could be estimated in 56-71% (Table 4.3).

Table 4.3 Production results based on SLs crude extract HPLC-UV quantification.

Run	Combination	SLs crude extract		SLs mixture present in the crude	Diacetylated lactonic C18:1 in the SLs crude extract
		(Total g)	(g g^{-1} DM_i)	(g g^{-1})	(%)*
1	+++	1.788	0.153	0.628	55.56
2	-++	1.315	0.131	0.518	59.93
3	-0-	1.412	0.141	0.460	60.92
4	+00	1.885	0.162	0.638	61.17

5	0--	0.810	0.075	0.495	60.40
6	+0+	1.895	0.163	0.709	64.50
7	0-+	1.614	0.149	0.660	68.97
8	--+	1.451	0.145	0.454	66.88
9	000[a]	1.690	0.156	0.679	67.18
10	0-0	1.604	0.148	0.445	62.99
11	+0-	1.213	0.104	0.648	67.99
12	+--	1.509	0.130	0.317	69.61
13	++-	1.834	0.157	0.488	66.68
14	-+0	1.372	0.137	0.458	65.09
15	---	1.044	0.104	0.447	70.54
16	-0+	1.343	0.134	0.505	64.50
17	+-+	1.860	0.160	0.670	56.09
18	000[b]	1.561	0.144	0.673	69.52
19	000[c]	1.691	0.156	0.640	68.62
20	0+0	1.540	0.142	0.529	65.18
21	00+[a]	1.687	0.156	0.689	65.59
22	++0	1.866	0.160	0.595	56.42
23	-+-	1.034	0.103	0.508	59.24
24	--0	1.170	0.117	0.431	66.97

25	00-[a]	1.634	0.151	0.535	67.63
26	-00	1.214	0.121	0.406	68.62
27	00-[b]	1.673	0.154	0.500	70.19
28	00-[c]	1.481	0.137	0.367	71.43
29	0+-	1.620	0.149	0.466	70.08
30	+-0	1.905	0.164	0.522	68.85
31	00+[b]	1.532	0.141	0.615	65.42
32	00+[c]	1.726	0.159	0.709	68.51
33	0++	1.431	0.132	0.591	62.43

*Percentage values were calculated based on SLs relative area on the crude extract. *Abbreviations:* DM_i, initial dry matter; [a, b,c] biological replicates at the central point for the corresponding fermentation time.

In contrast to the partially purified yellowish honey-like viscous product typically described in the literature and obtained in this study, pure SLs exhibits a colorless appearance and transforms into a white powder when completely dried (Claus and Van Bogaert, 2017; Jiménez-Peñalver et al., 2020; Kashif et al., 2022). This honey-like texture could indicate that the extract still contains impurities such as long-chain fatty acids (LCFA) originating from the WOC during fermentation. As the fermentation proceeds, higher SLs titers and lower LCFA concentrations are observed, thus increasing the purity of SLs crude extracts. Moreover, a variety of SLs congeners was observed (Figure 4.3).

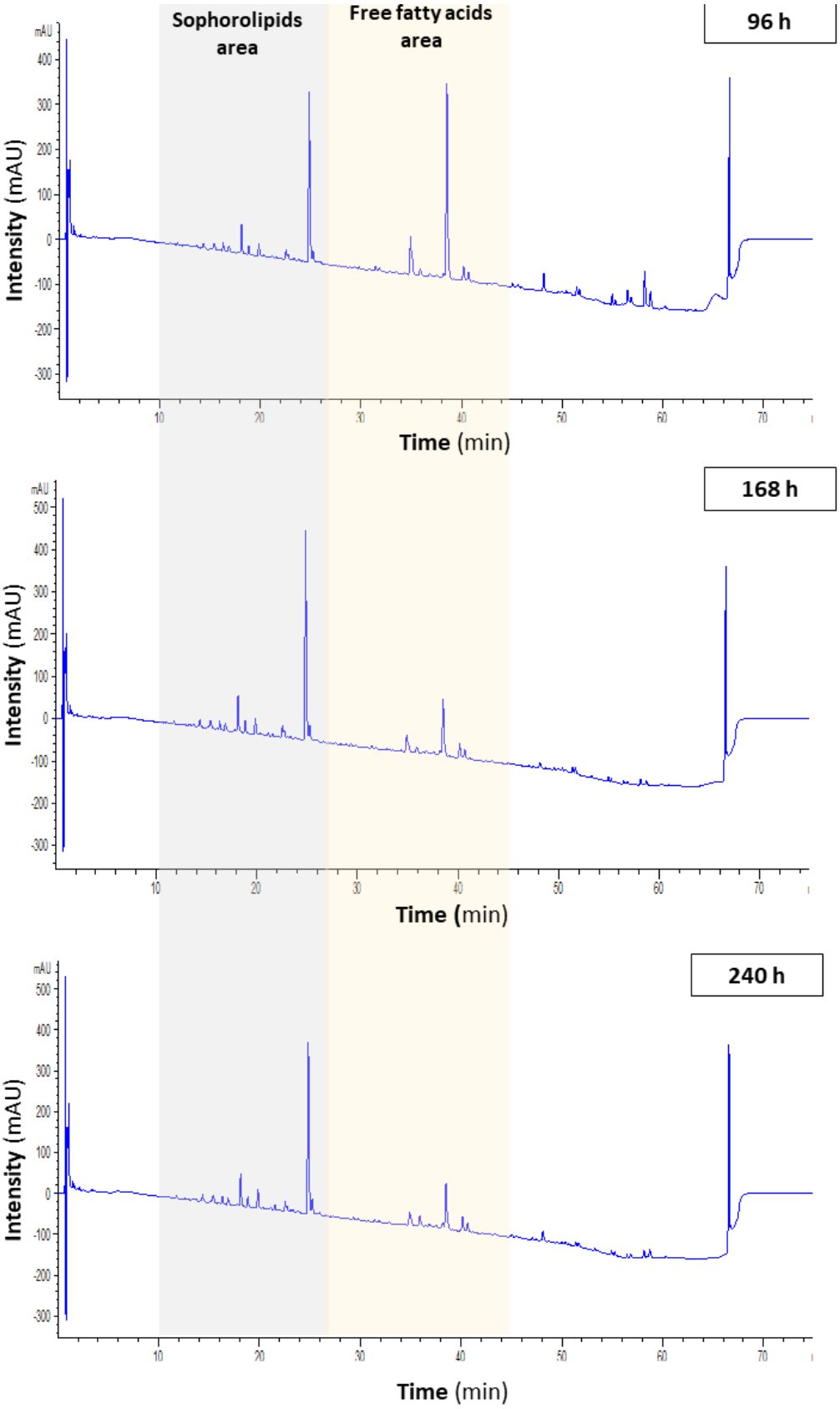

Figure 4.3 Central point HPLC-UV spectra at 198 nm. Sample belongs to a glucose:urea ratio of 125:1 (w w^{-1}) at 96, 168 and 240 h. Grey color shows SLs mixture relatively area in the crude extract while yellow represents the free fatty acids area.

4.4.3 *Diacetylated lactonic C18:1 production and productivity models and optimization*

The data obtained from the fermentation processes were analyzed using Design-Expert 12® software. The report of the diacetylated lactonic C18:1 measure was used in the design to clarify the process and the influence of the analyzed factors. Table 4.4 summarizes the ANOVA for the main responses used for the optimization process.

Table 4.4 ANOVA for the surface quadratic model when diacetylated lactonic C18:1 production and productivity were used as outcomes.

		Diacetylated lactonic C18:1 production (R^2 = 84.31%)		**Diacetylated lactonic C18:1 productivity** (R^2 = 86.23%)	
Source	**DF**	**Mean Square**	**p-value**	**Mean Square**	**p-value**
Model	9	0.0856	< 0.0001*	0.0002	< 0.0001*
X_1 -Glucose	1	0.2923	< 0.0001*	0.0003	< 0.0001*
X_2 -Nitrogen	1	0.0054	0.3610	0.0000	0.0679
X_3 -Time	1	0.1765	< 0.0001*	0.0007	< 0.0001*
$X_1 X_2$	1	0.0010	0.6952	0.0000	0.3424
$X_1 X_3$	1	0.0189	0.0951	1.218×10^{-6}	0.7394
$X_2 X_3$	1	0.0597	0.0051*	0.0001	0.0028*
X_1^2	1	0.0416	0.0166*	0.0000	0.0432*
X_2^2	1	0.1145	0.0003*	0.0002	0.0006*
X_3^2	1	0.0090	0.2420	0.0000	0.2844
Residual	23	0.0062		0.0000	
Lack of Fit	17	0.0068	0.3377	0.0000	0.0513

*Significant parameters (p < 0.05). Units: Diacetylated lactonic C18:1 production (total g); Diacetylated lactonic C18:1 productivity (g L^{-1} h^{-1}).

The lack of fit was not significant (p > 0.05), which is reliable in terms of prediction, showing that the variation between replicates is acceptable (Haber and Runyon, 1973). The statistical analysis for diacetylated lactonic C18:1 response and productivity resulted in a second-order polynomial approach (p-value <0.0001 in both outcomes), with glucose ($\boldsymbol{X_1}$) and fermentation time ($\boldsymbol{X_3}$) being the most significant parameters in terms of the p-value. As reported by Haber and Runyon (1973), the smaller the p-value, the more significant the corresponding coefficient.

Regarding diacetylated lactonic C18:1 production, the standard least squares regression (R^2) could explain 84.31% of the variability present in the analyzed response. Moreover, the model based on the predicted R^2 can also explain 64.57% of the variations in new observations, which is in reasonable agreement with the adjusted R^2 of 78.18% (difference less than 0.2). Despite the main influencing parameters, the term $\boldsymbol{X_2 X_3}$ (nitrogen and time) had a significant effect on diacetylated lactonic C18:1 production when the maximum glucose ratio was applied, demonstrating a quadratic curvature and an interaction between both terms. The importance of this interaction is supported since these parameters are related to microorganism growth and survival. Finally, the resulting normalized regression produced by the model for diacetylated lactonic C18:1 production is presented in the following equation:

$$Diacetylated\ lactonic\ C18{:}1\ (total\ g) = 0.6852 + 0.1274X_1 + 0.0173X_2 + 0.0896X_3 + 0.0090X_1X_2 + 0.0397X_1X_3 - 0.0705X_2X_3 - 0.0740{X_1}^2 - 0.1227{X_2}^2 - 0.0350{X_3}^2 \quad \text{(Eq. 21)}$$

Where: X_1 represents the glucose ratio, X_2 represents the nitrogen ratio and X_3 represents the fermentation time (h).

For productivity response, R^2 was of 86.23% with an adjusted and predicted R^2 of 80.84% and 67.34%, respectively. The ANOVA shows the same behavior as the diacetylated lactonic C18:1 outcome. The regression equation for the normalized data was as follows:

$$Productivity\ (g\ L^{-1}h^{-1}) = 00231 + 0.1274X_1 + 0.0173X_2 + 0.0896X_3 + 0.0090X_1X_2 + 0.0397X_1X_3 - 0.0705X_2X_3 - 0.0740{X_1}^2 - 0.1227{X_2}^2 - 0.0350{X_3}^2 \quad \text{(Eq. 22)}$$

Where: X_1, X_2 and X_3 are glucose ratio, nitrogen ratio and fermentation time (h), respectively.

To demonstrate the interaction between hydrophilic carbon (glucose) and nitrogen and fermentation time, surface plots in 3D were generated to show their effect on diacetylated lactonic C18:1 production and productivity (Figure 4.4). When the glucose: nitrogen ratio is modified, changes in the shape and contour of the RSM can be analyzed. Figure 4.4a shows that at the maximum fermentation time (240 h), a high glucose ratio (between 186.5 and 199.0) promotes the production of the lactonic SLs. Nitrogen causes a lower impact compared to glucose, as can be deduced by the model parameters. Moreover, when the highest glucose ratio was set, the interaction between time and nitrogen was demonstrated (Figure 4.4b). The highest productivity was achieved at 96 h and decreased afterward. Figure 4.4c presents productivity evolution at 96 h,

showing that maximum productivity was obtained when nitrogen and glucose ratios were at the highest values.

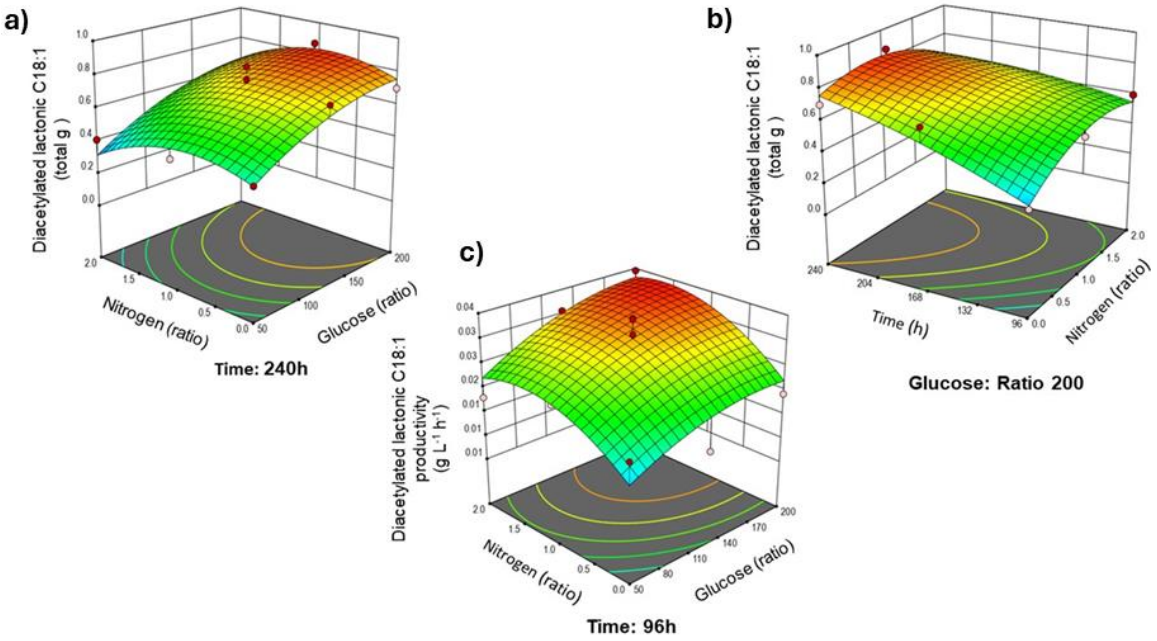

Figure 4.4 Combined effect of the analyzed factors for diacetylated lactonic C18:1 production response: a) glucose and nitrogen ratio at 240 h; b) time and nitrogen at glucose maximum ratio of 200 and c) productivity response at 96 h based on glucose and nitrogen ratio. Surface plots are colored from low (blue) to high (red).

As reported in the literature, medium optimization and experimental designs for SLs production in liquid cultures have been carried out by several research groups (Casas and García-Ochoa, 1999; Saerens et al., 2009; Rispoli, et al., 2010; Parekh and Pandit, 2012). Nevertheless, the scarcity of studies focused on optimizing nutrients in SSF has hindered meaningful comparisons within the field. Ingham and Winterburn (2022) developed a central composite experimental design to understand how nitrogen, glucose and oil sources influence sophorolipid production via SmF. Their findings support that nitrogen and oil were significant, but glucose did not demonstrate a significant effect on SLs production in the analyzed concentration range (15.9-184 g L^{-1}). In contrast, our research demonstrated the significant influence of glucose on the production of

diacetylated lactonic C18:1. These findings are aligned with those of the study conducted by Minucelli et al. (2017), who reported a reduction of 83% in SLs production when the glucose concentration was decreased from 100 g L^{-1} to 10 g L^{-1}. In addition, in our experiment, the hydrophobic carbon source was kept constant, which can also show the effect of glucose in the process.

4.4.4 *Experimental validation of the optimized conditions*

Utilizing the generated model, the production of diacetylated lactonic C18:1 was optimized using a numerical method provided by Design-Expert 12® software. Considering the associated costs of extended fermentation time, productivity becomes a crucial factor for prospective industrial process implementation and scale-up. Therefore, the objective was to maximize diacetylated lactonic C18:1production and productivity regardless of the yeast growth and SLs crude extract. The optimal point was a glucose: nitrogen ratio of 181.75:1.43 (w w^{-1}) or equivalently 127:1 (w w^{-1}) that corresponds to a glucose and nitrogen concentration of 94 and 0.74 g kg^{-1}, respectively, of the wet mixture initial weight. The optimal fermentation time was 100 h with a prediction of 0.612 diacetylated lactonic C18:1 total gram and a productivity of 0.033 g L^{-1} h^{-1} (Figure 4.5). It is worth mentioning that the optimal glucose concentration obtained in our study is comparable to that reported for SmF (50–100 g L^{-1}). While SSF offers the advantage of significantly reduced water volumes, it is essential to acknowledge that mass transfer limitations can affect the availability of nutrients for microorganisms (Kumar et al., 2021b; Chilakamarry et al., 2022; Al-Kashef et al., 2023).

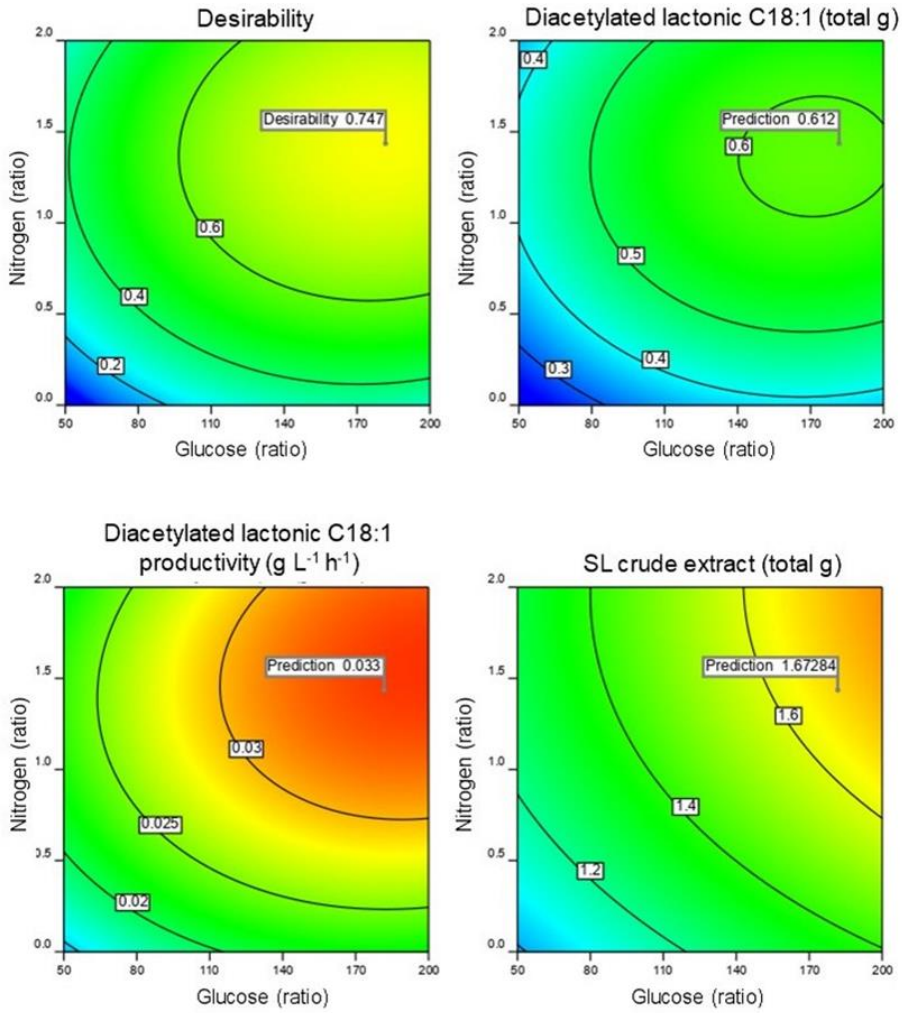

Figure 4.5 Contour profile of the predicted optimized point obtained with the achieved model.

The optimal conditions (WOC-O) were validated experimentally and tested in triplicate applying the scale-down methodology (section 3.3.1). The obtained results were 0.535 ± 0.007 diacetylated lactonic C18:1 total gram, which corresponds to 0.047 ± 0.001 g g^{-1} DM_i, with a productivity of 0.030 ± 0.001 g L^{-1} h^{-1}. This result fits in the 95% confidence interval provided by the software. As before, HPLC-UV analysis of the SLs crude extract showed that other SL congeners were present (Figure 4.6). The WOC-O combination yielded 1.617 ± 0.031 SLs crude extract total gram, with a SLs mixture equivalent area of 0.42 ± 0.031 g g^{-1} present in the crude extract being the most representative congener the diacetylated lactonic C18:1 by 76.9%.

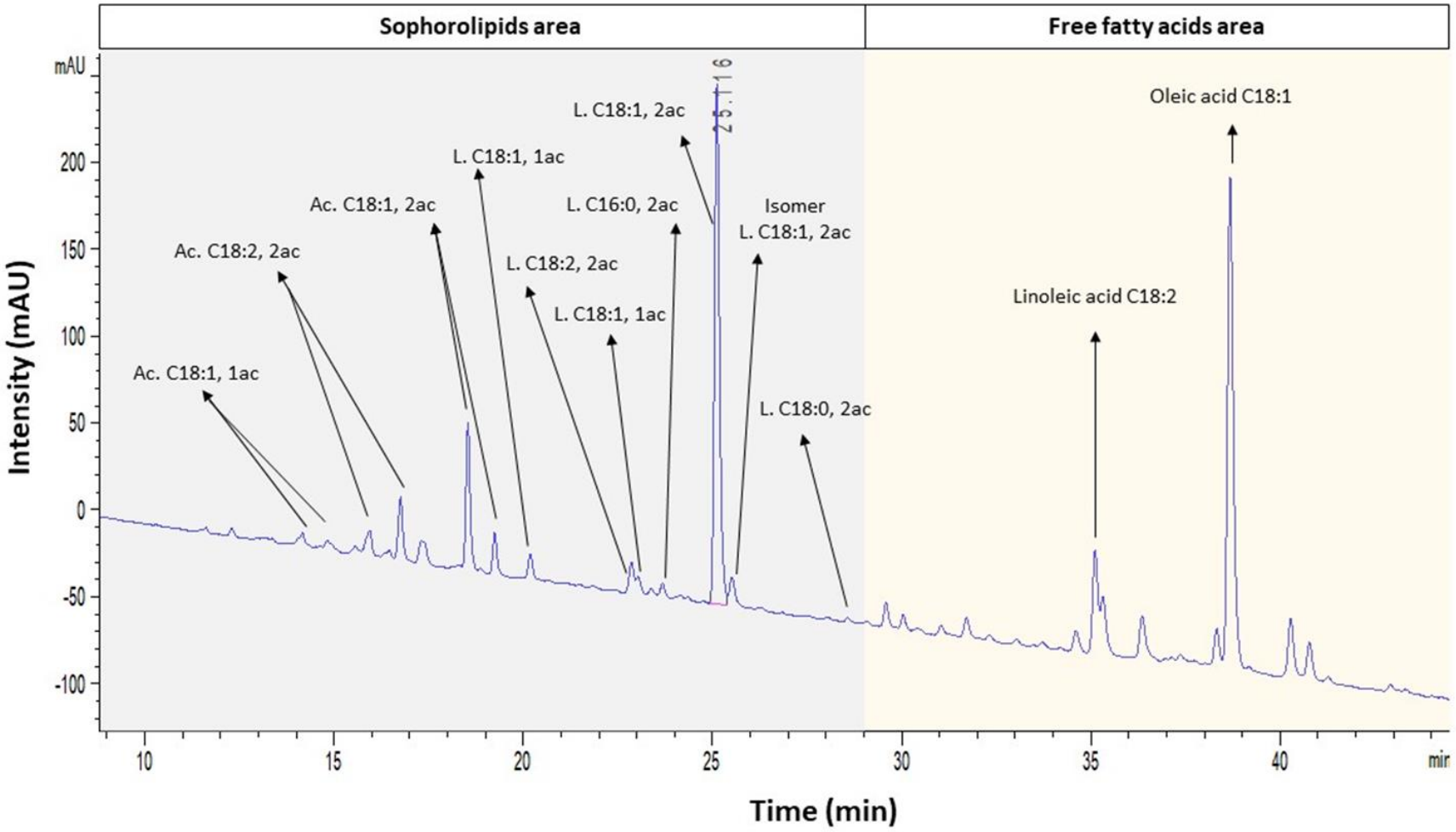

Figure 4.6 Optimized condition (WOC-O) HPLC-UV spectra at 198 nm. Identification of SLs congeners and other compounds was developed based on HPLC-MS.

4.4.5 *Time course comparison of optimized and reference conditions in 0.5 L packed bed bioreactor*

A time course experiment was conducted to evidence SLs crude extract and diacetylated lactonic C18:1 increase as well as to assess process behavior. Similar fermentation profiles were obtained when comparing the optimized (WOC-O) ratio and the reference conditions (WOC-R). As shown in Figure 4.7, samples were analyzed over 48, 96, 168 and 240 h at a 0.5 L reactor scale.

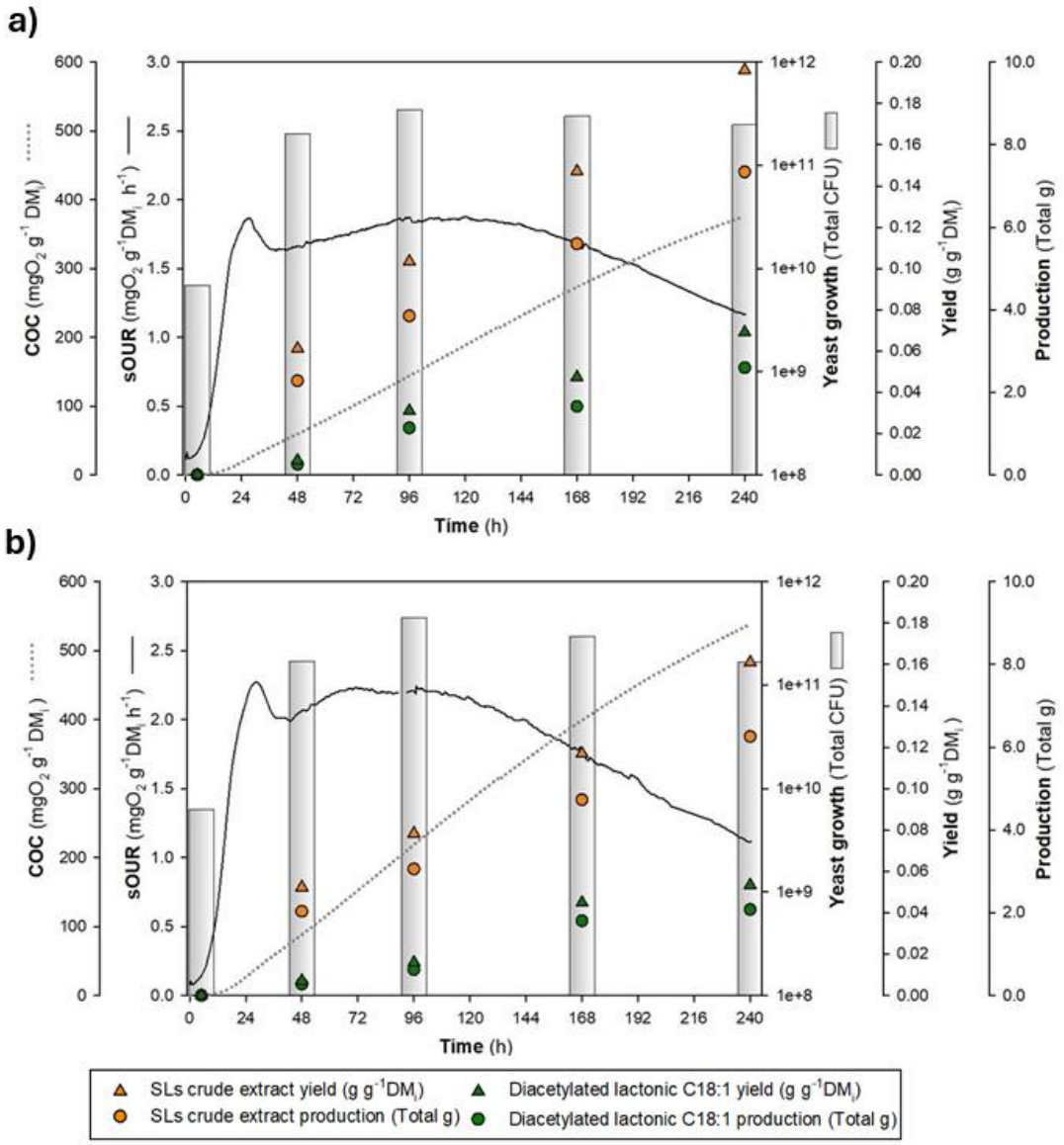

Figure 4.7 Time course comparison of different glucose:nitrogen ratios for SLs production at 240 h in a 0.5 L reactor. a) WOC-O and b) WOC-R.

The outcomes revealed the presence of SLs (crude extract and diacetylated lactonic C18:1) from the initial sampling at 48 h in both combinations. After 240 h of fermentation, a maximum SLs crude extract of 0.196 and 0.161 g g^{-1} DM_i was achieved by WOC-O and WOC-R, respectively. In addition, diacetylated lactonic C18:1 showed differences between both treatments with values of 0.069 g g^{-1} DM_i and 0.053 g g^{-1} DM_i, respectively. These findings highlighted that WOC-O produced a 22% increase in the SLs crude extract and a 30% increase in diacetylated lactonic C18:1 production compared to WOC-R. Moreover, WOC-O presented the highest volumetric productivity of the SLs crude extract and diacetylated lactonic C18:1 (0.082 and 0.029 g L^{-1} h^{-1}, respectively). These values are higher than those reported by Jiménez-Peñalver et al. (2016), who achieved a SLs crude extract yield of 0.179 g g^{-1} DM_i after 240 h using WOC and sugar beet molasses as substrates.

The HPLC–UV analysis showed a higher SLs mixture equivalent area in the WOC-O combination (6607.34 mAU*s) compared to WOC-R (5417.751 mAU*s), which represents a production of 0.461 and 0.365 g of SLs per gram of crude extract, respectively. In this sense, the main produced congener was diacetylated lactonic C18:1 which represents 51.5% in WOC-O and 55.9% in WOC-R of the SLs mixture. As stated in Table 4.5, diacetylated acidic C18:1 was the second abundant congener, which presented an increase in the concentration over time, with an area ratio of 1:5 compared to diacetylated lactonic C18:1 (Figure 4.8).

Throughout the fermentation process, in both treatments, the total CFU exhibited a notable increase by two orders of magnitude (10^{11}) when compared to the initial concentration (10^{9}), which is aligned with the observations reported by Rodríguez et al. (2021). Moreover, glucose analysis showed the presence of

a residual content of 0.010 g g^{-1} DM_i, followed by its depletion in WOC-O, while for WOC-R, glucose depletion after 48 h sampling was observed. The fermentation profile revealed a maximum sOUR peak achieved in both treatments at 28 h with values consistent with our previous published results (Jiménez-Peñalver et al., 2016; Rodríguez et al., 2020).

Table 4.5 Sophorolipid total area comparison between WOC-O and WOC-R.

Sophorolipid congeners	**Retention time** (min)	**Area** (mAU*s)		**Area** (%)	
		WOC-O	**WOC-R**	**WOC-O**	**WOC-R**
Ac. C18:1, 1ac	13.66	136.46	106.26	2.07	1.96
Ac. C18:1, 1ac*	14.33	75.36	45.91	1.14	0.85
Ac. C18:2, 2ac	15.47	245.50	177.182	3.72	3.27
Ac. C18:2, 2ac*	16.31	220.29	126.101	3.33	2.33
Ac. C18:1, 2ac	18.09	707.70	597.65	10.71	11.03
Ac. C18:1, 2ac*	18.80	209.22	173.846	3.17	3.21
L. C18:1, 1ac	19.64	160.48	113.83	2.43	2.10
L.C18:2, 2ac	22.30	104.69	88.120	1.58	1.63
L. C18:1, 1ac	22.48	67.72	58.631	1.03	1.08
L. C16:0, 2ac	23.23	173.94	124.015	2.63	1.08
L. C18:1, 2ac	24.54	3400.93	3029.802	51.47	55.92
L. C18:1, 2ac*	24.93	254.86	242.558	3.86	4.48
Others		850.21	533.845	12.87	11.06
Total		**6607.34**	**5417.75**	**100**	**100**

*Data belongs to SLs isomers; others group, involve no identifying SLs. *Abbreviations:* Ac, acidic; L, lactonic and ac, amount of acetylation.

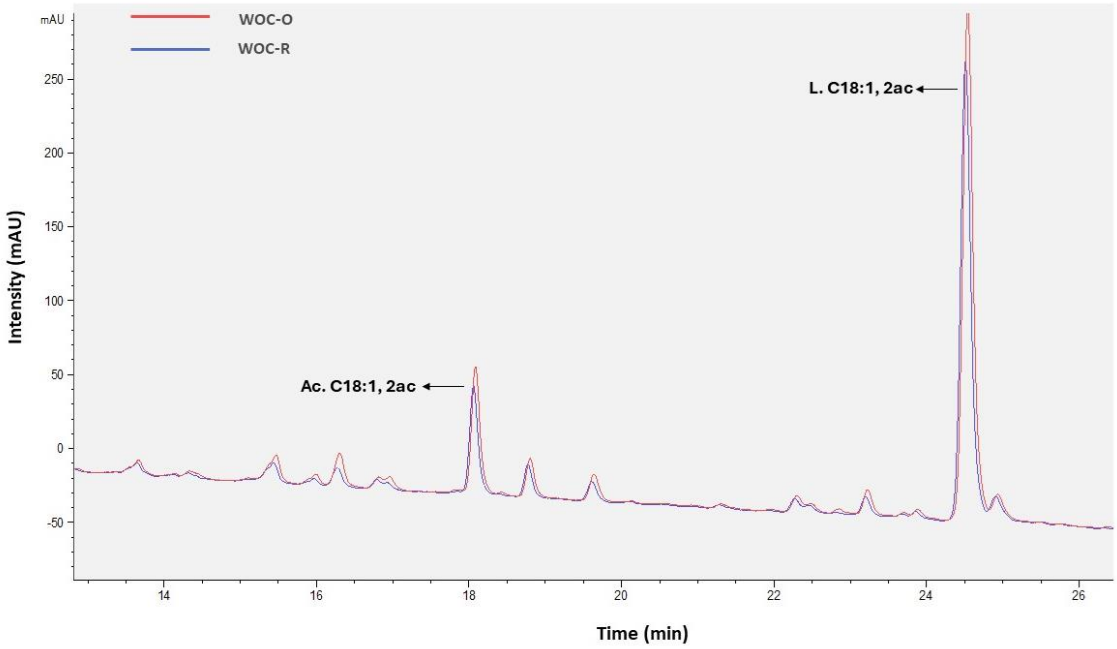

Figure 4.8 HPLC-UV chromatogram. SLs mixture area present in the crude extract when comparing WOC-O and WOC-R.

In summary, optimized glucose and nitrogen concentrations led to an SLs crude extract yield of approximately 0.19 g g^{-1} DM_i, exceeding yield values obtained under reference conditions herein and in previous publications with the same wild-type *S. bombicola* in SSF.

4.4.6 *Nitrogen source and yeast growth*

As our hypothesis was based on nitrogen as a growth-limiting factor and we were attempting to understand growth-production dynamics under SSF, yeast growth was also evaluated as a response on the same data obtained in section 4.4.1. The initial *S. bombicola* seed for this experiment was 1.89×10^9 total CFU, which represents 1.7×10^8 CFU g^{-1} DM_i. Subsequently, the analyzed results showed that CFU increased one order of magnitude after fermentation. The central point (000) presented a yeast growth mean of $4.9 \times 10^{10} \pm 5.61 \times 10^9$ total CFU ($4.5 \times 10^9 \pm 5.18 \times 10^8$ CFU $g^{-1}DM_i$), with an initial concentration of 0.649 g L^{-1} total nitrogen in the aqueous phase, which corresponds to a concentration of

0.001 g g^{-1} DM_i of urea. The highest growth was achieved in run 32 (00+) at 240 h ($8.5 \times 10^{10} \pm 4.6 \times 10^9$ total CFU). Nevertheless, the lowest growth was reached by combinations without the addition of urea, regardless of the glucose ratio in runs 15 (---), 10 (0–0) and 17 (+-+) at 96, 168 and 240 h, respectively (2.1×10^{10}, 2.2×10^{10} and 2.3×10^{10} total CFU, respectively).

The statistical analysis for yeast growth response was fitted to a second-order polynomial approach with base 10 logarithm data transformation (p-value 0.0019). As shown in Table 4.6, the influencing factors were nitrogen (p-value 0.0005) and fermentation time (p-value 0.0005).

Table 4.6 Yeast growth ANOVA for a surface quadratic model

		Yeast growth (R^2 = 63.33%)	
Source	**DF**	**Mean Square**	**p-value**
Model	9	0.0598	0.0019*
X_1 -Glucose	1	0.0000	0.9741
X_2 -Nitrogen	1	0.2261	0.0005*
X_3 -Time	1	0.2221	0.0005*
$X_1 X_2$	1	0.0053	0.5368
$X_1 X_3$	1	0.0033	0.6276
$X_2 X_3$	1	0.0130	0.3377
${X_1}^2$	1	0.0208	0.2278
${X_2}^2$	1	0.0159	0.2896
${X_3}^2$	1	0.0183	0.2568
Residual	23	0.0135	
Lack of Fit	17	0.0174	0.0122*

*Significant parameters ($p < 0.05$).

For this response, the lack of fit was significant (p-value 0.0122), which deemed that the model is not reliable in terms of prediction (R^2 of 63.33%). The regression equation for the normalized data was as follows:

$$Log_{10}(Growth) = 10.63 + 0.0009\,X_1 + 0.1121\,X_2 + 0.1005\,X_3 + 0.0211\,X_1X_2 - 0.0165\,X_1X_3 + 0.0329\,X_2X_3 - 0.0523\,{X_1}^2 - 0.0458{X_2}^2 + 0.0500{X_3}^2 \quad \text{(Eq. 23)}$$

Where: X_1 represents the glucose ratio, X_2 represents the nitrogen ratio and X_3 represents the fermentation time (h).

Although the model is not reliable in terms of prediction, some conclusions can be obtained from the observed trends (Figure 4.9). To maximize yeast growth, the optimal glucose: nitrogen ratio was 128.9:2 (w w^{-1}) at 240 h, reaching 7.51 × 10^{10} total CFU and a production of 0.57 total g of diacetylated lactonic C18:1. As expected, this implies lower glucose and higher nitrogen and time than optimal values for production and productivity and leads to slightly lower total production but far lower productivity due to increased process time. As reported by Daverey and Pakshirajan (2010), decreasing the nitrogen source in the fermentation broth can result in a lower biomass concentration, thereby negatively impacting SLs production. In their study, the optimum nitrogen concentration for biomass growth was 10 g L^{-1}, while for SLs production, it was 2 g L^{-1}.

Moreover, Gao et al. (2013) reported that at a flask scale, the cell density increased proportionally to the increase in standard Yeast Malt Broth used as the nitrogen source, which aligned with our findings. In contrast, the authors also emphasized that a high glucose concentration suppressed growth, which differs from our findings, where glucose was not a significant parameter for this response

in the range studied. In addition, Ma et al. (2011) found that the highest cell dry weight (12.90 g L^{-1}) was achieved using yeast extract (3 g L^{-1}) and (NH4)2SO4 ammonium sulfate (4 g L^{-1}) as nitrogen sources. Nevertheless, it negatively influenced SLs synthesis (29.75 g L^{-1}) when compared with the control group of each nitrogen source (73.10 and 71.00 g L^{-1}, respectively). This highlights the significant influence that accessible nitrogen can have on the process, emphasizing the importance of identifying the optimal nitrogen concentration to enhance SLs production. Furthermore, these findings also imply the necessity of evaluating the influence that nitrogen source combinations can exert on the process.

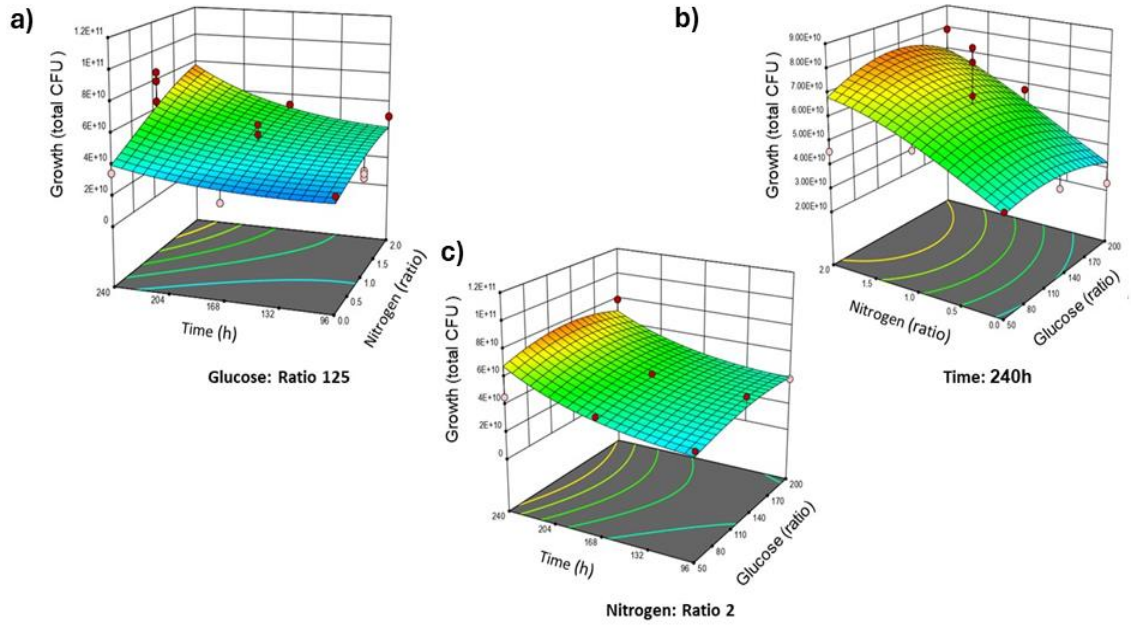

Figure 4.9 Response surface methodology for yeast growth. a) Influencing factors interaction at glucose central point (ratio 125); b) Effect of time level +1 (240 h) on glucose and nitrogen parameters and c) Nitrogen level +1 (ratio 2) influence on the evaluated parameters. Surface plots are colored from low (blue) to high (red).

From the results presented herein (Table 4.2), no clear relationship was observed between total growth and total diacetylated lactonic C18:1 production considering either all data or specific production times in the correlation analysis.

This illustrates the complexity of dynamics under solid-state fermentation where higher cell growth does not necessarily mean higher SLs production (crude extract and diacetylated lactonic C18:1). In addition, SLs production profiles obtained in batch, packed bed SSF reveal a significant production of SLs in the initial days of the process simultaneous to cell growth. This confirms that in solid heterogeneous matrices, cell growth and metabolite production are not two sequential phases and different metabolic phases co-exist. Operation strategies allowing for matrix homogeneity should improve growth and, hence, SLs production.

4.4.7 *Alternative hydrophilic carbon and nitrogen sources for SL production*

With the achieved optimal glucose: nitrogen ratio (181.75:1.43 w w^{-1} initial dry weight) and time value (100 h), pure substrate substitution was assayed using industrial organic residues. Table 4.7, summarizes the characteristics of the different industrial residues used in this study. Based on residue characterization, RHP and RAC present potential to be used as a nitrogen source. However, large differences were found for pH and TN values related to their origin. RSC and ROF characteristics framed these residues as potential hydrophilic carbon sources due to their glucose content. Moreover, it is important to highlight that ROF could also be used as a nitrogen source, making it a versatile residue, as reported by Kaur et al. (2019) and To et al. (2023). Additionally, RSO corresponds to a mix of residues, where sugar candy industry wastewater (6.96 mL) was used as a hydrophilic carbon source and OFMSW hydrolysate (0.037 mL) as a nitrogen source, to evaluate the influence of combined residues on the process.

Table 4.7 Main characteristics of the different industrial residues used as feedstock

Substrate	DM (%)	MC (%)	OM (%, db)	pH	Glucose* (g L^{-1} or g Kg^{-1})	TC* (g L^{-1} or g kg^{-1})	TN* (g L^{-1} or g kg^{-1})
Winterization oil cake (WOC)	91.87	8.13	44.02	6.04	n.d	10.50	0.04
Cosmetic sludge (RHP)	11.12	88.88	93.87	6.53	n.d	21.24	3.89
Clean house products sludge (RAC)	8.39	91.61	92.38	11.75	n.d	41.60	1.05
Sugar candy industry wastewater (RSC)	12.76	87.24	94.68	4.38	2.08	57.30	0.02
OFMSW hydrolysate (ROF)	2.01	97.99	99.37	5.20	17.03	28.70	1.20

*Data units are expressed by volume or weight according to residue physical characteristics. *Abbreviations:* db, dry basis; n.d, not detected.

As Figure 4.10 shows, the tested residues allowed *S. bombicola* growth and SLs production at 100 h. The literature reported that fermentations using this microorganism are associated with a pH decrease (Van Bogaert et al., 2011; Jiménez-Peñalver et al., 2018). In these experiments, pH decreased from an initial value of approximately 5.5 ± 0.25 to values of approximately 2.6–3.0 after 100 h, except for RAC which had a pH of 4.6. Initial samples do not present significant differences in CFU content, as based on Tukey's test (p-value >0.05). After fermentation, the optimized values (WOC-O achieved a total yeast growth of 7.50 ± 0.87 × 10^{10} CFU. Moreover, when residues were compared, the combination RSC presented the highest growth (7.24 × 10^{10} ± 1.15 × 10^{10} total

CFU), while the lowest was achieved by RAC (2.19 × 10^{10} ± 0.11 × 10^{10} total CFU). Statistical Dunnett's multiple comparison test showed that combinations ROF (p-value 0.0070), RHP (p-value 0.0118), RAC (p-value 0.0003) and RSO (p-value 0.0154) presented significant differences in yeast growth at 100 h compared with WOC-O.

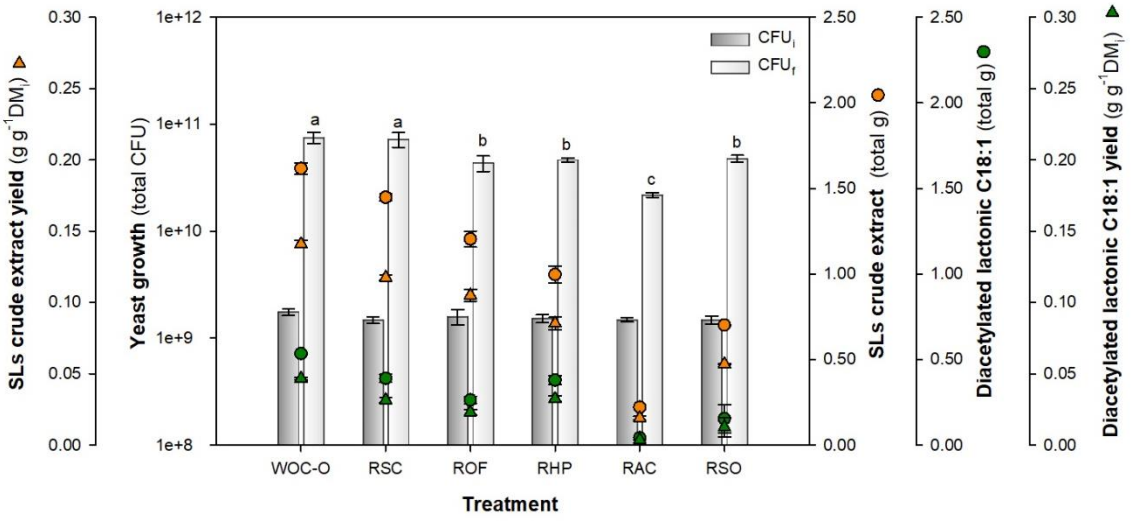

Figure 4.10 Second-generation feedstocks for SLs production and yeast growth at 100 h using the optimal glucose:nitrogen ratio of 181.75:1.43. Same letters indicate statistically insignificant differences at p-value <0.05 for yeast growth. WOC-O, optimized conditions group; RSC, sweet candy wastewater; ROF, OFMSW hydrolysate; RHP, cosmetic sludge; RAC, clean house product sludge; and RSO, sugar candy industry wastewater + OFMSW hydrolysate.

Regarding SLs production, statistical Tukey's test showed that the highest production of diacetylated lactonic C18:1 was achieved by RSC (0.389 ± 0.024 total g) and RHP residues (0.379 ± 0.026 total g) with a productivity of approximately 0.022 ± 0.001 g L^{-1} h^{-1} (p-value: 0.9869). Furthermore, RAC presented the lowest production (0.043 ± 0.001 total g). When comparing residues that used OFMSW (ROF and RSO), no significant difference was observed (p-value: 0.2786). The obtained SLs production results do not agree with those reported by Kaur et al. (2019), who used the OFMSW hydrolysate. For the ROF residue, the glucose present in the hydrolysate was used as a carbon

source, while RSO is based on a combination of two residues: sugar candy industry wastewater and OFMSW hydrolysate. Although these combinations maintained the evaluated nutrient ratio, it is clear that the hydrolysate also contains other types of sugars, fatty acids and nutrients due to its origin (Kaur et al., 2019; Pleissner and Peinemann, 2020). In this way, recent literature reported that autoclaving hydrolysates can lead to the formation of inhibitors in the media, which suggests that tangential filtration could be considered as a potential option for future investigations (Ingham et al., 2023).

In addition, SLs crude extract production presented significant differences (p-value <0.0001) between RSC (1.448 ± 0.023 total gram with a yield of 0.117 ± 0.002 g g^{-1} DM_i) and RHP (0.996 ± 0.051 total gram with a yield of 0.085 ± 0.004 g g^{-1} DM_i). As mentioned before, SL congeners were present in the crude extract mix for RSC 0.38 and for RHP 0.45 g of SL per gram of crude mix, with a diacetylated lactonic C18:1 having relative areas of 71% and 84%, respectively. When SLs crude extract productivity was analyzed, the best result was achieved by RSC, 0.080 ± 0.046 g L^{-1} h^{-1}; this result aligned with that obtained by Rashad et al. (2014), who achieved a titer of 10 g L^{-1} with a productivity of 0.107 g L^{-1} h^{-1} on SSF.

One of the major drawbacks associated with low-cost substrates is the selection of an appropriate waste with the precise balance of carbon and nitrogen that allows significant growth and product formation. The obtained results demonstrate the potential of RSC as a hydrophilic carbon source and RHP as a nitrogen source for diacetylated lactonic C18:1 production (Figure 4.11). Wadekar et al. (2012c) used sweet water supplemented with glycerol for SLs production on SmF, achieving a yield of 6.36 g L^{-1} composed of 18.9% acidic SLs, 19.6%

lactonic C18:1 and 60.8% lactonic C18:2. In the present study, the quantification results were focused on diacetylated lactonic C18:1, showing that in the optimized control group, this compound constituted 53.5% of the SLs crude extract, while for RSC and RHP, it is around 38.9% and 37.9%, respectively. However, when the SLs mixture area was analyzed, the diacetylated lactonic C18:1 represents 76.9% for WOC-O, 70.9% for RSC and 84.4% for RHP. This suggests that the composition of the SLs congeners is affected by the complex composition of residues used as the hydrophobic and nitrogen sources.

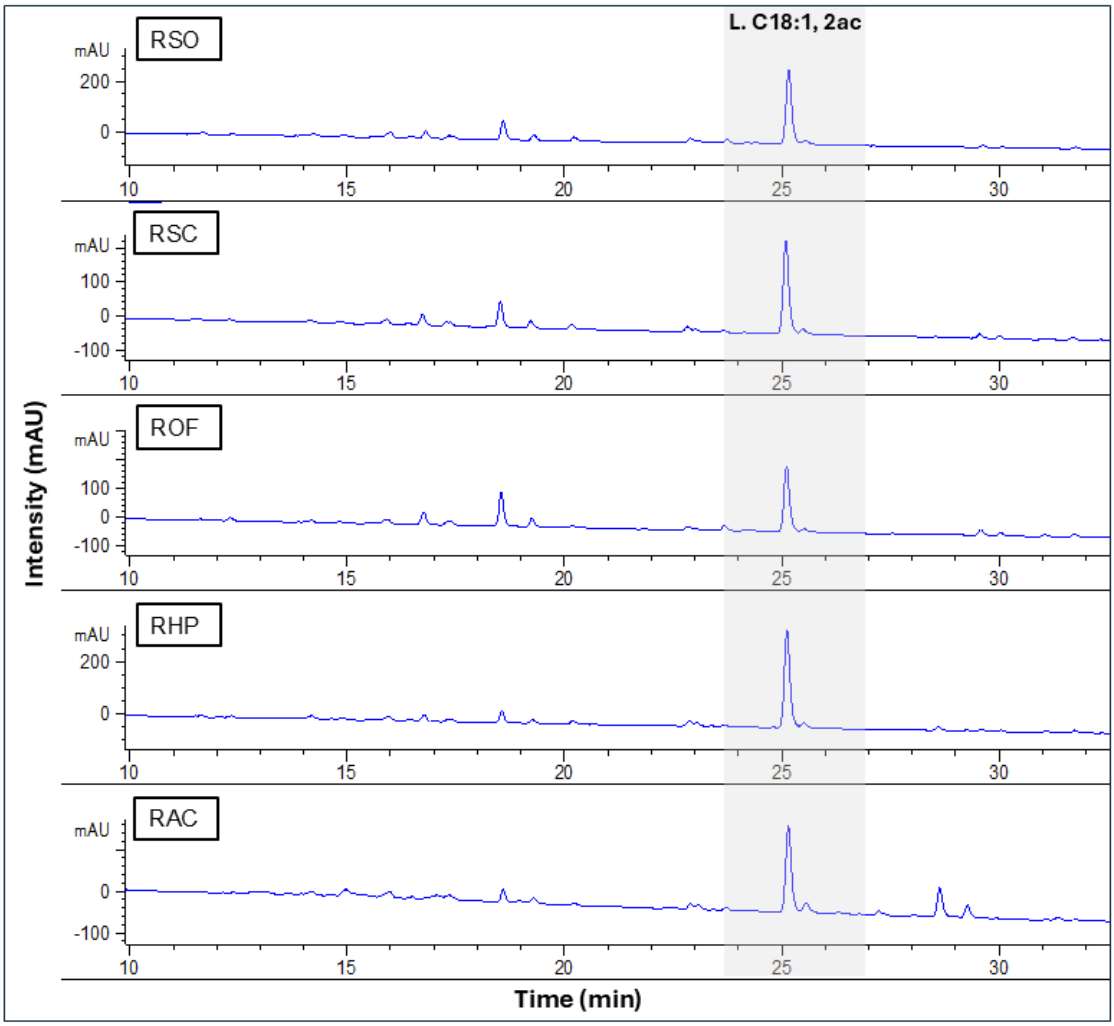

Figure 4.11 HPLC-UV chromatograms using waste as feedstock for SLs production. RSO, sugar candy industry wastewater + OFMSW hydrolysate; RSC, sugar candy industry wastewater; ROF, OFMSW hydrolysate; RHP, cosmetic sludge and RAC, clean house product sludge.

The results that focused on the residues used as nitrogen sources (RHP, RAC and RSO) are congruent with those reported by Ma et al. (2011), who reported that inorganic nitrogen sources, such as ammonium sulfate, promote the formation of acidic SLs, while organic nitrogen sources enhance the production of lactonic SLs, which is confirmed with RHP results. The literature reports that there is a knowledge gap regarding alternative nitrogen sources for biosurfactant production (Solaiman et al., 2007; Wongsirichot et al., 2021), which will be explored later in this thesis. In this context, our research outcomes contribute positively to waste valorization in the SLs production framework.

Although all residues showed a significantly lower production (p-value <0.05) compared to the WOC-O, it is important to highlight that, when using residues, they contribute with glucose, nitrogen and micronutrients, thus potentially increasing the process sustainability and reducing the amounts of pure substrates required. References that report diacetylated lactonic C18:1 yield on SSF processes could not be found in the literature. However, compared with the results obtained by Rodríguez et al. (2020), who reported a SLs crude extract yield of 0.2 g g^{-1} DM_i using WOC and molasses as feedstock at 22 and 100 L, respectively, the SL yields achieved in this study using second-generation residues are lower. Nevertheless, it should be considered that the present experiment was set-up at a flask scale and in this sense, the results could be improved in a scale-up process applying aeration and agitation (Raghavarao et al., 2003; Oiza et al., 2022).

It is important to emphasize the challenge in comparing research outcomes between SSF and SmF due to the presence of multiple differing parameters that can significantly influence production outcomes such as productivity and yield.

Therefore, it is essential to acknowledge that SSF and SmF could be complementary technologies for SLs industrial production when residue revalorization is the main purpose. Nevertheless, it must be highlighted that the low water and energy consumption of SSF suggests that it can be an effective and economically feasible technology for biosurfactant production.

4.5 Conclusion

To sum up, with the aim of providing knowledge about SLs production through solid-state fermentation, a Box-Behnken design and response surface methodology were applied. A quadratic model was adjusted and validated for the analyzed parameters, with glucose and time being the influencing factors for diacetylated lactonic C18:1 production and productivity, while nitrogen is the influencing factor for yeast growth, achieving the highest productivity at 100 h. A productivity of 0.033 g L^{-1} h^{-1} was achieved with a glucose:nitrogen ratio of 181.75:1.43 or 127:1 (w w^{-1} initial dry weight) reaching a yield of 0.047 g g^{-1} DM_i for the diacetylated lactonic C18:1 and 0.141 g g^{-1} DM_i for the SLs crude extract. Moreover, the time course comparison in a 0.5 L packed bed reactor using the optimal combination showed a production increase in the SLs crude extract (22%) and diacetylated lactonic C18:1 (30%) when compared with the reference medium combination. In addition, when using residues, sugar candy wastewater and nitrogenous cosmetic sludge showed good potential as alternative feedstocks to pure substrates. Finally, based on the outcomes achieved, it was clear that multiple yeast metabolic stages could coexist in the SSF process due to the heterogeneous nature of solid matrices. Future work could be focused on fed-batch and scale-up processes, in addition to the evaluation of alternative hydrophobic carbon source that can be exploited by SSF (Chapter 5).

Chapter 5

Alternative hydrophobic sources in SSF

Contents of this chapter are submitted for publication under the title "*A comparative study of oilseed cakes as hydrophobic feedstocks for sophorolipid production by solid-state fermentation*" in *Industrial Crops and Products*.

Summary

The sustainable production of sophorolipids (SLs) has promoted the use of alternative agricultural byproducts and residues as nutrient sources. Corn, sunflower, soybean and rapeseed oilseed cakes are studied in this Chapter as newer hydrophobic feedstocks for SLs production through solid-state fermentation (SSF) using the wild-type *S. bombicola*. Maximum SLs crude extract production at 0.5 L bioreactors was found to be reached by corn cake (0.071 ± 0.001 g g^{-1} DM_i) followed by rapeseed cake (0.058 ± 0.003 g g^{-1} DM_i) at 96 h. The HPLC analysis revealed that the composition of the hydrophobic substrate strongly influences the profile and distribution of SL congeners. Due to SLs properties being highly dependent on the hydrophobic substrate used for their production, emulsification and oil displacement tests were investigated in the obtained crude extracts showing that emulsification stability is influenced by SLs crude extract concentration. Moreover, the best-performing oilseed cakes were scaled-up in a 22 L bioreactor and although no significant increase in production was observed, temperature influence was discussed and the reproducibility of the production method was confirmed. Finally, outcomes highlighted a linear relation between initial fat concentration and SLs crude extract production (R^2=0.93). The chapter scheme is represented in Figure 5.1.

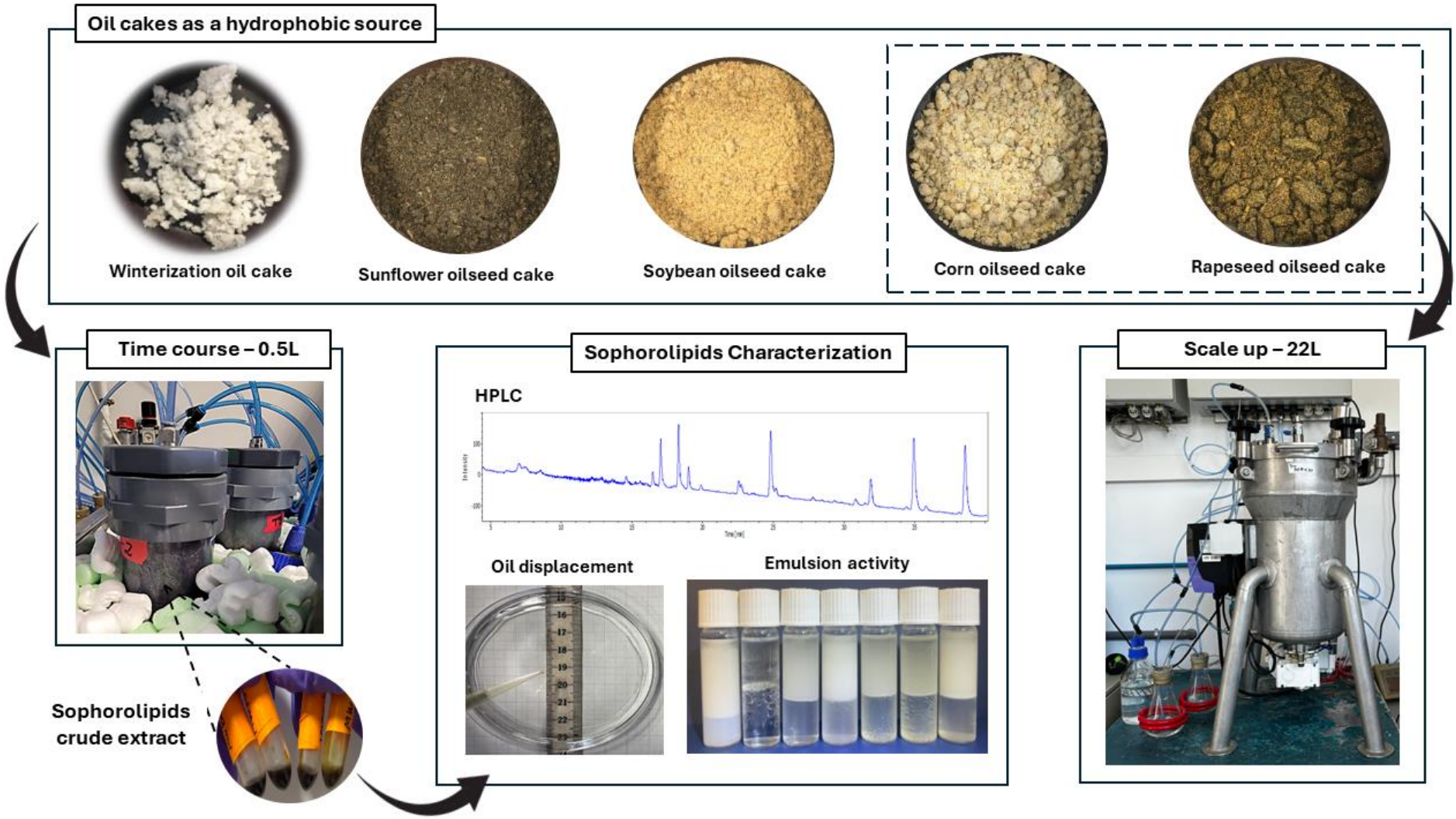

Figure 5.1 Schematic overview of the chapter.

5.1 Introduction

Alternative substrates for the commonly used pure hydrophobic carbon sources (vegetable oils or fatty acids) in SLs production include residues and byproducts from vegetable oil processing. Despite these byproducts are primarily used as an ingredient in animal feed, their abundant production, accumulation and improper disposal results in a negative environmental impact (Venkatramanan et al., 2021). Notably, oilseed cakes are nutrient-rich and contain approximately 8-10% of oils or fatty acids (Popović et al., 2020). In this context, an alternative application focuses on utilizing them for SLs production through SSF, avoiding the need for pre-treatments and providing knowledge about poorly described alternative hydrophobic sources and their influence in the SSF process. In addition, it's been proven that hydrophobic carbon source composition influences the structure and congeners profile of the obtained SLs crude extract (Jiménez-Peñalver et al., 2018). Thus, using alternative substrates can potentially lead to the synthesis of new SLs structures with varying properties such as emulsification, solubilization, toxicity, among others (Pal et al., 2023).

In this chapter, corn, sunflower, soybean and rapeseed oilseed cakes were evaluated for SLs production through SSF and compared with winterization oil cake, as a reference hydrophobic substrate used in previous SSF studies by the group. Additionally, SLs congener profiles were analyzed and the obtained SLs crude extract was characterized to demonstrate the influence of the hydrophobic carbon source in the process and in the final product. The better-performing fermentations were scaled up to a 22 L bioreactor to confirm oilseed cakes suitability.

5.2 Materials

Several hydrophobic carbon sources were assayed in this chapter. Corn (CNC), sunflower (SFC), soybean (SBC) and rapeseed (RPC) oilseed cakes were obtained after seed pressing for vegetable oil extraction (*Aceites de Semillas S.A;* Barcelona) as described in section 3.2.2. Their characterization is presented in Table 5.1. In addition, WOC was used as the model hydrophobic substrate and wheat straw was kept as the organic support. Finally, pure substrates, such as glucose as a hydrophilic carbon, urea as a nitrogen source and yeast extract as a nutrient source were all analytical grade (section 3.1.2).

5.3 Experiments

0.5 L batch reactor fermentations were carried out with a fermentation solid weight of 78.01 ± 0.42 g. The solid matrix of the fermented solid was prepared as described in section 3.3.2 and the aqueous phase which constituted 45.77 ± 2.40% (w w^{-1}) of the solid mixture. It consisted of 26.45 ± 0.47 mL of a nutrient solution which ratio was determined based on optimization results outlined in Chapter 4 and remained constant for all experiments.

Process modifications were developed before mixture preparation, for that oilseed cakes and wheat straw were autoclaved twice at 121°C for 30 minutes, with an additional autoclaving step after the assembly of the solid matrix while the nutrient solution was autoclaved separately before initial solid mixture preparation. Six reactors were prepared and started for each oilseed cake/WOC mixture (Figure 5.2) and were sacrificed in duplicate at 96, 168 and 240 h for post-fermentation analysis. For monitoring the process, SLs production was analyzed as described in sections 3.4 and 3.10**.** Moreover, the obtained SLs

crude extracts were characterized by emulsification and oil displacement tests as described in section 3.5.

Figure 5.2 Solid mixture appearance using different oilseed cakes.

Based on the obtained outcomes, fermentations with corn oilseed cake (CNC) and rapeseed oilseed cake (RPC) were scaled up in a 22 L bioreactor as described in section 3.3.3 and samples were analyzed at 96 h at different reactor heights (top, middle and bottom).

5.3.1 *Oil supplementation*

The impact of the fat content from the hydrophobic source on the process was examined by supplementing several ratios of CNC supplemented with artificial winterization corn oil cake (WCNC). This WCNC was prepared by mixing perlite with corn oil, mainly composed of linoleic acid (49 g in 100 mL), to

saturation, to emulate a real WOC. The blends of CNC to WCNC were formulated at ratios of 100:0, 75:25 and 50:50 (w w^{-1}), respectively (Figure 5.3). The solid mixture composition and fermentation were carried out as described above. Samples were analyzed in biological duplicates at 96 and 168 h.

Figure 5.3 Appearance of the solid mixture using different fat ratio combinations of CNC and WCNC.

5.4 Results and Discussion

5.4.1 *Solid-state fermentation: initial comparison*

Five different alternative substrates were assessed as hydrophobic sources for SLs production. Time course fermentations using WOC, CNC, SFC, SBC and RPC were conducted to assess their potential. Figure 5.4a, illustrates the sOUR profiles obtained using 0.5 L SSF fixed bed reactors. sOUR works as an indirect measure of biological activity (Ponsá et al. 2010). As can be observed, sOUR maximum values were reached during the first 48 h of fermentation for all treatments. However, CNC showed the highest respiration level (3.04 ± 0.18 $mgO_2\ g^{-1}\ DM_i^{-1}\ h^{-1}$) while RPC (2.85± 0.20 $mgO_2\ g^{-1}\ DM_i\ h^{-1}$) and SFC (2.88 ± 0.35 $mgO_2\ g^{-1}\ DM_i\ h^{-1}$) presented similar values. The lowest values were achieved by SBC (2.40 ± 0.13 $mgO_2\ g^{-1}\ DM_i\ h^{-1}$) and WOC (1.73 ± 0.16 $mgO_2\ g^{-1}\ DM_i\ h^{-1}$).

Nonetheless, when comparing the respirometry profiles, WOC presented an extended stationary phase compared to the oilseed cakes, probably due to its higher fat content (Table 5.1). This could influence the production of SLs because it is a secondary metabolite (Van Bogaert et al., 2007).

Additionally, the double peak in the respirometric profiles of the oilseed cakes appears to indicate cometabolism, characterized by the depletion of glucose followed by the consumption of oil. There were significant differences in glucose consumption among the processes (p-value 0.011). In the fermentations with oilseed cakes, glucose was depleted before 96 h, whereas when using WOC, glucose depletion occurred after 96 h. According to Ingham and Winterburn (2022), once glucose is depleted, hydrophobic substrates begin to be consumed. When glucose is eventually exhausted, a minimum amount of oil is necessary to produce enough glycerol to sustain the consumption of hydrophilic carbon sources until harvest. With longer fermentation periods or lower initial oil levels, glycerol would likely become fully depleted and SLs production would drop.

Concerning yeast growth (Figure 5.4b), initial samples do not demonstrate significant differences between the different fermentations (p-value 0.056). However, at 96 h significant differences were found between WOC and the assessed oilseed cakes. Moreover, at 240 h a decline in CFU concentration was observed in CNC and RPC. This could be due to fungal contamination, an occurrence during prolonged SSF which would explain the late increase in sOUR values after 192 h (Rodríguez et al., 2021a). Although no significant fungal growth was detected in plates, some fungal growth was visually observed in the sampled fermented solids.

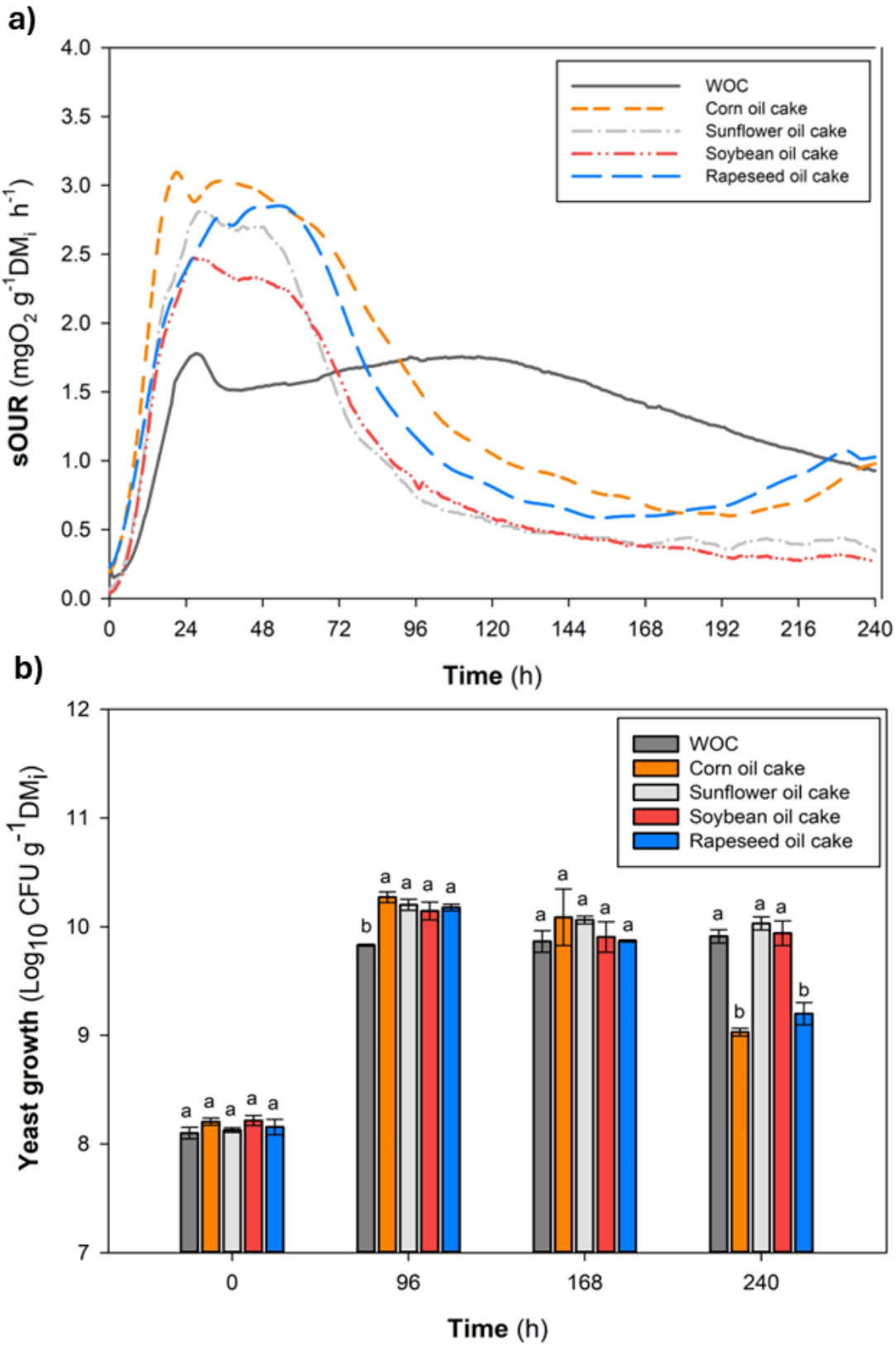

Figure 5.4 Time course comparison using second-generation hydrophobic substrates as feedstock. a) Specific respirometry index (sOUR) and b) Yeast growth. Error bars represent the standard deviation of biological replicates (n=2). Means that do not share a letter are significantly different for each fermentation time (p-value < 0.05).

Table 5.1 Characterization of the oilseed cakes used as hydrophobic substrates.

Parameter / Oilseed cake	WOC	CNC	SFC	SBC	RPC
DM (%)	91.87 ± 0.14	94.33 ± 0.34	95.48 ± 0.25	93.57 ± 0.20	96.45 ± 0.51
MC (%)	8.13 ± 0.14	5.67 ± 0.34	4.52 ± 0.25	6.43 ± 0.20	3.55 ± 0.51
OM (%,db)	50.51 ± 0.06	93.31 ± 0.11	93.97 ± 0.33	93.41 ± 0.03	93.43 ± 0.04
Glucose (g Kg^{-1})	n.d	2.68 ± 0.14	6.00 ± 0.13	0.83 ± 0.06	0.88 ± 0.15
C (%,db)	33.55 ± 2.61	46.78 ± 1.06	49.07 ± 1.38	47.87 ± 0.40	48.80 ± 0.40
H (%,db)	4.90 ± 2.19	7.02 ± 1.93	6.75 ± 0.82	6.94 ± 0.27	6.92 ± 0.14
N (%,db)	n.d	2.54 ± 11.42	5.28 ± 1.80	7.43 ± 5.04	4.11 ± 14.05
S (%,db)	n.d	0.14 ± 23.69	0.25 ± 9.61	0.26 ± 7.81	0.57 ± 2.21
pH	6.04	6.47	6.01	6.44	6.00
Fat content (%, db)	47.82 ± 1.09	16.60 ± 0.01	14.30 ± 0.29	10.61 ± 0.04	14.29 ± 0.12
Fatty acid profile (% on fat content)					
C16:0 (palmitic acid)	3.96	18.89	5.88	12.35	5.86
C18:0 (stearic acid)	3.19	0.92	1.54	1.66	1.53
C18:1 (oleic acid)	83.62	13.84	54.14	27.91	54.65
C18:2 (linoleic acid)	6.25	58.68	23.14	55.39	23.01
C18:3 (linolenic acid ALA)	0.14	3.81	6.88	1.14	6.9
C20:1 (gondoic acid)	0.28	0.44	1.06	0.26	1.03
C22:0 (behenic acid)	1.03	0.11	0.37	0.14	0.14
C24:0 (lignoceric acid)	0.37	0.12	0.19	0.18	0.19
Oleic acid cis isomers (C18:1)	84.34	14.86	59.27	28.53	59.52
Saturated fatty acids	8.88	20.35	8.62	14.69	8.43
Monounsaturated cis fatty acids	84.72	16.13	61.22	28.79	61.43
Polyunsaturated cis fatty acids	6.39	63.14	30.02	56.53	29.91

*Calculated using the response factors for short-chain fatty acids and conversion factors from fatty acid methyl ester (FAME) to short-chain fatty acids. Values are the average of independent samples and their standard deviation. *Abbreviation*: n.d, not detected; DM, dry matter, MC, moisture content; OM, organic matter.

Regarding SLs crude extract, WOC achieved the highest production (0.165 ± 0.009 g $g^{-1}DM_i$) at the end of the fermentation (240 h). In comparison, the lowest production was achieved by SFC (0.013 ± 0.001 g $g^{-1}DM_i$) and SBC (0.014 ± 0.001 g $g^{-1}DM_i$) with no significant difference (p-value 0.589). CNC (0.061 ± 0.006 g $g^{-1}DM_i$) and RPD (0.046 ± 0.011 g $g^{-1}DM_i$) led to intermediate values with no significant differences (p-value 0.298) among them. In addition, the highest SLs productivity was reached at 96 h for all the treatments (Table 5.2). These results may be related to the fat content of each substrate due to WOC presented the highest fat content, while the other oilseed cakes had a significantly lower fat content (29.17 ± 5.18% less, Table 5.1) which values are aligned with those described by Popović et al. (2020).

Table 5.2 Time course outcomes using alternative hydrophobic sources.

Time	Growth	SLs crude extract production		SLs crude extract productivity	SLs mixture*
h	Log_{10} CFU $g^{-1}DM_i$	g $g^{-1}DM_i$	gSL $g^{-1}Fat_i$	g g^{-1}	g g^{-1}
WOC - Winterization oil cake					
0	8.100 ± 0.053 ^{f}	-	-	-	-
96	9.830 ± 0.009 ^{d}	0.086 ± 0.008 ^{c}	0.360 ± 0.034 ^{d,e}	0.089 ± 0.008 ^{a}	0.46
168	9.865 ± 0.099 ^{c,d}	0.141 ± 0.005 ^{b}	0.613 ± 0.033 ^{a,b,c}	0.086 ± 0.005 ^{a}	0.47
240	9.912 ± 0.060 ^{b,c,d}	0.165 ± 0.009 ^{a}	0.693 ± 0.042 ^{a,b}	0.068 ± 0.004 ^{b}	0.52
CNC - Corn oil cake					
0	8.205 ± 0.032 ^{f}	-	-	-	-
96	10.272 ± 0.050 ^{a}	0.071 ± 0.001 ^{c,d}	0.743 ± 0.038 ^{a}	0.065 ± 0.003 ^{b,c}	0.62
168	10.086 ± 0.260 ^{a,b,c,d}	0.069 ± 0.006 ^{c,d}	0.717 ± 0.065 ^{a,b}	0.036 ± 0.003 ^{d}	0.73
240	9.029 ± 0.035 ^{e}	0.061 ± 0.006 ^{d,e}	0.648 ± 0.025 ^{a,b}	0.023 ± 0.001 ^{e,f}	0.65
SFC - Sunflower oil cake					
0	8.128 ± 0.022 ^{f}	-	-	-	-
96	10.201 ± 0.051 ^{a,b}	0.019 ± 0.001 ^{f}	0.210 ± 0.017 ^{e,f}	0.021 ± 0.002 ^{e,f,g}	0.16
168	10.063 ± 0.036 ^{a,b,c,d}	0.016 ± 0.003 ^{f}	0.182 ± 0.032 ^{f}	0.010 ± 0.002 ^{f,g,h}	0.14
240	10.031 ± 0.061 ^{a,b,c,d}	0.013 ± 0.001 ^{f}	0.146 ± 0.014 ^{f}	0.006 ± 0.001 ^{h}	0.14
SBC - Soybean oil cake					
0	8.215 ± 0.045 ^{f}	-	-	-	-
96	10.144 ± 0.082 ^{a,b,c,d}	0.012 ± 0.000 ^{f}	0.163 ± 0.002 ^{f}	0.013 ± 0.000 ^{f,g,h}	0.14
168	9.906 ± 0.140 ^{b,c,d}	0.012 ± 0.001 ^{f}	0.168 ± 0.008 ^{f}	0.008 ± 0.000 ^{g,h}	0.18
240	9.940 ± 0.113 ^{b,c,d}	0.014 ± 0.001 ^{f}	0.192 ± 0.020 ^{f}	0.006 ± 0.001 ^{h}	0.22
RPC - Rapeseed oil cake					
0	8.157 ± 0.071 ^{f}	-	-	-	-
96	10.178 ± 0.029 ^{a,b,c}	0.058 ± 0.003 ^{d,e}	0.607 ± 0.029 ^{a,b,c}	0.053 ± 0.003 ^{c}	0.41
168	9.869 ± 0.004 ^{c,d}	0.056 ± 0.005 ^{d,e}	0.575 ± 0.070 ^{b,c}	0.029 ± 0.004 ^{d,e}	0.45
240	9.199 ± 0.103 ^{e}	0.046 ± 0.011 ^{e}	0.469 ± 0.073 ^{c,d}	0.016 ± 0.003 ^{e,f,g,h}	0.42

*SLs mixture represent the area accumulation in the SLs crude extract analysis by HPLC. Productivity was calculated using reactor working volume (0.38 L). Values are the average of biological replicates (n=2) and their standard deviation. ^{a,b,c,d,e,f,g,h} Means that do not share a letter are significantly different for each response.

In the present study, at the end of the fermentation, the highest total oxygen consumption was reached by WOC and CNC (336.71 and 347.06 mgO_2 $g^{-1}DM_i$, respectively) followed closely by RPC (308.74 mgO_2 $g^{-1}DM_i$). Conversely, SBC and SFC presented the lowest values (224.19 and 239.73 mgO_2 $g^{-1}DM_i$, respectively). Jiménez-Peñalver et al. (2016) reported that SLs crude extract yield and COC presented a significant correlation (R^2=0.86) which is aligned with our findings (R^2=0.98).

As reported in our previous research focused on SLs production through SSF, fat content in WOC is variable (around 40-80%) (Eras-Muñoz et al., 2024a; Jiménez-Peñalver et al., 2016; Rodríguez et al., 2021a). Also, the hydrophobic substrates used in this study present different fat content. Consequently, in the present chapter SLs production is also reported per gram of initial fat to facilitate comparisons among the different oilseed cakes used (Figure 5.5). At 96 h, CNC reached the highest yield (0.743 ± 0.038 gSL g^{-1} Fat_i), whereas, at 168 h, no significant difference was observed among WOC, CNC and RPC (p-value 0.182), with this trend persisting for WOC and CNC at 240 h (p-value 0.318).

As mentioned before, the presence and concentration of hydrophobic carbon sources in the SLs production medium significantly influence the process (Daverey and Pakshirajan, 2010). This is attributed to the fact that triglyceride breakdown can also provide glycerol to the yeast, which can be used as a hydrophilic carbon source (Ingham and Winterburn, 2022). Furthermore, Shah et al. (2017) reported that a high saturated fat concentration in alternative substrates is favorable for SLs production. During production, saturated fat is more easily hydrolyzed into fatty acids; therefore, higher saturate levels result in a greater

concentration of fatty acids and, ultimately, a higher SLs yield. These findings align with the results obtained, where CNC presented the highest production related to initial fat content, being the CNC the oilcake with the highest saturated fatty acid content (20.35%).

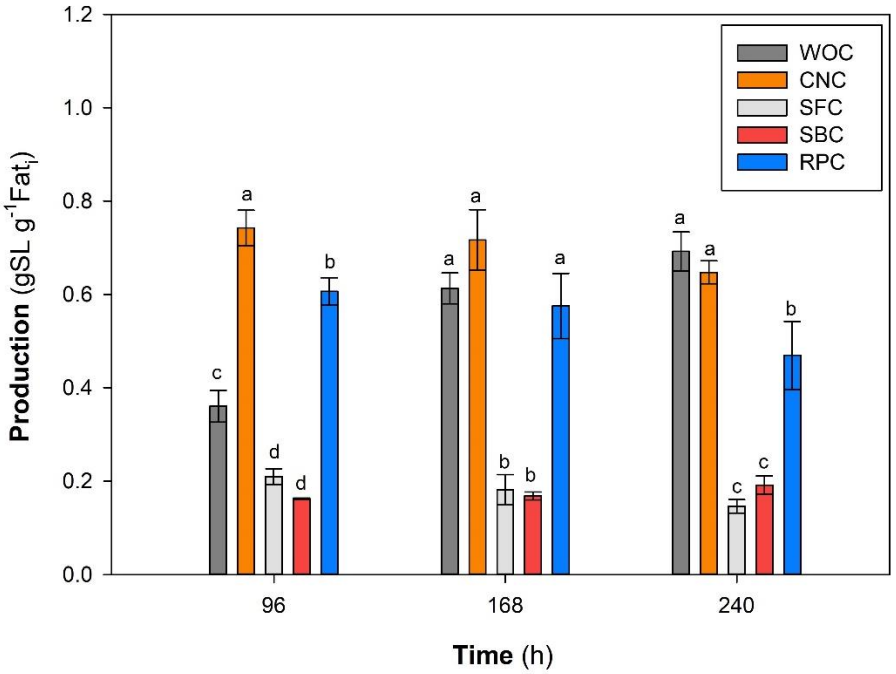

Figure 5.5 SLs crude extract production by hydrophobic source initial fat content. Error bars represent the standard deviation of biological replicates (n=2). Means that do not share a letter are significantly different for each fermentation time (p-value < 0.05).

When comparing product/hydrophobic substrate yields (Y_{P/S_Fat}) based on SLs crude extract total gram per gram of hydrophobic substrate fat content, WOC and CNC presented similar yields of 1.69 and 1.60 g g^{-1}, respectively. RPC reached 1.48 g g^{-1}, while SFC and SBC exhibited the lowest values of 0.50 and 0.28 g g^{-1}, respectively. Moreover, growth/hydrophobic substrate yields (Y_{X/S_Fat}) based on CFU per gram of hydrophobic substrate fat content showed that WOC reached 0.88 total CFU g^{-1} while oilseed cakes yielded around 1.34 total CFU g^{-1}. These results are consistent with the results of previous Chapter 4 (Eras-Muñoz et al. 2024a), where no correlation was found between growth and SLs production

in SSF. Shah et al. (2017) reported that the hydrophobic substrate composition influences the SLs metabolic pathway due to the nitrogen content present in the substrate that promotes yeast growth and decreases yield. Specifically, this trend was reported when palm, tapis and sunflower oil were used as hydrophobic sources. Therefore, the literature supports the results obtained with SBC and SFC, as they demonstrated the highest nitrogen content, in comparison with the other oilseed cakes with 7.43% and 5.28% nitrogen (calculated on a dry basis, not necessarily soluble nitrogen), respectively.

As extensively documented in the literature, pH levels decreased during SLs fermentation processes due to yeast metabolic activity (Van Bogaert et al., 2011). Indeed, starting from an initial pH of around 5.5, the pH at the end of the fermentation (240 h) was 3.5 for WOC, 4.5 for CNC and 4.1 for RPD. In contrast, SFC and SBC exhibited pH values of 6.4 and 6.6, respectively. The pH rise observed in these fermentations is in concordance with the lower SLs production achieved using those oilseed cakes. To et al. (2022) highlighted that pH is the most influential parameter in the SLs production process.

5.4.2 *Sophorolipids characterization*

Previous studies focused on SLs production emphasized that the use of alternative substrates could result in new SLs congeners (Chapter 4) that may differ in their characteristics and properties (Amiri and Habibi, 2024). From the studied substrates, the fatty acid profile shows that WOC is mainly composed of oleic acid (C18:1) in 83.62%, followed by SFC (54.14%) and RPC (54.65%). Meanwhile, CNC and SBC cakes are mostly composed of linoleic acid (C18:2) at 58.68 and 55.39%, respectively. Researchers reported a decrease in SLs

production when using shorter (≤C16) or longer (≥C20) fatty acids, as well as fatty acids with multiple unsaturation sites (Felse et al., 2007). In the oilseed cakes used herein, free fatty acids of C20, C22 and C24 were present in low quantities.

In addition, the SLs crude extract is a mixture of congeners that could contain impurities mainly of a hydrophobic nature such as lipids or residual oil (Baccile et al., 2013; Eras-Muñoz et al., 2024a). Therefore, their analytical quantification should be complemented with more precise techniques such as HPLC. Further, HPLC-UV analysis reflects that even diacetylated lactonic C18:1 is reported as the main produced congener other types of SLs were produced when using oilseed cakes (Figure 5.6).

According to the literature, peaks appearing during the first 10 minutes correspond to impurities followed by acidic forms due to their lower hydrophobicity compared to lactonic ones (Jiménez-Peñalver et al., 2020). When comparing the peak area, the main peak of SLs derived from WOC belonged to diacetylated lactonic C18:1 with a retention time of 25 min. Even diacetylated lactonic C18:1 was also produced in different ratios when oilseed cakes were used, CNC and RPC presented higher peak variability in comparison with SBC and SFC. RPC main peaks belonged to congeners such as acetylated acidic C18:2 and diacetylated acidic C18:1, while CNC main peak corresponded to acetylated acidic C18:2 at a retention time of 17.2 min. Based on oilseed cake characterization, these profiles and congeners production is related to the oilseed cake composition due to both oilseed cakes present mainly oleic and linoleic acid, respectively.

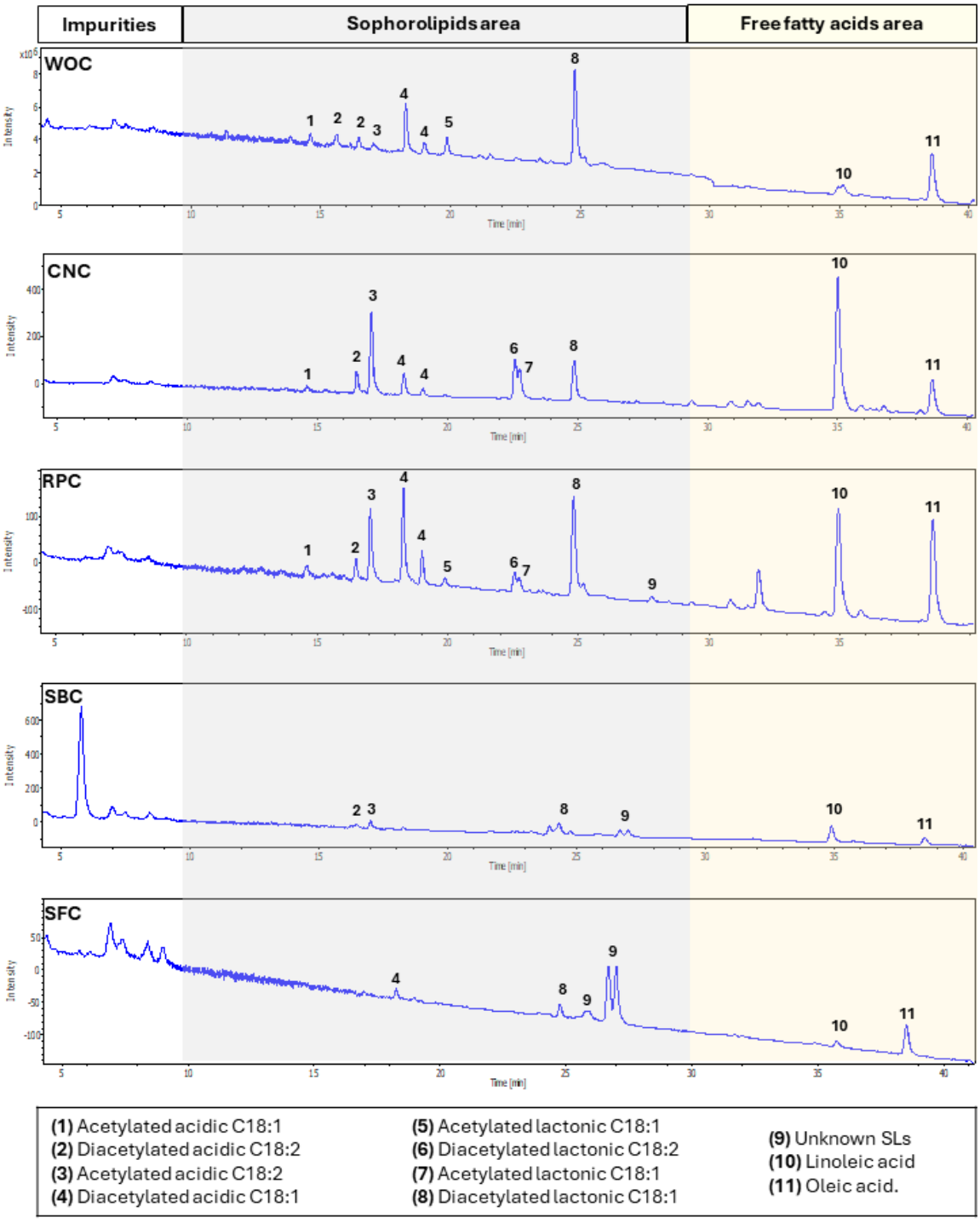

Figure 5.6 HPLC-UV spectra coupled with HPLC-MS chromatograms for the identification of the obtained SLs. Abbreviation: WOC, winterization oil cake; CNC, corn oilseed cake; RPC, rapeseed oilseed cake, SBC, soybean oilseed cake; SFC, sunflower oilseed cake.

5.4.3 *Biosurfactants properties*

Regarding previous works, SLs can effectively emulsify and stabilize emulsions with various types of hydrocarbons and oils such as motor oils, crude oil, vegetable oils among others (da Silva et al., 2021; Shah and Daverey, 2021). Moreover, these properties are a decisive characterization for their application in food and cosmetic industry (Pal et al., 2023). Indeed, biosurfactant emulsification and oil displacement capacity depend on the carbon sources used for their production. The obtained results are presented in Table 5.3.

Despite the different SL congeners distribution observed in the different crude extracts, the emulsification index in the oil-in-water system does not show significant differences (p-value>0.05) between the assessed oilseed cakes. However, E24 showed that Triton X-100 presented a higher emulsification index at 1 mg mL^{-1} while at 0.1 mg mL^{-1}, no significant differences were found (p-value 0.74) compared to the obtained SL crudes. Emulsification stability seems to be influenced by SLs crude extract concentration. Findings derived from the emulsion test revealed that emulsions containing biosurfactants exhibited stability persisting up to 168 h post-test. Conversely, at the same analyzed time, the emulsion with the non-ionic surfactant Triton X-100 (0.1 mg mL^{-1}) experienced a decrease of 18.11% in its stability. The oil displacement test revealed that the commercial Triton X-100 exhibited a superior dispersion capacity compared to the crude extracts. However, the dispersion capacity of the obtained SLs crude extracts aligns consistently with values reported in the literature for promising biosurfactants produced from yeasts. As reported by da Silva et al. (2021) a formulated commercial biosurfactant produced by *S. bombicola* can generate a

clear zone diameter in motor oil of 8.55 ± 0.32 cm when cotton oil (50 g L^{-1}) was used as the hydrophobic source.

It must be highlighted that literature in both SmF and SSF report the use of SLs crude extract for emulsion and characterization purposes. Nevertheless, is important to note that SLs crude extract contains impurities and residual free fatty acids, which can influence the assay and potentially generate false positives. Therefore, further work must be focused on implementing a partial purification method for SLs crude extracts obtained mainly from SSF to eliminate these interfering compounds. Nonetheless, the comparable results obtained for the different crude extracts suggest similar potential general applications for the SLs produced through SSF, regardless of the substrate cake used.

Table 5.3 Properties of SLs crude extract produced using alternative hydrophobic sources and compared with the commercial Triton-X 100.

Hydrophobic carbon source/ SLs concentration	Emulsification index (%)				Oil displacement (cm)
	24 h		168 h		
	1 mg mL^{-1}	0.1 mg mL^{-1}	1 mg mL^{-1}	0.1 mg mL^{-1}	10 mg mL^{-1}
SBC	58.63 ± 1.84 ^{b}	56.83 ± 1.40 ^{a}	55.87 ± 1.99 ^{b}	52.94 ± 1.45 ^{a}	6.40 ± 0.57 $^{a, b}$
SFC	58.81 ± 0.68 ^{b}	57.64 ± 3.65 ^{a}	57.38 ± 1.53 ^{b}	56.15 ± 1.96 ^{a}	5.85 ± 0.49 ^{b}
RPC	61.93 ± 0.17 ^{b}	56.53 ± 3.50 ^{a}	59.14 ± 3.07 ^{b}	56.73 ± 4.32 ^{a}	7.75 ± 0.35 ^{a}
CNC	61.52 ± 1.52 ^{b}	58.60 ± 1.78 ^{a}	60.64 ± 3.09 ^{b}	56.05 ± 0.53 ^{a}	7.40 ± 0.57 $^{a, b}$
WOC	60.84 ± 3.11 ^{b}	57.34 ± 2.16 ^{a}	60.24 ± 3.82 ^{b}	58.65 ± 2.99 ^{a}	7.50 ± 0.14 ^{a}
Triton-X 100	77.21 ± 2.43 ^{a}	55.01 ± 3.55 ^{a}	71.30 ± 6.42 ^{a}	45.05 ± 1.58 ^{b}	8.00 ± 0.01 ^{a}

$^{a, b}$ Means that do not share a letter are significantly different for each SLs crude extract concentration at the same analyzed time (p-value < 0.05).

5.4.4 *Scale-up comparison*

CNC and RPC show good potential as hydrophobic substrates presenting competitive yields when compared with WOC as a reference substrate. Their effect on SLs production was investigated at 22 L and validated at 0.5 L. The lack of temperature control in 22 L bioreactor led to a temperature increase due to fermenting solids self-heating because of metabolic heat release. Temperature ranged from 23.5 to 43.5 ºC for CNC and from 20.0 to 44.0 ºC for RPC, while it remained constant at 30 ºC in the validation scale.

Even though the 22 L respirometry profile for CNC could not be shown due to technical issues, in the validation scale (Figure 5.7a) a maximum sOUR of 3.49 ± 0.34 $mgO_2\ g^{-1}DM_i\ h^{-1}$ was reached with a COC of 203.21 ± 18.88 $mgO_2\ g^{-1}DM_i$ which is similar to the results obtained in section 5.4.1. Regarding SLs crude extract production by CNC, it was significantly different between the scales (p-value 0.089) obtaining a yield of 0.052 ± 0.011 g $g^{-1}DM_i$ at 22 L which was 22.39% lower than the obtained at the validation scale (0.067 ± 0.004 g g^{-1} DM_i, statistically equal from the reported in section 5.4.1, p-value 0.20).

On the other hand, RPC profiles were similar despite the scale (Figure 5.7b). At 22 L, the maximum sOUR achieved was 2.86 $mgO_2\ g^{-1}DM_i\ h^{-1}$, which was similar to that obtained at the validation scale (2.88 ± 0.29 $mgO_2\ g^{-1}DMi\ h^{-1}$). Moreover, COC presented a negligible difference of 2.14% between both scales (for 22 L, 172.90 and for 0.5 L 176.65 ± 17.17 $mgO_2\ g^{-1}DM_i$, respectively). SLs crude extract production using RPC at both scales was similar (p-value 0.05). At 22 L, 0.042 ± 0.008 g $g^{-1}DM_i$ were achieved while at 0.5 L 0.059 ± 0.007 g $g^{-1}DM_i$ (statistically equal to the reported in section 5.4.1, p-value 0.96).

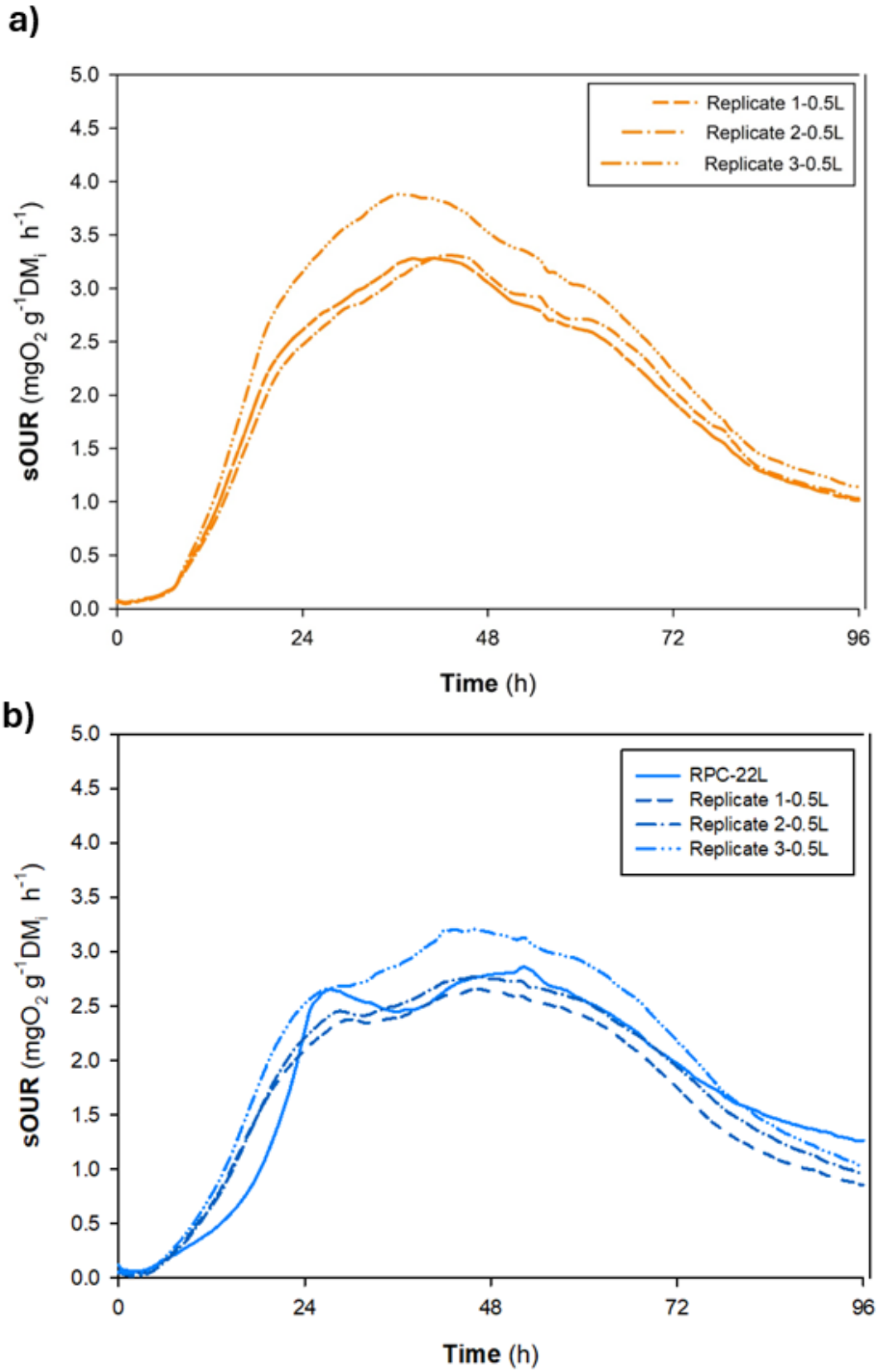

Figure 5.7 Respirometry profiles of SLs production using alternative hydrophobic substrates at different working scales (22 and 0.5 L). a) Corn oilseed cake (CNC) and b) Rapeseed oilseed cake (RPC).

As a consequence of the low production, diacetylated lactonic C18:1 was poorly quantified at the 22 L scale reaching 0.006 ± 0.004 and 0.005 ± 0.003 g $g^{-1}DM_i$ for CNC and RCP, respectively. As previously spotlighted, the SLs crude extract could contain impurities. The SLs mixture present in the SLs crude extracts obtained at 22 L scale represents 28.2% and 32.7% of the total crude mixture for RPC and CNC, respectively.

Even though the obtained outcomes validate the solid matrix preparation and the reproducibility of the fermentation process at 0.5 L scale, the SSF scale-up, as well documented, presents some drawbacks that mainly rely on the heterogeneity and complexity of the matrix, as well as on the mass and heat transfer limitations (Oiza et al., 2022; Rodríguez et al., 2021a). For that reason, to better understand the process, results are also discussed based on reactor heights (Figure 5.8) comparing values from the top, middle and bottom of the reactor.

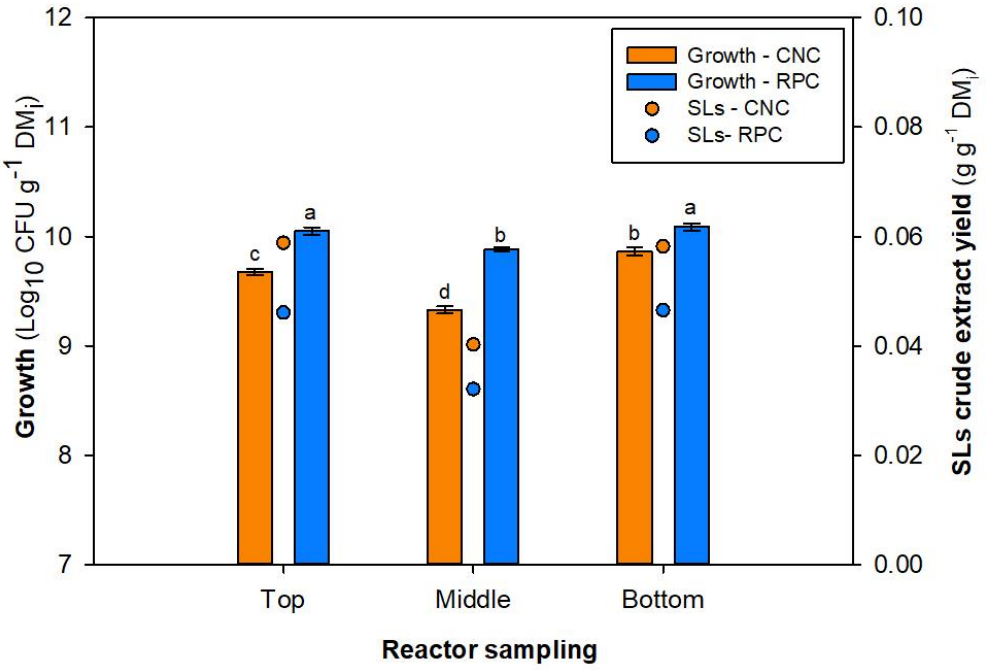

Figure 5.8 Scale-up main outcomes for SLs crude extract production based on reactor heights. Means that do not share a letter are significantly different for yeast growth (p-value < 0.05).

When comparing yeast growth based on reactor heights significant differences were found in CNC fermentation (p-value 0.001) while for RPC the middle of the reactor presented a significant difference (p-value 0.011) in comparison with top and bottom samples. SLs production in the center of the reactor decreased by 46.52% and 46.88% for CNC and RPC, respectively. Temperature values in the center of the reactor were around 44 ºC, 7.14% and 8.28%, higher than the top and bottom of the reactor for CNC and RPC, respectively (Figure 5.9).

Rodríguez et al. (2021a) reported a temperature over 35 ºC when using winterization oil cake and proposed the strategy used in this work, increasing aeration to decrease temperatures. The obtained temperature profiles confirm the performance of this strategy as a decrease from 44 ºC to 37 ºC was observed in both reactors. It is well documented that the optimal growing temperature for *S. bombicola* is around 25-30 ºC (Yang et al., 2019; Van Bogaert et al., 2011). The dynamic profile of temperatures obtained by Rodriguez et al. (2021a) exceeded 30 ºC however SLs crude extract production was slightly higher than that obtained at lab scale. Contrary, Vedaraman and Venkatesh, (2010) reported that increasing the temperature from 25 ºC to 35 ºC has adverse effects on SLs production, which aligns with our findings.

Based on the findings, it is evident that the combination of elevated temperature and reduced fat content in the fermentation medium diminishes the production of SLs. Therefore, forthcoming research in SSF should explore alternative methods to control temperature increases, such as implementing agitation or evaluating the effectiveness of different bioreactor configurations, like

tray reactors that have been used successfully for conidia production (Sala et al., 2022).

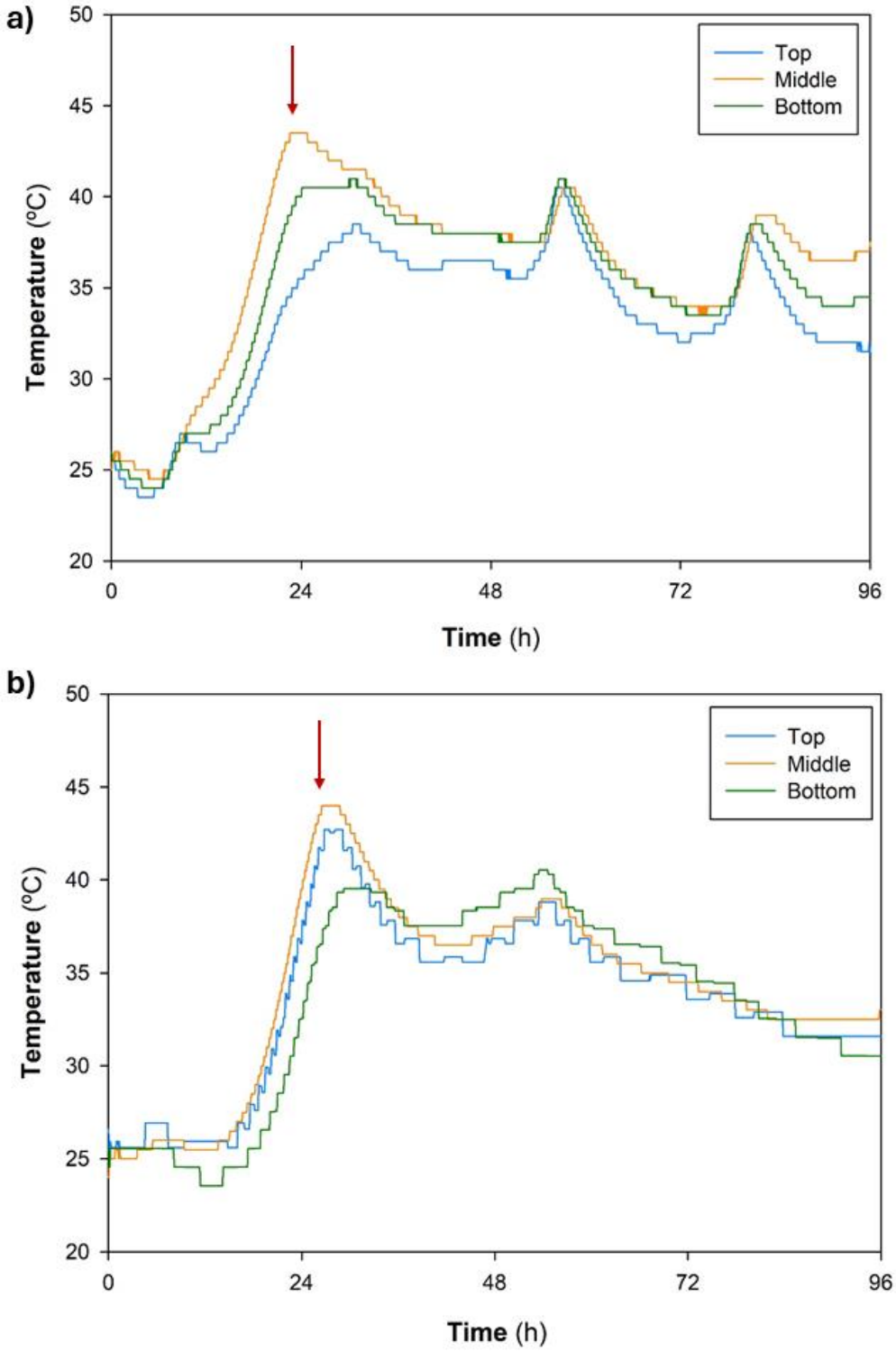

Figure 5.9 Temperature profiles in the 22 L bioreactor at the top, middle and bottom. a) Corn oilseed cake (CNC) and b) Rapeseed oilseed cake (RPC).Red arrow represents the point at which the airflow was increased.

5.4.5 *Fat concentration influence*

Based on the ratio of fatty acids in SLs crude extract and in the total oil consumption, Yang et al., (2019) estimated that only 40% of the oils that entered the cells are used to generate SLs when using the *S. bombicola* wild-type in the fermentation process. Also, as discussed before, the fat concentration is linked with fermentation performance. Therefore, to explore the influence of fat concentration in the process, preliminary experiments were performed (as described in section 5.3.1), using CNC as a hydrophobic substrate because of CNC reported excellent results at 96h and 168 h (gram per gram of initial fat). Fermentations were allowed for 96 and 168 h using different ratios of fat by supplementing CNC with WCNC. As shown in Table 5.4, no significant differences (p-value 0.063) were found in yeast growth between the combinations at each fermentation time. The highest SLs crude extract production was achieved at 168h by combination 50:50 (0.13 g g^{-1} DM_i) which also achieved the highest productivity (0.076 ± 0.005 g L^{-1} h^{-1}).

As highlighted in section 5.4.2, other SLs congeners were produced. In this context, HPLC chromatograms revealed that at retention times of 25 and 17.02 min, the most representative peaks were identified, corresponding to diacetylated lactonic C18:1 (LC18:1, 2ac) and acetylated acidic C18:2 (Ac. C18:2, 1ac), respectively. Although the highest diacetylated lactonic C18:1 production was achieved with the 50:50 ratio (0.013 g g^{-1} DM_i), it should be highlighted that production efficiency cannot be reported based solely on a single congener when using second-generation feedstocks, as this would introduce bias into the production report. Accordingly, SLs mixture present in the SLs crude extract was also quantified by adding the areas as described in section 3.10.1. Results

demostrated that combination 100:0 which presents the lower initial fat concentration (0.07 g g^{-1} DM_i) reached the higher SLs mixture present in the crude extract (0.64 g g^{-1} and 0.78 g g^{-1} at 96 and 168 h, respectively) in comparison with the other combinations

Furthermore, results demonstrated that at 96 h, comparing the different fat ratios, no relationship was found between SLs crude extract production and fat initial concentration (R^2=0.08). However, a linear relationship (R^2=0.93) was found at 168 h, reporting 0.48 g of SLs crude extract produced per g of initial fat (Figure 5.10). Nonetheless, it should be noted that no correlation was observed between diacetylated lactonic C18:1 or the SLs mixture and the initial fat content (R^2 = 0.04 and 0.01, respectively). This behavior could be attributed to the SLs crude extract containing impurities, such as long-chain free fatty acids. Consequently, as can be observed in Table 5.4 a higher initial fat content (0.13 g g^{-1} DM_i) results in more impurities being carried over into the final product (~44%) while a lower initial fat content (0.07 g g^{-1} DM_i) results in less impurities (~29%). Thus, future work should focus on optimizing the initial fat concentration required for SLs production through SSF.

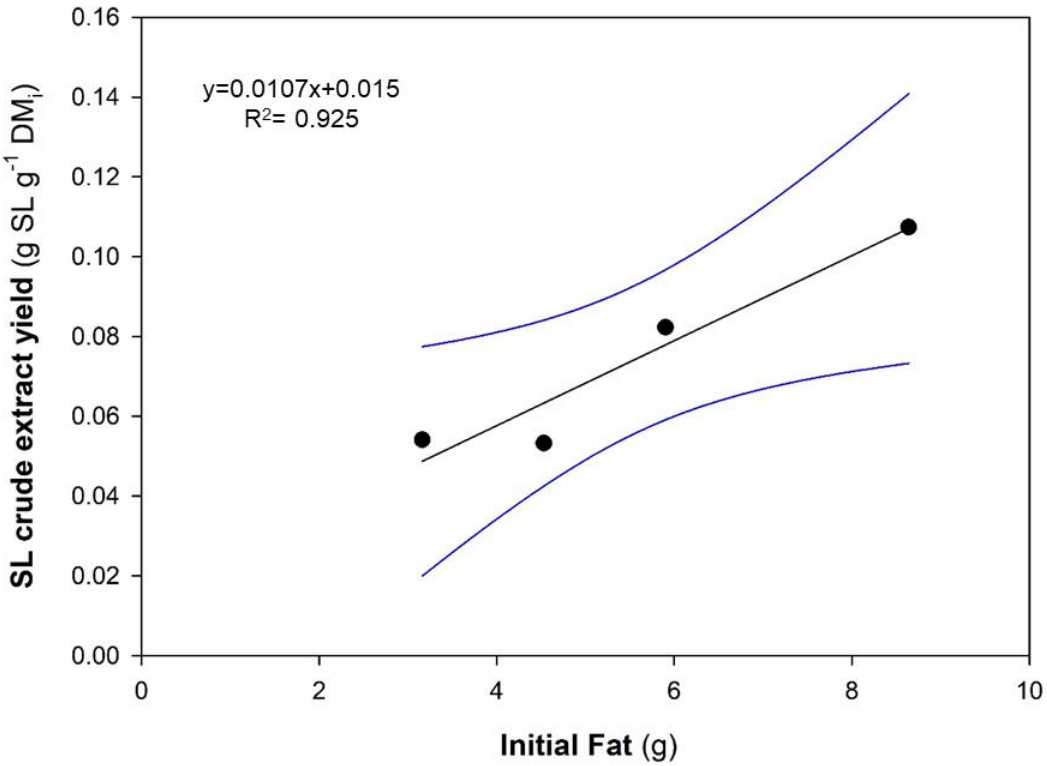

Figure 5.10 Initial fat content vs SLs crude extract production at 168 h of fermentation.

Table 5.4 Influence of fat ratio on yeast growth and SLs production by fat supplementation using artificial WCNC

Ratio	Time	Fat content	Growth	SLs crude extract	Productivity	LC18:1, 2ac production			SLs mixture in the crude extract	Relevance peak Area	
										LC18:1, 2ac (25 min)	Ac. C18:2, 1ac (17.02 min)
CNC: WCNC	h	g g^{-1} DM_i	Log_{10} CFU$g^{-1}DM_i$	g g^{-1} DM_i	g L^{-1} h^{-1*}	g g^{-1}SL	g $g^{-1}DM_i$	%	g g^{-1}	mAU*min	
	0	0.07	8.17 ± 0.03	-	-	-	-	-	-	-	-
100:0	96	0.05	10.18 ± 0.06[a]	0.051 ± 0.003	0.057 ± 0.002	0.139	0.007	17.72	0.640	1,513	2,713
	168	0.04	9.97 ± 0.16[a]	0.054 ± 0.006	0.032 ± 0.003	0.200	0.011	20.83	0.781	2,189	3,128
	0	0.09	8.14 ± 0.01	-	-	-	-	-	-	-	-
75:25	96	0.05	10.07 ± 0.09[a]	0.053 ± 0.006	0.060 ± 0.006	0.165	0.009	21.86	0.614	1,793	1,958
	168	0.05	9.98 ± 0.20[a]	0.053 ± 0.007	0.034 ± 0.006	0.197	0.011	22.38	0.715	2,147	2,890
	0	0.13	8.08 ± 0.01	-	-	-	-	-	-	-	-
50:50	96	0.06	10.04 ± 0.06[a]	0.064 ± 0.006	0.076 ± 0.005	0.146	0.009	21.04	0.574	1,591	1,581
	168	0.05	9.86 ± 0.13[a]	0.082 ± 0.005	0.055 ± 0.004	0.160	0.013	23.80	0.551	1,742	1,610

*SLs mixture belongs to areas addition from the SLs crude extract HPLC-UV analysis; Productivity values were calculated based on reactor working volume (0.38 L). Results are presented as mean ± standard deviation of biological replicates (n=2).

5.5 Conclusion

In summary, novel hydrophobic substrates were able to produce SLs through SSF, expanding the range of second-generation hydrophobic substrates studied in this process. Corn oilseed cake and rapeseed oilseed cake demonstrated the most promising results at the laboratory scale. The significance of hydrophobic carbon sources as essential substrates was underscored, emphasizing their impact on SLs yield, the composition of new congeners and their properties. Even though scaling up did not increase SLs production, process reproducibility at 0.5 L scale was demonstrated and challenges such as temperature increase were highlighted. Moreover, it was demonstrated that fat concentration in the initial mixture influences process performance and the quality of SLs crude extract, with higher fat content leading to more impurities. Thus, future research should focus on optimizing fat content and scaling up, as well as implementing a partial purification process to remove impurities from SLs crude extract, thereby promoting the use of SSF-produced SLs in industrial applications.

Chapter 6

Alternative nitrogen sources in SmF

This chapter was developed as part of an international stay at The University of Manchester in collaboration with the research group of Dr. James Winterburn.

Contents of this chapter were published in *Waste Management*, 186, 23-34.
Screening of alternative nitrogen sources for sophorolipid production through submerged fermentation using *Starmerella bombicola*.
Eras-Muñoz, E., Wongsirichot, P., Ingham, B., Winterburn, J., Gea, T. and Font, X. (2024b).

Summary

Once analyzed alternative hydrophilic, hydrophobic and nitrogen sources for SLs production via SSF, in this chapter, several hydrolysates from agricultural byproducts, such as wheat feed, rapeseed meal, coconut waste and palm waste were used as nitrogen sources to explore the sustainable SLs production via SmF. This was done in collaboration with Dr. James Winterburn during a research stay in The University of Manchester, to gain knowledge on this fermentation technology. The four hydrolysates overperformed the controls after 168 h of fermentation using *Starmerella bombicola* ATCC 22214. Wheat feed and coconut waste hydrolysates were the most promising feedstocks presenting a linear relationship between yeast growth and diacetylated lactonic C18:1 production at total nitrogen concentrations below 1.5 g L^{-1} (R^2= 0.90 and 0.83, respectively). At 0.31 g L^{-1} total nitrogen, wheat feed hydrolysate achieved the highest production, yielding 72.20 ± 1.53 g L^{-1} of SLs crude extract and 60.05 ± 0.56 g L^{-1} of diacetylated lactonic C18:1 at shake flask scale with productivities of 0.43 and 0.36 g L^{-1} h^{-1}, respectively. Results were confirmed in a 2 L bioreactor increasing 15% diacetylated lactonic C18:1 production. Moreover, wheat feed hydrolysate supplemented only with a hydrophobic carbon source was able to produce mainly diacetylated lactonic C18:1 congener (88.5% wt.), suggesting that the composition of the hydrolysate significantly influences the congeners profile. Overall, this study provides valuable insights into agricultural byproduct hydrolysates as potential nitrogen feedstocks for SLs production and their further application in industrial biotechnology. The use of N-rich hydrolysates can be easily extrapolated to SSF systems according to results of Chapter 4. A scheme of the chapter is provided in Figure 6.1.

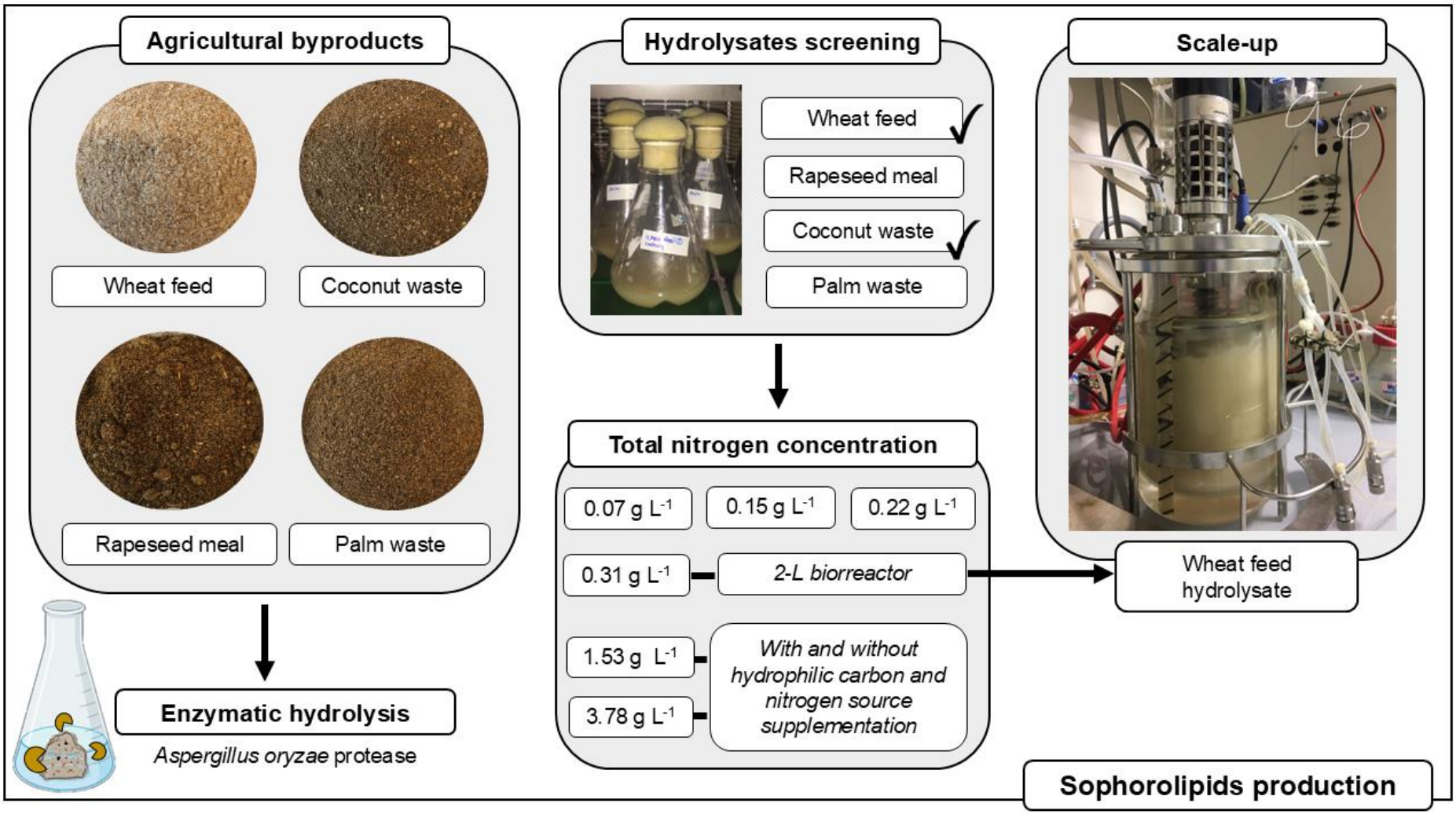

Figure 6.1 Scheme of the chapter

6.1 Introduction

Literature reported the use of several second-generation feedstocks for SLs production (section 1.8). Nevertheless, literature in this field has focused on exploring alternative substrates that can serve as hydrophilic and/or hydrophobic carbon sources, creating a gap in terms of alternative nitrogen sources. Indeed, this thesis contributes to filling this gap in SSF, as detailed in Chapter 4, highlighting that nitrogen sources in SmF remain a relevant area of study, which is the focus of this chapter.

In this regard, agro-industrial byproducts are characterized by their abundant nutrient content, including sugars and lipids, making them excellent nutrient sources for microbial growth in fermentation processes (Banat et al., 2014). They can be used directly on SSF as a supporting material (Rodríguez et al., 2021) or as substrates as demonstrated in Chapter 5; or they can be pretreated to increase the availability of nutrients and enable their use in either SSF or SmF processes. Enzymatic hydrolysis has emerged among other pretreatments due to higher specific activities, thermal stability, better resistance to environmental factors and inhibitors and improved combinations of various enzymes (Yang et al., 2011). Indeed, enzymatic hydrolysis has been applied successfully as pretreatment for SLs production by authors such as Kaur et al. (2019) and Wongsirichot et al. (2022a) using food waste and agricultural residues, respectively.

The goal of this chapter is to assess enzymatic hydrolysates derived from agricultural byproducts such as wheat feed, rapeseed meal, coconut waste and palm waste as nitrogen sources for SLs production and to select the best hydrolysate for subsequently scaling up the process. To the best of our

knowledge, this is the first report of using these agricultural biomasses as nitrogen feedstocks for SLs production.

6.2 Materials

As described in section 3.6.2, coconut waste (CW), palm waste (PW), rapeseed meal (RM) and wheat feed (WF) hydrolysates were used as alternative nitrogen sources and were prepared using the commercial *Aspergillus oryzae* protease as detailed in section 3.7 and were characterized following section 3.10.3-3.10.6. Moreover, rapeseed oil was used throughout this chapter as the hydrophobic carbon source (section 3.1.2).

6.3 Experiments

To assess the feasibility of using hydrolysates as a nitrogen source, the CSL and $(NH_4)_2SO_4$ used in the control groups (section 3.8.1) were substituted by WF, RM, CW and PW hydrolysates. The TN concentration was standardized at 0.15 g L^{-1} while the concentration of rapeseed oil and total glucose were maintained at 100 g L^{-1} each, consistent with the control group.

Figure 6.2, illustrates the appearance of the obtained fermented broths. While some visual conclusions about the fermentation process can be deduced from the accumulation of residual oil and the precipitation of SLs. The resulted fermented broth was analyzed for quantification and cell dry weight as described in Sections 3.9 and 3.10.1.

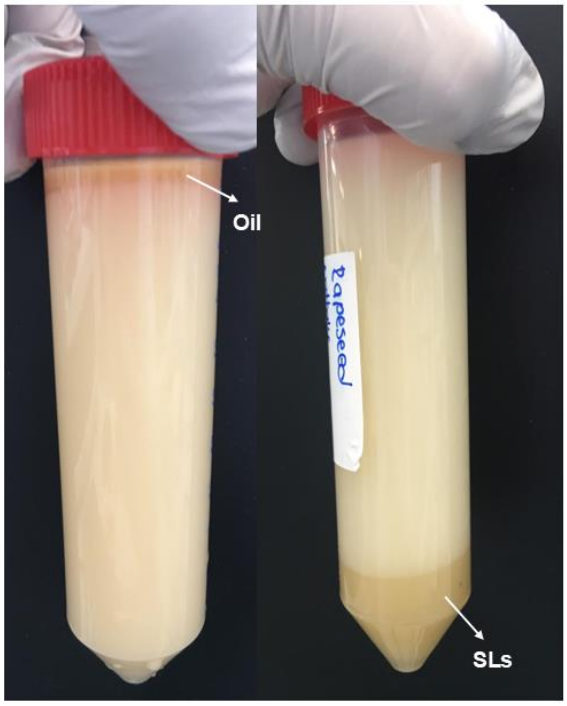

Figure 6.2 Fermented broth before their analysis.

6.3.1 *Evaluation of different nitrogen concentrations*

The hydrolysates that showed the best outcomes (SLs production) were used to assess a wider range of nitrogen concentrations. Initially, a TN concentration of 0.15 g L^{-1} was employed, with the impact on the SL-producing fermentation evaluated at half (0.07 g L^{-1}), one- and a half-fold (0.23 g L^{-1}) and two-fold (0.31 g L^{-1}) concentrations (Figure 6.3).

The maximum TN hydrolysates (1.53 g L^{-1} and 3.78 g L^{-1}) were also evaluated with (CW-1.53; WF-3.78) and without (CW-1.53B; WF-3.78B) glucose supplementation. Once the fermentation media was prepared, it was filter sterilized followed by rapeseed oil addition and seed inoculation. Finally, shake flask fermentations were carried out according to section 3.8.1.

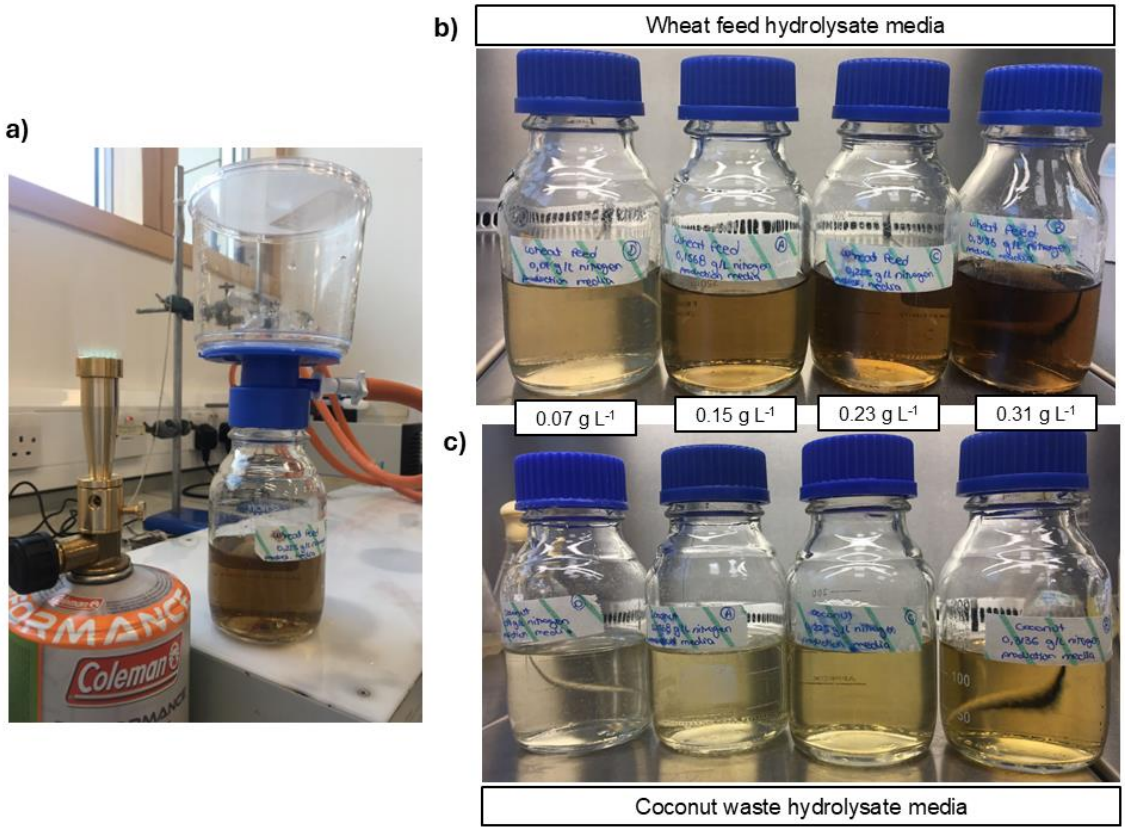

Figure 6.3 Fermentation media appearance using several nitrogen concentrations. a) Media vacuum filtration, b) Wheat feed hydrolysate (WF) dilutions (WF) and c) Coconut waste hydrolysate (CW).

6.3.2 *Batch bioreactor fermentation*

Scale-up was developed in UAB facilities (*Planta Pilot de Fermentació, Escola d'Enginyeria*). The process was carried out as described in section 3.8.2. Moreover, due to the utilization of a second batch of WF hydrolysate, the fermentation medium for the scale-up was also assessed in triplicate using 4-bottom baffled Erlenmeyer flasks (250 mL) at 30 ºC, 200 rpm for 168 h. Figure 6.4, shows the evolution of the fermented broth over time. Samples were taken each 24 h and were analyzed as described in section 3.9 and 3.10.1.

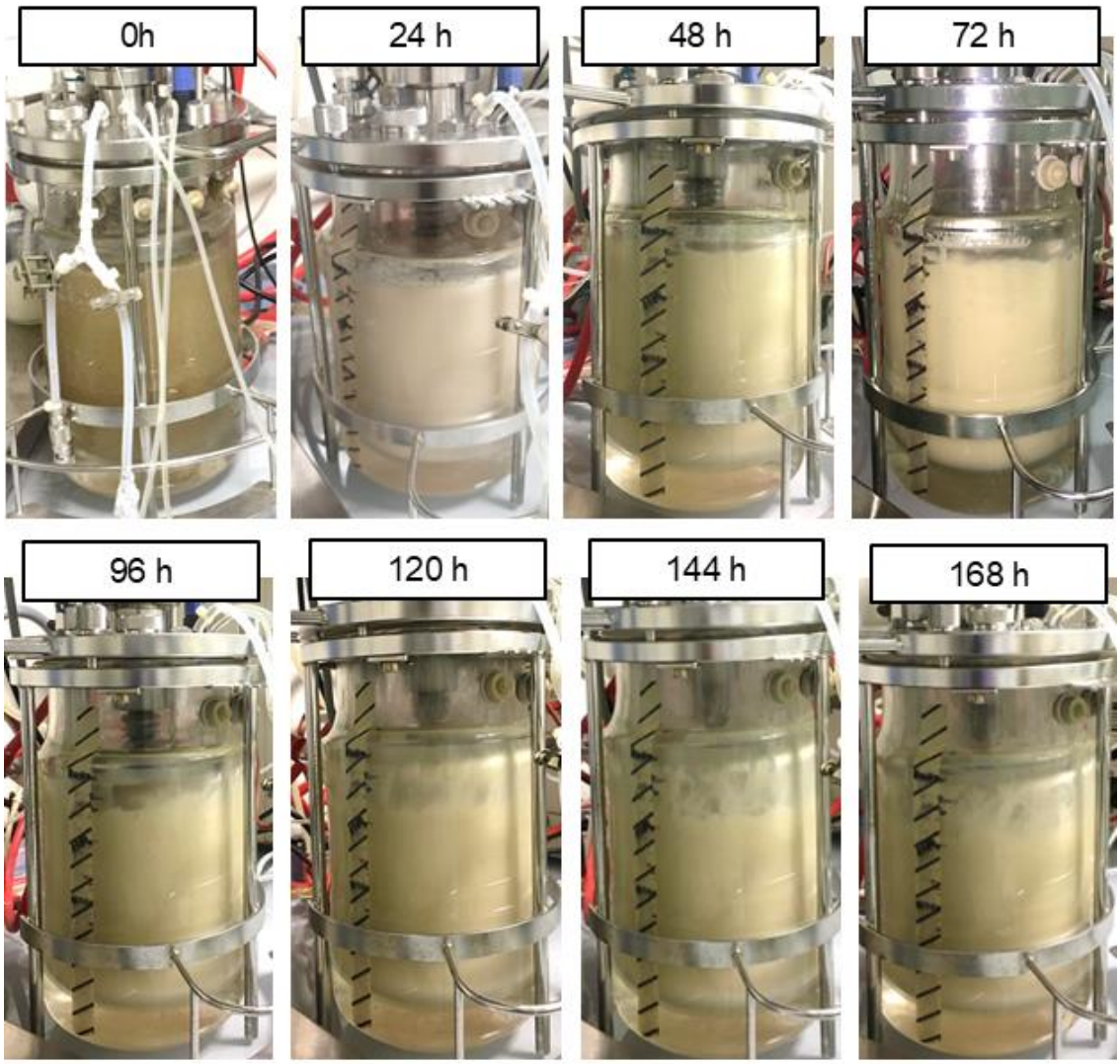

Figure 6.4 Evolution of the fermentation medium used for the 2 L scaling up over time.

6.4 Results and Discussion

6.4.1 *Feedstock and hydrolysate characterization*

Biomass hydrolysates have the potential to serve as nutrient-rich substitutes as alternatives to pure substrates whilst providing the nutrients required for SLs production. Table 6.1 provides a summary characterization of each agricultural byproduct and the corresponding hydrolysates obtained using *A. oryzae* protease. Results reveal that WF and RM hydrolysates exhibited the highest TN values (3.78 ± 0.28 g L^{-1} and 2.47 ± 0.51 g L^{-1}, respectively). Conversely, PW

hydrolysate presented the lowest TN content (0.69 ± 0.09 g L^{-1}). The TN value is a critical parameter in the formulation of media for bioproducts, particularly when nitrogen limitation is required and it influences the overall success of the process (e.g. SLs). Moreover, nitrogen-containing compounds are involved in microorganism's metabolism (for amino acids and proteins building) and optimized TN levels support the production of necessary enzymes for specific metabolic pathways and bioprocesses (Nurfarahin et al., 2018).

Sugar profile revealed notable distinctions among the four hydrolysates (Figure 6.5). Specifically, WF hydrolysate exhibited a variety of sugars, including cellobiose, mannose, xylose, arabinose and fructose. Additionally, WF hydrolysate demonstrated the highest glucose concentration (6.95 ± 0.05 g L^{-1}), followed by CW (1.48 ± 0.03 g L^{-1}) and PW (1.30 ± 0.02 g L^{-1}) hydrolysates, whereas no detectable glucose content was observed in RM hydrolysate. Conversely, other sugars such as fructose were identified in both RM and PW hydrolysates, while in CW hydrolysate mannose. Literature reports that the mean composition of RM includes oil, proteins, lignocellulosic fibers and phenolics the last one can act as a microbial inhibitor during the fermentation process (Lomascolo et al., 2012). Nonetheless, the remarkable variability of these hydrolysates concerning their glucose/nitrogen content underscores their strong potential as promising feedstocks for SLs production (Rivera et al., 2019; Wongsirichot et al., 2021).

Regarding biomass pretreatment, the selection of enzymatic hydrolysis was determined by its effectiveness in biomass transformation and previous research conducted in the context of SLs production (Nayak and Bhushan, 2019). As reported by De Castro and Sato (2014), the biochemical characterization of *A.*

oryzae protease revealed that the enzyme exhibited maximum activity within a pH range of 5.0–5.5 and an optimal temperature for enzymatic activity of between 55 °C and 60 °C. In this study, the reported enzyme conditions were applied to achieve a considerable degree of hydrolysis. This approach ensured the thorough validation of the biomass hydrolysate's suitability as media for the subsequent steps. It's important to keep in mind that using proteases releases peptides while some other biopolymers such as cellulose will remain in the solid fraction after hydrolysis, to be valorized in subsequent steps.

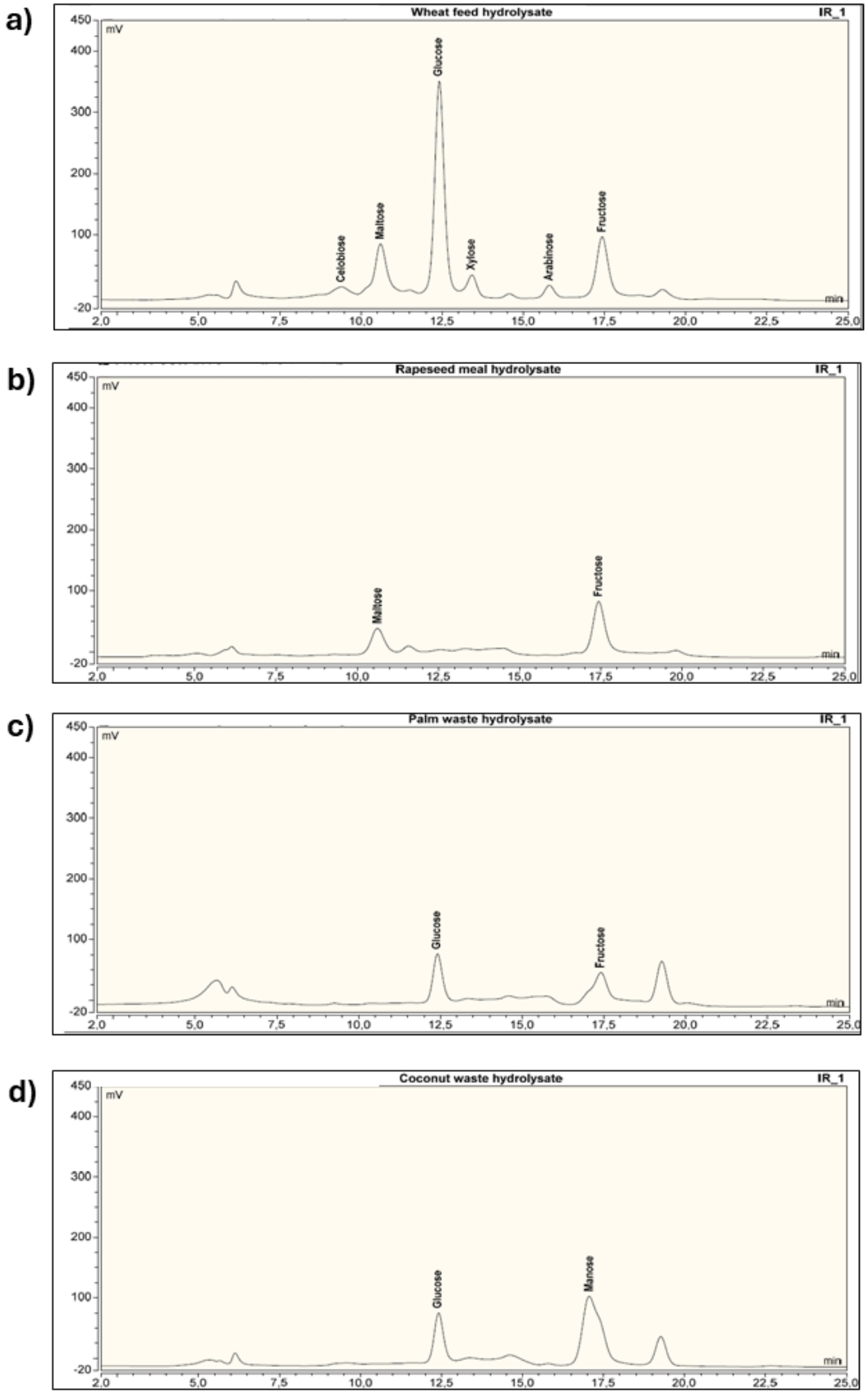

Figure 6.5 Hydrolysates sugar profile. a) Wheat feed (WF) and b) Rapeseed meal (RM), c) Palm waste (PM) and d) Coconut waste (CW).

Table 6.1 Characterization of the alternative substrates and their corresponding hydrolysates used in this chapter.

Feedstock	Biomass characterization									Hydrolysate characterization				
	pH	Glucose ($g\ kg^{-1}$)	DM (%)	MC (%)	OM (%, db)	C (%, db)	H (%, db)	N (%, db)	S (%, db)	C:N		pH	Glucose ($g\ L^{-1}$)	TC ($g\ L^{-1}$)	TN ($g\ L^{-1}$)
Wheat feed (WF)	6.25	5.21 ± 0.14	89.71 ± 0.14	10.29 ± 0.14	95.35 ± 0.05	43.69 ± 0.10	6.34 ± 0.01	2.58 ± 0.10	0.14 ± 0.01	16.93	Batch 1	5.34	6.23 ± 0.03	24.04 ± 0.25	3.78 ± 0.28
											Batch 2	5.51	7.67 ± 0.07	18.64 ± 0.40	4.19 ± 0.12
Rapeseed meal (RM)	5.67	n.d	85.18 ± 0.15	14.82 ± 0.15	92.55 ± 0.01	44.80± 0.03	6.20 ± 0.06	6.17 ± 0.16	0.67 ± 0.01	7.26		4.50	n.d	18.46 ± 0.61	2.47 ± 0.51
Coconut waste (CW)	5.46	0.73 ± 0.01	91.72 ± 0.79	8.28 ± 0.79	95.44 ± 0.25	44.57 ± 0.17	6.51 ± 0.11	2.57 ± 0.02	0.18 ± 0.02	17.34		5.62	1.48 ± 0.03	13.20 ± 0.36	1.53 ± 0.14
Palm waste (PW)	4.92	0.16 ± 0.01	91.73 ± 0.29	8.27 ± 0.29	95.84 ± 0.79	45.41 ± 0.06	6.54 ± 0.09	1.73 ± 0.10	0.10 ± 0.01	26.25		5.02	1.30 ± 0.02	10.78 ± 1.91	0.69 ± 0.09

Abbreviations: db, dry basis; n.d, not detected; DM, dry matter; MC, moisture content; OM, organic matter; C, carbon, H, hydrogen; N, nitrogen; S, sulfate; C:N, carbon and nitrogen ratio; TC, total carbon; TN, total nitrogen. Data presented as mean values ± standard deviation of the sample analysis (n=3).

6.4.2 *Evaluation of biomass hydrolysates supplemented with glucose and rapeseed oil*

The results obtained from the biomass screening demonstrate that the hydrolysates tested supported the growth of *S. bombicola* and led to the production of SLs (Figure 6.6). Despite having the same nutrient concentration (Section 3.8.1), Control 1 exhibited a higher dry cell weight (p-value = 0.02) and SLs crude production than Control 2 (p-value = 0.01). These findings are aligned with those of To et al., (2022) who reported that acidic SLs were produced when using ammonium salts, though an improvement in cell growth was observed. Zhou et al. (2022) report that the crude proteins, amino acids, minerals, vitamins, reducing sugars and organic acids present in CSL contribute to a better yeast fermentation performance, something that was observed in this study when comparing the two control groups. Moreover, a comparison between the control groups reveals no significant differences in glucose consumption (p-value = 0.37). Nevertheless, Control 1 group, presented the highest nitrogen consumption which is aligned with the highest dry cell weight achieved whilst the tested hydrolysates showed no statistically significant differences (p-value > 0.05) in their nitrogen consumption.

Ma et al. (2011) and Nurfarahin et al. (2018) underscore that as a consequence of the inherent complexity of organic nitrogen sources they may also contain a carbon component in their structure that could act as a metabolic precursor which promotes cell growth, enzymes production (such as lactone esterase) and polysaccharide formation. In this sense, Control 1 exhibited a cell dry weight of 7.43 ± 0.30 g L^{-1}, whereas Control 2 attained 4.29 ± 0.58 g L^{-1}.

Concerning the hydrolysates, WF, RM, PW and CW yielded 5.47 ± 0.83 g L^{-1}; 3.65 ± 0.83 g L^{-1}; 5.31 ± 0.07 g $L^{-1}$and 5.08 ± 0.79 g L^{-1}, respectively. Growth statistical analysis revealed significant differences between both control groups (p-value 0.02) and RM compared to the Control 1 group (p-value 0.01), while the remaining treatments did not exhibit significant differences (p-value > 0.05). The difference between the control groups seems to be associated with a low concentration of nutrients in the fermentation medium of Control 2 due to the absence of CSL. Whereas in the case of RM a high concentration of inhibitory compounds (e.g. phenolic derivates, tannins, protease inhibitors, among others) could be present as reported by Wongsirichot et al. (2022b).

Ma et al. (2020) reported that SLs production is known to be significantly enhanced when both hydrophilic and hydrophobic carbon sources are present in the fermentation medium. In our case, both glucose (hydrophilic carbon source) and rapeseed oil (hydrophobic carbon source) were used, contributing to the notable increase in SLs production. Concerning SLs crude extract production, the tested hydrolysates gave a higher concentration compared to Control 1 (20.78 ± 0.57 g L^{-1}) showing their potential to serve as viable sources of glucose, nitrogen and micronutrients that can be used by *S. bombicola* and incorporated into SL metabolic pathway. Notably, the highest production was achieved using WF hydrolysate (59.12 ± 0.17 g L^{-1}), followed by CW (57.11 ± 0.49 g L^{-1}). Moreover, RM and PW hydrolysates do not exhibit significant differences (p-value 0.99) in SLs production levels (54.44 ± 0.06; 54.59 ± 0.30 g L^{-1}, respectively). In a prior study conducted by Zhu et al. (2013), RM was used for biosurfactants production, specifically surfactin, through SSF, in which the role of RM was predominantly limited to acting as a support and co-substrate. In this context, the current findings

illustrate that WF, RM, PW and CW hydrolysates enable SL production through SmF. Nevertheless, despite the minor discrepancy between the production ranges, WF and CW yielded superior result.

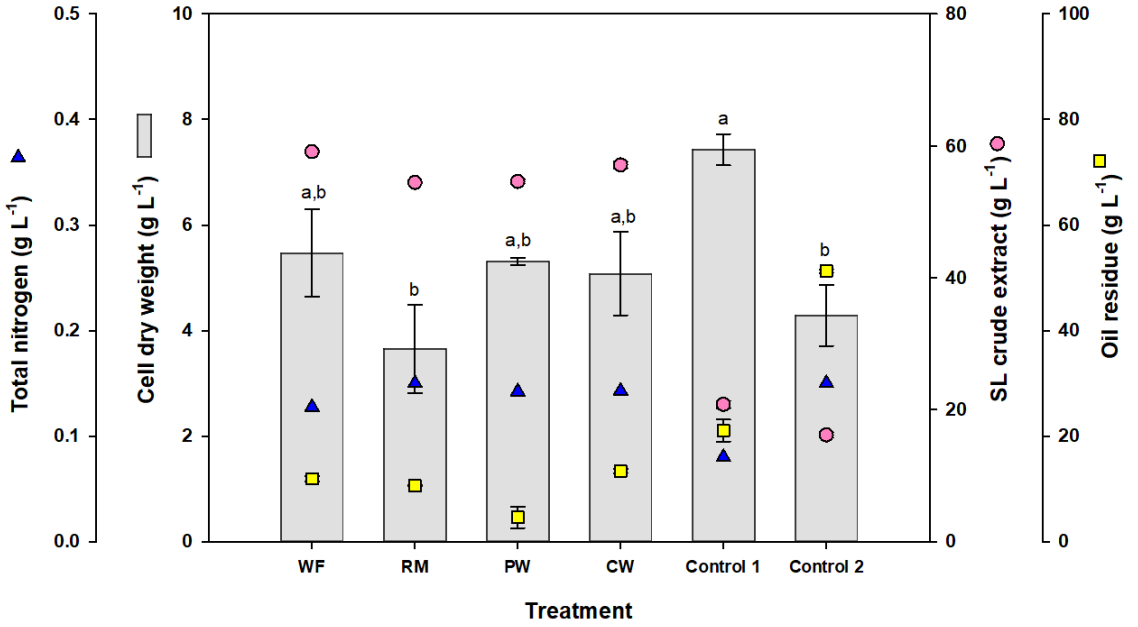

Figure 6.6 Shake flask fermentations using agricultural byproducts hydrolysates as a nitrogen source at 168 h. Hydrolysate abbreviations: WF (wheat feed), RM (rapeseed meal), PW (palm waste), CW (coconut waste). Control 1 (ammonium sulphate + CSL) and Control 2 (ammonium sulphate). Error bars represent the standard deviation of biological replicates (n=2). Cell dry weight means that do not share a letter are significantly different (p-value < 0.05).

It is well known that SLs crude extract is a mixture of congeners and that the partially purified product exhibits a yellowish honey-like viscous appearance that can be attributed to the higher water content (40%-60% residual water) and the presence of other impurities, such as fatty acids. Dierickx et al. (2022) and Ingham et al. (2023) reported that SLs gravimetric quantification through solvents extraction may co-extract unidentified compounds inherent in the feedstock potentially resulting in an overestimation of production levels. In this sense, to provide more meaningful SL results, the analytical quantification of diacetylated lactonic C18:1 by HPLC-UV is also reported and used as a determinant parameter in this study. The production of diacetylated lactonic C18:1 on the

tested hydrolysates (Figure 6.7) ranges from 34.90 to 47.40 g L^{-1} which represents a four-fold increase compared to Control 1 (11.64 ± 0.44 g L^{-1}). Furthermore, the diacetylated lactonic C18:1 congener was predominantly synthesized when using the hydrolysates with a purity ranging from 65% to 80%, whereas in Control 1 and Control 2 purity was around 56% and 42%, respectively. Importantly, to assess significant differences among the tested hydrolysates, Tukey's test was used. When comparing WF and CW hydrolysates, no significant difference was observed in the results (p-value 0.09). This suggests that both hydrolysates are promising feedstocks and support their potential use in subsequent steps.

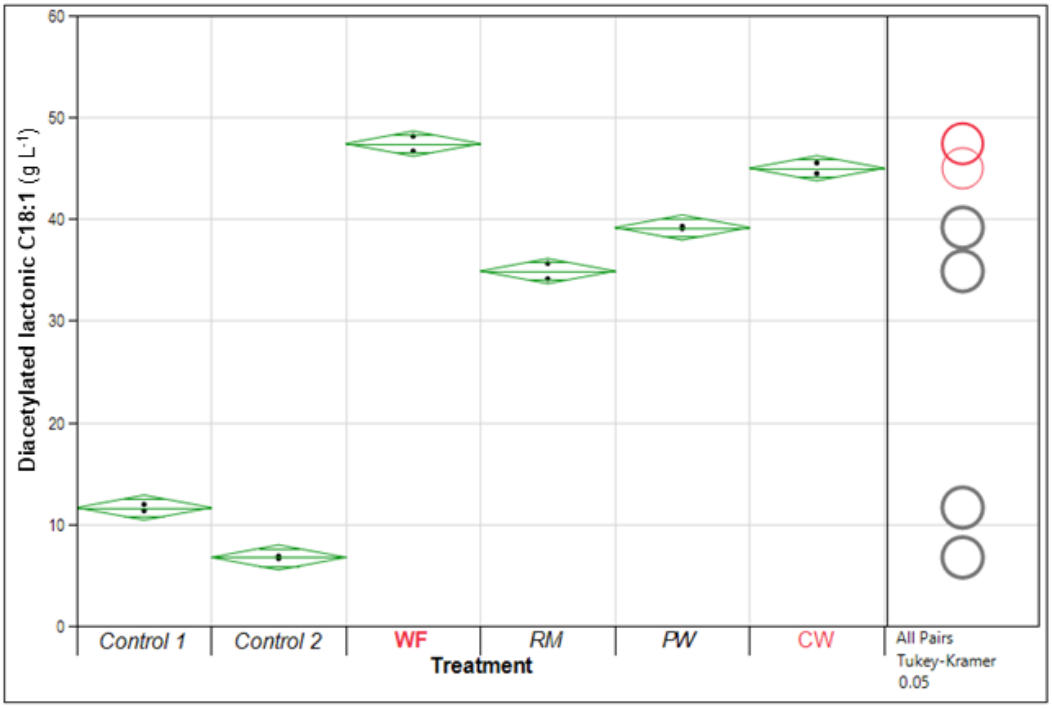

Figure 6.7 Comparison of diacetylated lactonic C18:1 production at 168 h using agricultural byproducts hydrolysates as nitrogen source. The box graph displays individual samples as dark points (biological replicates for each treatment n=2). Diamonds indicate the 95% confidence interval, with the central line denoting the group mean and the overlap marks at the top and bottom the standard deviation. Tukey's test is visually presented through the circle comparison, in red groups which means are significantly similar and in grey groups which means are different from the selected group (WF).

6.4.3 *Effect of nitrogen concentrations on SL production*

Several nitrogen concentrations were evaluated using WF and CW hydrolysates due to their capacity of producing diacetylated lactonic C18:1. The results indicated that the tested concentrations exhibited similar behavior for both hydrolysates (Figure 6.8).

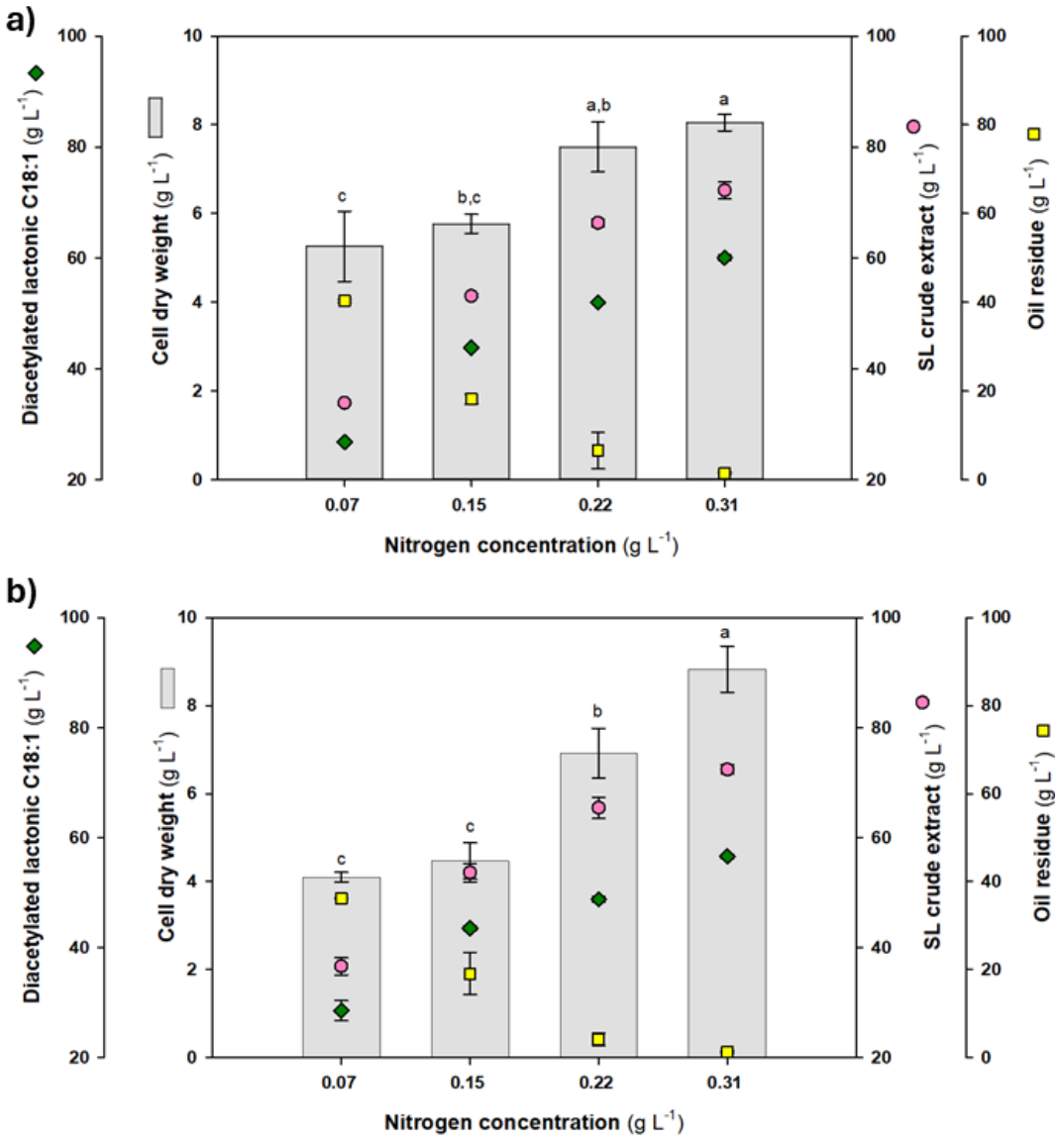

Figure 6.8 Influence of total nitrogen concentration on SLs production. Main fermentation outcomes using agricultural byproducts hydrolysates at total nitrogen concentrations range from 0.07 to 0.31 g L^{-1}. a) Wheat feed hydrolysate (WF) and b) Coconut waste hydrolysate (CW). Error bars represent the standard deviation of biological replicates (n=2). Cell dry weight means that do not share a letter are significantly different (p-value < 0.05).

The highest SLs crude extract production using WF hydrolysate (72.20 ± 1.53 g L^{-1}) was achieved at 0.31 g L^{-1} TN. Conversely, the lowest (33.84 ± 0.17 g L^{-1}) was obtained at 0.07 g L^{-1} TN. Furthermore, CW hydrolysate displayed a similar production pattern, achieving the highest (72.40 ± 0.82 g L^{-1}) and the lowest (36.60 ± 1.56 g L^{-1}) SLs crude extract production under the same TN concentrations. Moreover, it is noteworthy that both hydrolysates exhibited a SLs crude extract volumetric productivity of 0.43 g L^{-1} h^{-1} at 0.31 g L^{-1} TN concentration.

At nitrogen concentrations of 0.07 g L^{-1}, no significant differences (p-value 0.31) were observed between WF and CW in the final diacetylated lactonic C18:1 concentration (26.81 ± 0.20 and 28.55 ±1.82 g L^{-1}, respectively). Similarly, at 0.15 g L^{-1} TN, an insignificant difference (p-value 0.59) was found in the final SLs crude extract concentrations (43.78 ± 0.27 and 43.54 ± 0.45 g L^{-1}, respectively). However, WF and CW showed significant differences at 0.22 g L^{-1} TN (51.94 ± 0.25 and 48.79 ± 0.54 g L^{-1}, respectively) and 0.31 g L^{-1} TN (60.05 ± 0.56 and 56.63 ± 0.37 g L^{-1}, respectively) with p-values of 0.02 for both cases. These findings may be associated with the composition of the used hydrolysates. Consequently, future research could focus on identifying potential amino acids within such byproducts or hydrolysates that could serve as supplementary nitrogen source and evaluate whether their presence positively impacts SLs production.

Furthermore, as reported by Ingham and Winterburn (2022), a direct relationship between SLs production and oil consumption was observed. In this study, oil consumption was directly assessed by quantifying the remaining oil via hexane extraction. Remarkably, a direct relation (R^2 = 0.91) was observed

between TN concentration and oil consumption. In the current study, an initial oil concentration of 100 g L^{-1} was used in all the treatments. After 168 h of fermentation, oil concentrations neared depletion when 0.31 g L^{-1} TN for both types of hydrolysates. Residual oil quantities of 1.42 ± 0.23 g L^{-1} and 1.29 ± 0.21 g L^{-1} were observed for WF and CW hydrolysates, respectively. Importantly, these concentrations were concurrent with the highest SLs reported production, as previously described.

Our findings validate a strong correlation between yeast growth and SLs production at the tested concentrations in a 168-h fermentation. SmF technology ensures the homogeneity of the culture medium, leading to cell growth and secondary metabolite production occurring as sequential stages. In contrast, SSF in static packed-bed bioreactors does not exhibit this behavior, as multiple metabolic stages coexist. This allows for better understanding and control of the process when using this fermentation strategy. Notably, both WF and CW hydrolysates exhibited a linear relationship in the range studied, with R^2 values of 0.90 and 0.83, respectively. This linear correlation was also reported by Marcelino et al. (2019). Thus, it is essential to undertake an extensive investigation covering a wider range of nitrogen levels and focusing on process kinetics to enable a comprehensive assessment of the correlation between yeast growth and SLs production. In this context, TN was increased to 1.53 g L^{-1} and 3.78 g L^{-1}, the uppermost limits of CW and WF hydrolysates and were evaluated with and without supplementation (Figure 6.9).

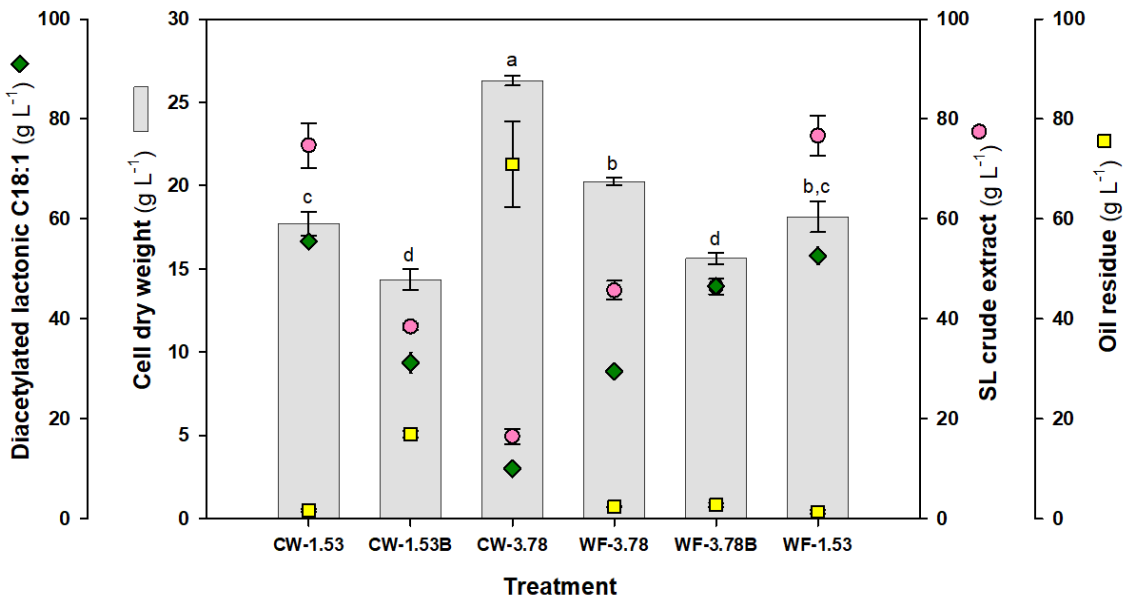

Figure 6.9 Maximum hydrolysate total nitrogen (TN) evaluation with and without glucose supplementation. Error bars represent the standard deviation of biological replicates (n=2). Cell dry weight means that do not share a letter are significantly different (p-value < 0.05). Abbreviations: CW-1.53, CW-3.78, WF-1.53 and WF-3.78 represent coconut waste (CW) and wheat feed (WF) hydrolysates at TN concentrations of 1.53 and 3.78 g L^{-1} supplemented with glucose;CW-1.53B and WF-3.78B represents CW and WF hydrolysates without glucose supplementation at 1.53 and 3.78 g L^{-1} TN.

Significant differences (p-value 0.01) in SLs crude extract production were observed when comparing CW-1.53 and CW-1.53B, while WF-3.78 and WF-3.78B showed similar outcomes (p-value 0.71). Moreover, when comparing based on TN concentration, non-significant differences were found between CW-1.53 and WF-1.53 (p-value 0.93), whereas significant differences were observed for CW-3.78 and WF-3.78 (p-value < 0.01). These variations may be attributed to the sugar profiles of each hydrolysate (section 6.4.1) and to the inorganic nitrogen source used to supplement CW to reach 3.78 g L^{-1} TN. Indeed, HPLC-UV analysis revealed that CW-3.78 mainly produces acidic SL congeners instead of lactonic ones (Figure 6.10). Additionally, CW-3.78 presented the highest oil residue content (70.96 ± 8.63 g L^{-1}) which is directly correlated with a low SLs (16.46 ±

1.50 g L^{-1}) and diacetylated lactonic C18:1 production (9.99 ± 0.06 g L^{-1}). When comparing results in terms of glucose consumption, CW-1.53 (3.23 ± 0.58 g L^{-1}) and WF-1.53 (2.22 ± 0.10 g L^{-1}) do not present significant differences (p-value 0.14). However, when the hydrolysate was employed as the sole source of nutrients significant distinctions emerged (p-value < 0.01) between CW-1.53B and WF-3.79B (2.32 ± 0.09 and 0.75 ± 0.07 g L^{-1}, respectively). This discrepancy implies that the C:N ratio within the WF hydrolysate facilitated the highest glucose consumption after 168 hours of fermentation, consequently increasing SLs production.

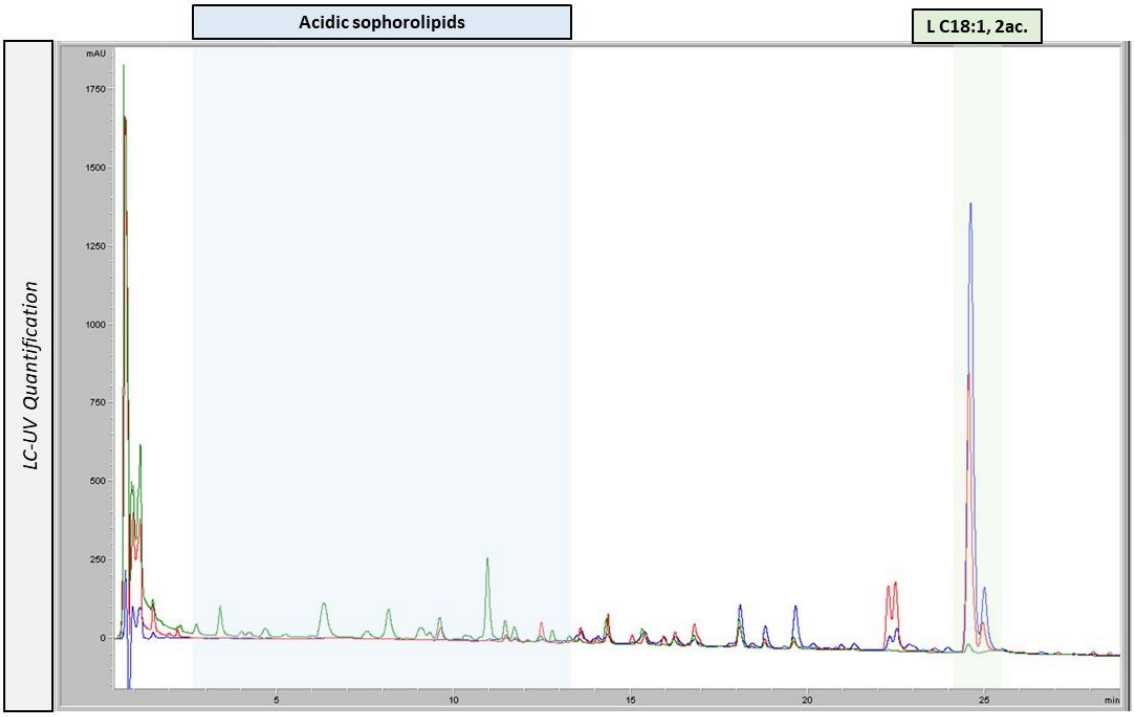

Figure 6.10 HPLC-UV sophorolipid congeners profile using WF and CW hydrolysates. Blue chromatogram: standard diacetylated lactonic C18:1 (LC18:1). Red chromatogram: Wheat feed hydrolysate at 3.78 g L^{-1} TN supplemented with glucose (WF-3.78). Green chromatogram: coconut waste hydrolysate at 3.78 g L^{-1} TN supplemented with glucose and ammonium sulfate (CW-3.78).

Gao et al. (2013) reported that the presence of high nitrogen levels in the broth foster a rapid growth rate and enable high cell density. In this way, results show that CW-1.53B and WF-3.78B do not present significant differences (p-

value 0.13) in growth (14.36 ± 0.62; 15.63 ± 0.35 g L^{-1}, respectively). In contrast, when supplemented with glucose, CW-3.78 achieved the highest cell dry weight (26.32 ± 0.31 g L^{-1}), significantly (p-value < 0.01) surpassing WF-3.78 (20.25 ± 0.21 g L^{-1}). Despite both combinations presenting higher yeast growth, there was a noteworthy decline in SL production and diacetylated lactonic C18:1 (Figure 6.11). Literature spotlight that SL production is non-growth-related and increases when the culture reaches the stationary phase (Daverey and Pakshirajan, 2010; Kaur et al., 2019). Consequently, treatments characterized by high yeast growth, such as observed in the case of CW-3.78, may suggest that the stationary phase has not been reached influencing SL production.

The evidence from this study indicates that SLs production is not correlated with biomass concentration at higher nitrogen concentrations (> 1.53 g L^{-1}). Similarly, Daverey and Pakshirajan (2010) reported that the optimal TN concentration for yeast growth was 10 g L^{-1}, while for SL production was 2 g L^{-1} which is consistent with the findings presented here. In this process fermentation time should also be considered, as prolonged fermentation periods tend to result in increased biomass, potentially leading to higher secondary metabolite production. Nevertheless, it is important to acknowledge that an extended exponential phase may delay the stationary phase required in this process so lower SL yields could be observed for the same timeframe. It is important to highlight that the standard duration of the 168-hour fermentation used in this research, as extensively documented in the literature, reflects a balance between enhanced secondary metabolite production and practical considerations stemming from achieving a high cell titer during the growth phase and cost-effectiveness (Samad et al. 2017; Wang et al., 2020; Ingham et al., 2022).

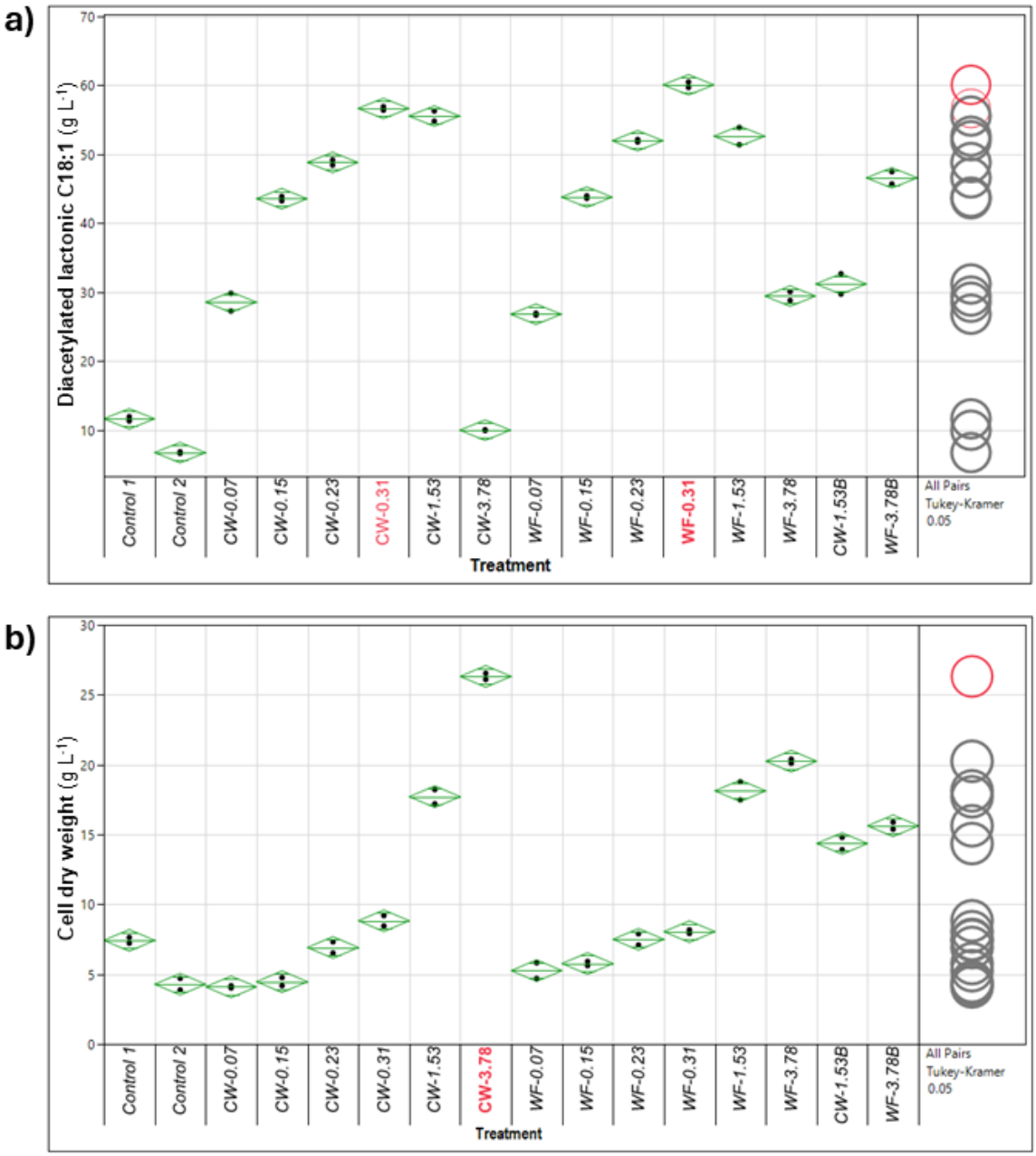

Figure 6.11 Influence of TN concentration on diacetylated lactonic C18:1 production and cell dry weight. a) Diacetylated lactonic C18:1 production (g L^{-1}) and b) Cell dry weight (g L^{-1}). The box graph displays individual samples as dark points (biological replicates for each treatment n=2). Diamonds indicate the 95% confidence interval, with the central line denoting the group mean and the overlap marks at the top and bottom the standard deviation. Tukey's test is visually demonstrated through circles comparison, in red groups which means are significantly similar and in grey groups which means are different from the selected group. Abbreviations: Control 1 (ammonium sulphate + CSL) and Control 2 (ammonium sulphate), coconut waste (CW), wheat feed (WF), treatment belongs to the tested hydrolysate followed by the nitrogen concentration (0.07; 0.15; 0.23; 0.31; 1.53 and 3.78 g L^{-1}) and the letter B represents hydrolysates without glucose supplementation.

SLs crude extract contains different SL molecules/congeners and may also contain some impurities such as long-chain fatty acids. In this way, at the end of the fermentation diacetylated lactonic C18:1, constituted approximately from 68.61% to 81.16% of the SLs crude extract. From a global perspective, CW-0.31 and WF-0.31 produced the highest concentration of diacetylated lactonic C18:1. However, WF hydrolysate resulted in a higher purity of the congener. Surprisingly, WF-3.78B yielded a broth with a dough-like texture (Figure 6.12). This result is directly associated with the achieved values of SLs crude extract (46.46 ± 1.56 g L^{-1}) and diacetylated lactonic C18:1 (46.57 ± 1.26 g L^{-1}) in this treatment. As mentioned by Yang et al. (2012), SLs in their pure form are colorless and, upon complete drying, assume the appearance of a white powder. Furthermore, the presence of white crystalline residues in fermentation broths suggests the possibility of lactonic SLs crystallization. HPLC-UV quantification revealed a higher ratio of diacetylated lactonic C18:1 production when employing WF-3.78B, comprising 88.5% of the SLs crude extract (Figure 6.13). These ratio values are higher than those achieved by Zhang et al. (2018) applying gravity separation at lab scale (74%).

Moreover, this finding is aligned with Solaiman et al. (2007), who reported that the exclusion of yeast extract and urea from the fermentation media, coupled with the use of soy molasses as the nutrient source, resulted in the attainment of pure SL in lactone form, with a purity of 87% and a volumetric productivity of 53 ± 3 g L^{-1}. As reported by Ahalliya et al. (2023), the synthesis of the hydrophilic and hydrophobic moiety and the composition of biosurfactant congeners will change and rely on the carbon source used by the microorganism. Even though glucose is the main hydrophilic source used for SLs production, it was clear that

WF hydrolysate also contains other sugars (section 6.4.1) that could potentially be used by the yeast (Samad et al., 2015; Minucelli et al., 2017). Ingham et al. (2023) highlight that *S. bombicola* could not use xylose and galactose as hydrophilic carbon; nevertheless, when a sugar mix is applied, SLs production ranges up to 40.15 g L^{-1} which is in concordance with our findings.

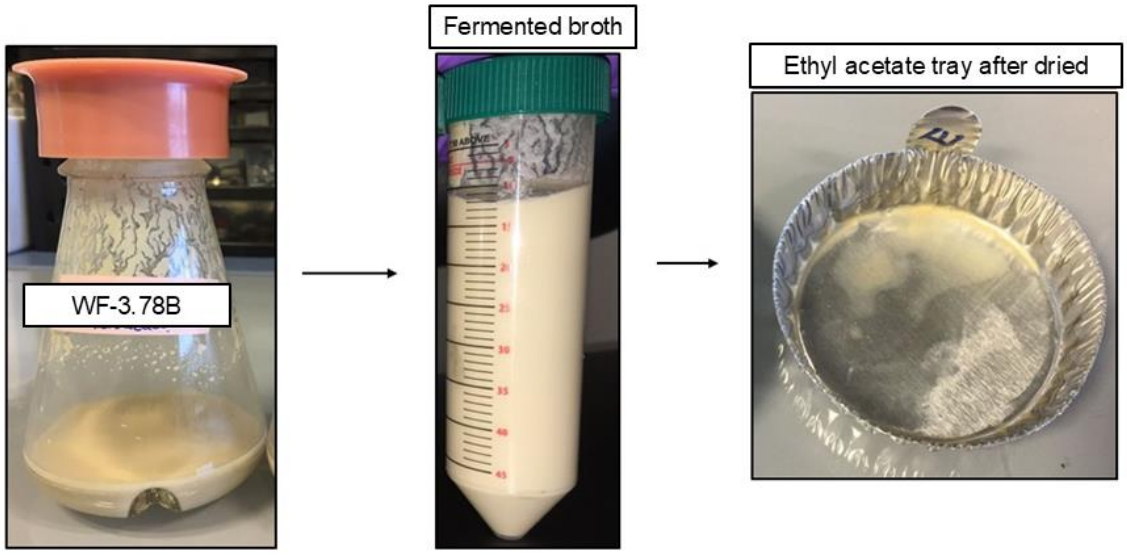

Figure 6.12 Wheat feed hydrolysate (WF-3.78B) fermented broth appearance after 168 h. Left: Erlenmeyer flask after fermentation. Middle: Fermented broth before solvents extraction. Right: Overnight dry tray belonging to ethyl acetate extraction, sophorolipid appear as a white powder.

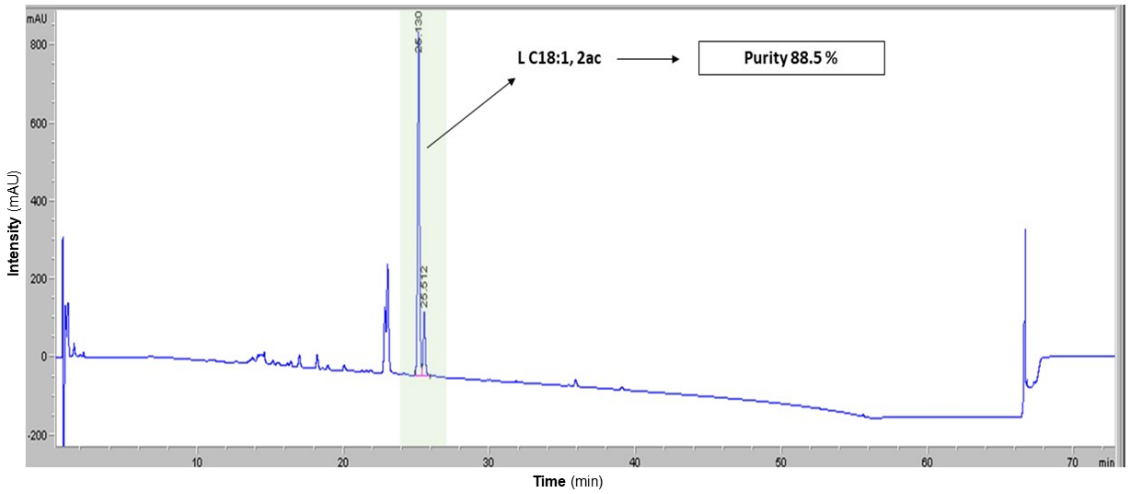

Figure 6.13 Diacetylated lactonic C18:1 quantification via HPLC-UV at 198 nm using wheat feed hydrolysate without glucose supplementation (WF-3.78B).

From an industrial perspective, our findings are promising in terms of reducing costs associated with raw substrates and aligning with the principles of the circular economy. These findings illustrate the utilization of WF hydrolysate as a dual nitrogen and carbon source which contains a C:N ratio of 6.36 that promotes SLs production. Additionally, our results showed that a low C:N ratio could influence the production of a specific SL congener. To et al. (2022) reported that an optimal C:N ratio falls within the range of 44 to 64 for lactonic SLs synthesis based only on glucose as the carbon source. Indeed, literature reported that an elevated C:N ratio facilitates the initiation of SLs biosynthesis, while a lower ratio could result in unmetabolized SLs or lead to the metabolism of previously synthesized which consequently decreases SLs titer (Van Bogart et al., 2011). It should be highlighted that SLs, as secondary metabolites, are produced in the stationary growth phase in this way an excess of nitrogen could prevent the limiting conditions required to maximize their production. Studies where alternative feedstocks were used reported that the amount of available nitrogen will also influence the choice of the agricultural byproducts due to microbial requirements (Albrecht et al., 1996; Wongsirichot et al., 2022a).

Several genetic engineering studies have been carried out to obtain pure SLs, exemplifying efforts in this direction for future application studies (Van Bogaert et al., 2016; Pala et al., 2023). However, this research supports an alternative pathway by utilizing residues such as WF. Notably, the findings reported here are promising in terms of substituting pure substrates, maximizing the potential of byproducts hydrolysates and developing a competitive and sustainable process. Even though the final SL concentrations achieved using hydrolysates as the sole nutrient source are lower compared to those obtained

with glucose supplementation, the increased costs associated with pure substrates and downstream processes at an industrial scale, combined with the demand for a high-purity final product, promote their utilization. Further sustainability assessment should help choosing between only biomass-derived, purified feedstock or the combined usage of both.

6.4.4 *Batch bioreactor fermentation*

In the existing literature SLs production using agricultural-derived hydrolysates as nitrogen source is not extensively reported. Nonetheless, processes involving hydrolysates and batch fermentations allow for the closest comparison with the results achieved in our study (Table 6.2). The results of the batch cultivation of *S. bombicola* in a bioreactor are illustrated in Figure 6.14 and operational control parameters are shown in Figure 6.15.

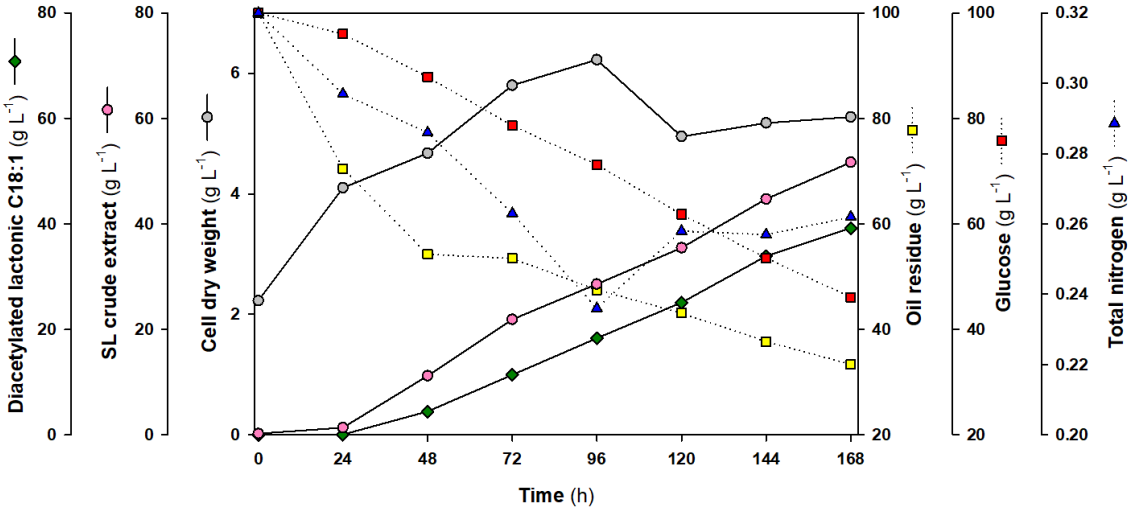

Figure 6.14 Time course fermentation in a 2 L batch bioreactor by *S. bombicola* using wheat feed hydrolysate (WF) at a TN concentration of 0.31 g L^{-1}.

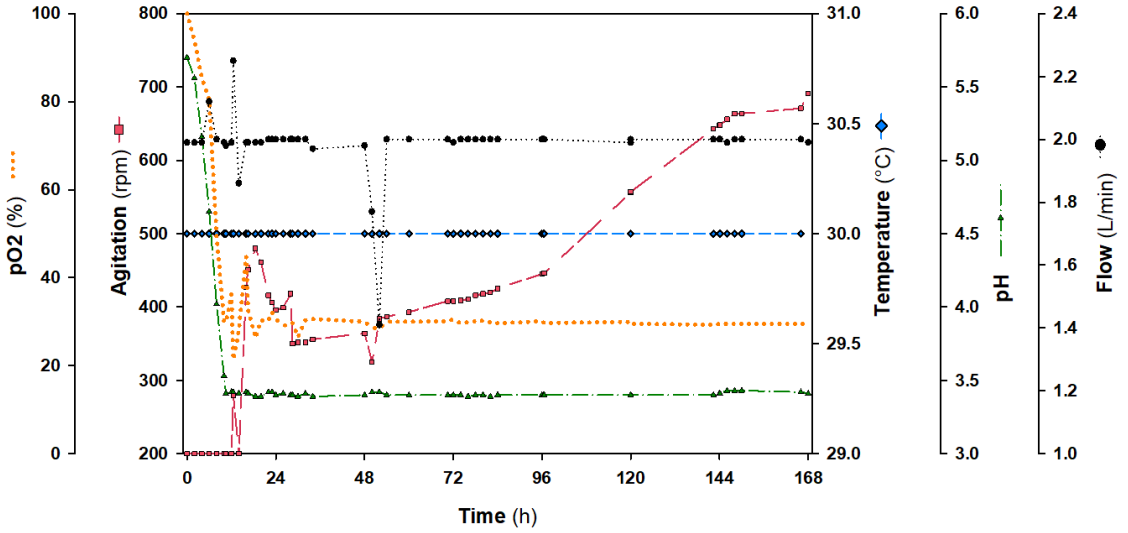

Figure 6.15 Bioreactor control parameters for SLs production using wheat feed hydrolysate at 168 h.

During the fermentation, a maximum cell growth of 6.23 ± 0.39 g L^{-1} was reach at 96 h, followed by a decrease in cell concentration to 5.28 ± 0.04 g L^{-1}. This decrease in biomass indicates the transition from the growth phase to the stationary phase, which is favorable for SLs production. Notably, significant SLs levels were observed at 48 h (11.20 ± 0.21 g L^{-1}) and at the end of the fermentation (168 h) SLs concentration was 51.70 ± 0.07 g L^{-1}, with a volumetric productivity of 0.31 g L^{-1} h^{-1} and a diacetylated lactonic C18:1 concentration of 39.24 ± 0.29 g L^{-1}. These results closely resemble those outlined by Samad et al. (2017) who achieved a SLs concentration of 52.1 g L^{-1} with a titer of 0.31 g L^{-1} h^{-1} using agricultural biomass hydrolysates as hydrophilic carbon source. Furthermore, Kaur et al. (2019) achieved a SLs concentration of 28.15 g L^{-1} with a volumetric productivity of 0.39 g L^{-1} h^{-1} at 72 h using food hydrolysates at batch scale. In contrast, in this research at the same fermentation time, a SLs crude extract concentration of 21.88 ± 1.03 g L^{-1} with a productivity of 0.30 ± 0.01 g L^{-1} h^{-1} was obtained which is related with the biomass/hydrolysate composition used in each study.

Results reveal that the highest TN consumption occurred at the 96 h of fermentation (0.23 g L^{-1}) which is within to the exponential growth phase. However, an increase in nitrogen was observed after 120 h during the stationary phase, something that could be attributed to yeast lysis. As expected, these results confirm that SLs biosynthesis is promoted during the stationary phase when a higher C:N ratio (185) was found and yeast is nitrogen limited as reported by some authors when phosphate is consumed (Roelants et al., 2019; Wang et al., 2019).

During the bioreactor fermentation glucose, oil and nitrogen concentrations exhibited a gradual decline, coinciding with the increase in both biomass formation and SLs production. However, it is important to acknowledge that the hydrophilic and hydrophobic substrates were not completely depleted, with residual quantities of 46.07 ± 2.86 g L^{-1} glucose and 33.33 ± 0.25 g L^{-1} oil at the end of the fermentation process. HPLC-MS chromatograms showed the presence of residual fatty acids which primarily belonged to derivatives of rapeseed oil, namely oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3) (Figure 6.16). Therefore, the presence of C18 fatty acids in the crude extract may be a result of triglyceride degradation by a yeast-produced lipase. However, the absence of their incorporation into sophorose could be linked to the SL synthesis pathway and enzymatic efficiency (e.g. glucose transferase I, II, among others) (Ahalaiya et al., 2023).

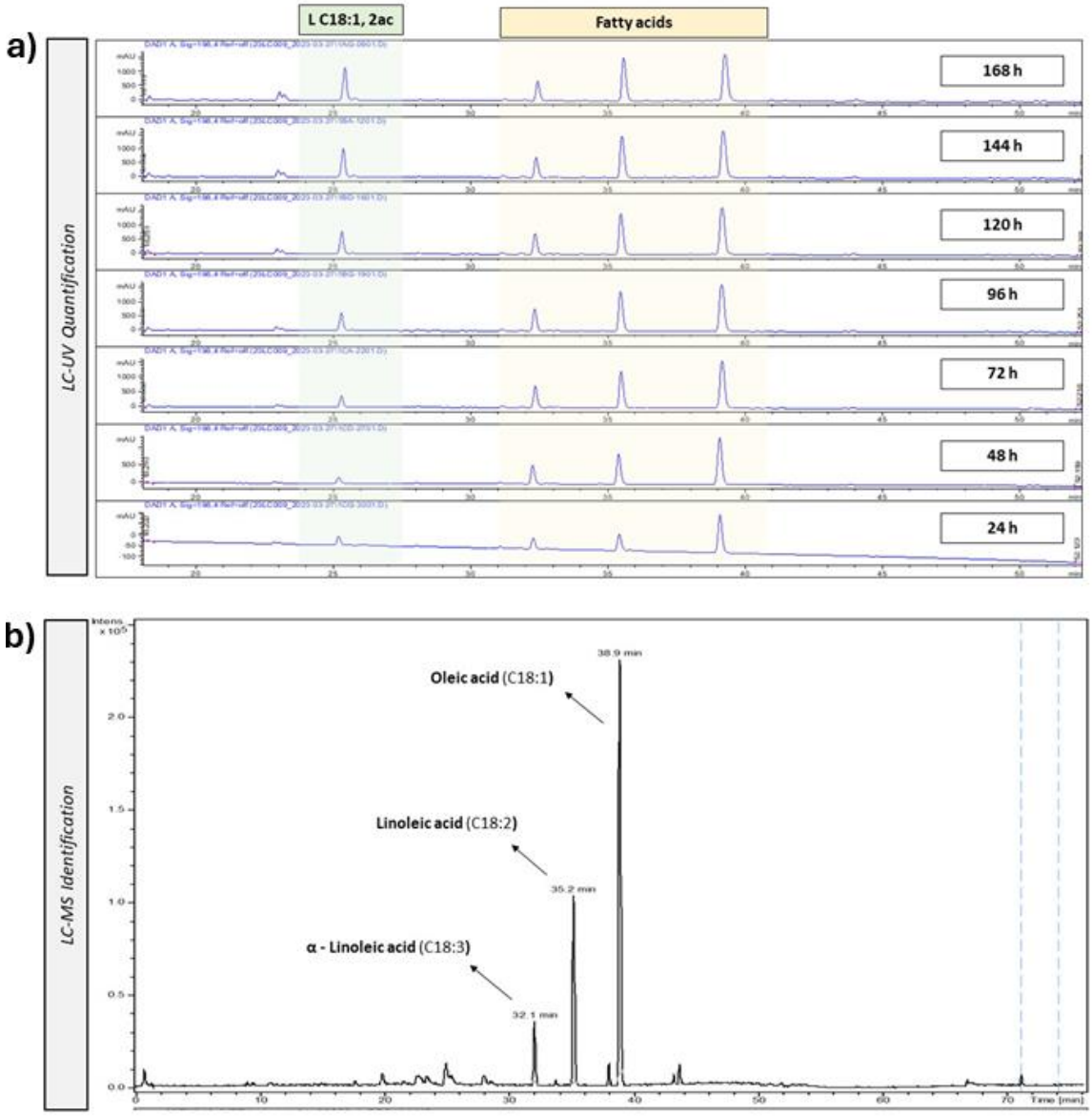

Figure 6.16 Time course fermentation broth using wheat feed hydrolysate at 0.31 g L^{-1} TN supplemented with rapeseed oil. a) HPLC-UV chromatogram at 198 nm from samples taken at each 24 h and b) HPLC-MS peaks identification belonging to the main free fatty acids found in the fermented broth.

As a new hydrolysate batch was used for the bioreactor experiment, a parallel fermentation in Erlenmeyer was performed simultaneously for a fair scale comparison. Erlenmeyer flasks exhibited a production of 56.98 ± 2.43 g L^{-1} and 34.24 ± 2.71 g L^{-1} for SLs crude extract and diacetylated lactonic C18:1, respectively. In comparison with 2 L bioreactor outcomes, there were no significant differences observed for SLs crude extract production (p-value = 0.06)

while diacetylated lactonic C18:1 production was notably lower (p-value = 0.03). Within the literature it is reported that fermentations carried out in a bioreactor or supplied with more air tend to have a greater demand for a nitrogen source and a higher capacity for converting carbon substrate compared to those carried out in a flask (Dolman et al., 2019; Wongsirichot et al., 2022a). From the literature, at flask scale SLs highest productivity was 8.46 g L^{-1} h^{-1} using bagasse sweet sorghum and soybean oil (Samad et al. 2015) while at bioreactor scale it was 1.25 g L^{-1} h^{-1} using food waste and oleic acid (Kaur et al. 2019). These results highlight the potential of alternative feedstocks and the challenges associated with SLs scaling up process. In operational procedures, maintaining a certain amount of oil prevents foam formation, which commonly causes mass transfer limitation in this kind of fermentation (Dolman et al., 2019).

Wongsirichot et al. (2022a) reported a SLs titer for a batch bioreactor over 10 times higher than the shake flask scale with a production of 40 g L^{-1} using potato media hydrolysate at 168 h. In the current chapter, at the same fermentation time, a 15% increase was observed in diacetylated lactonic C18:1 production using WF hydrolysate at 0.31 g L^{-1} TN at 2 L bioreactor scale. It is important to emphasize that the bioreactor fermentation demonstrates a linear correlation (R^2 = 0.99) between time and SLs production. Therefore, prolonging the fermentation duration could potentially result in a greater output of SLs which is also linked to the remaining substrate quantities found at the end of the fermentation. Akhlesh and Kannan (2009) reported that substrate limitation and product accumulation also decrease SLs production in bioreactors when using a fermentation medium containing sugarcane molasses, yeast extract, urea and soybean oil.

Van Bogaert et al. (2007) highlighted that the addition of nutrients such as phosphate or citrate has been found to positively influence SLs production due to their function in the biosurfactant pathway. In addition, literature on the topic reported that during the stationary phase, when SLs production begins, the addition of a lipid substrate/hydrolysate in a stepwise or continuous manner is practiced for improving SLs titer (Rocha et al., 2023). In this context, the scale-up results underline the limitations of the batch cultivation approach suggesting as future work hydrophobic substrate optimization and the application of fed-batch strategy after 96 h SLs fermentation.

Table 6.2 Summary of biomass hydrolysis for SLs production in SmF from literature and present study

Pre-treatment	Hydrophilic substrate	Hydrophobic substrate	Fermentation strategy	Microorganism	Time (h)	Yield (g L^{-1})	Productivity (g L^{-1} h^{-1})	Reference
Aqueous hydrolysis	Rice bran	Household cooking oil	Batch	*S. bombicola* Y-6419	216	51.0	0.24	Rocha et al. (2023)
Acidic hydrolysis	Bagasse sweet sorghum	Soybean oil	Flask scale	*S. bombicola* ATCC 22214	240	84.6	8.46	Samad et al. (2015)
	Corn fiber	Soybean oil	Flask scale	*S. bombicola* ATCC 22214	240	15.6	1.56	
Acidic hydrolysis	Sugarcane bagasse	-	Flask scale	*Cutaneotrichosporon mucoides* UFMG-CMY6148	72	12.5	0.17	Marcelino et al., (2019)
Acidic hydrolysis	Corn stover	Yellow grease	Fed-batch	*S. bombicola ATCC 22214*	168	52.1	0.31	Samad et al. (2017)
Acidic hydrolysis and Enzymatic hydrolysis: cellulose	Corncobs	Olive oil	Flask scale	*S. bombicola* NBRC 10243	96	49.2	0.51	Konishi et al. (2015)
Enzymatic hydrolysis: cellulose	Delignined corncob residue	Oleic acid	Flask scale	*Wickerhamiella domercqiae* CGMCC 1576	168	50.2	0.29	Ma et al. (2014)

Enzymatic hydrolysis: protease, lipase and glucoamylase	Food waste	Oleic acid	Batch	*S. bombicola* ATCC 22214	72	28.15	0.39	Kaur et al. (2019)
			Fed-batch		92	115.2	1.25	
Enzymatic hydrolysis: cellulase, α-amylase and glucoamylase	Potato byproduct	Rapeseed oil	Batch	*S. bombicola* ATCC 22214	312	60.5	0.19	Wongsirichot et al. (2022a)
			Fed-batch		216	77.8	0.68	
	Sugar beet	Rapeseed oil	Batch		312	25.6	0.08	
Enzymatic hydrolysis: protease, lipase and glucoamylase	Food waste	Oleic acid	Fed-batch	*S. bombicola* ATCC 22214	72	36.5	0.51	Wang et al. (2019)
Enzymatic hydrolysis: protease	Agricultural biomass waste	Rapeseed oil	Batch	*S. bombicola* ATCC 22215	168	51.7	0.31	Present study

*Acidic hydrolysis englobes the use of sulphuric acid.

6.5 Conclusion

In summary, this chapter expands the range of sustainable nitrogen sources that can be used for SLs production. Four agricultural byproducts have been successfully used as an alternative and novel feedstock which aligns with the principles of a circular bioeconomy process. Our results underscore a direct correlation between yeast growth and SLs production for TN concentrations between 0.07-0.31 g L^{-1}. Moreover, WF hydrolysate without hydrophilic carbon or nitrogen supplementation, exhibited a predominant production of diacetylated lactonic C18:1 congener (88.5% wt.). These results demonstrate that the use of WF has the potential to reduce purification steps and consequently decrease the costs associated with downstream. Given the limited literature on alternative nitrogen sources for SLs production, future research should explore a broader range of biomass hydrolysates, investigate enzymatic cocktails and assess alternative feedstocks without supplementation. Also, the validation of these N sources in SSF shall be considered.

Chapter 7

Global discussion

In this chapter, the challenges found throughout the thesis regarding the use of alternative feedstocks are discussed, focusing on residue selection, availability, fermentation process and final product characteristics.

7.1 Considerations for a sustainable biosurfactant production

Residues and byproducts are originated worldwide. Europe produces approximately 1.3 billion tonnes of waste annually, of which agricultural and food waste account for 700 million tonnes (Corrado et al., 2019; et al. 2017). Moreover, Bedoić et al. (2019) emphasized that the cereal, fruit and vegetable sectors produce higher quantities of agricultural waste, co-products and byproducts (AWCB) in countries with larger available land areas and favorable climate conditions. (Figure 7.1).

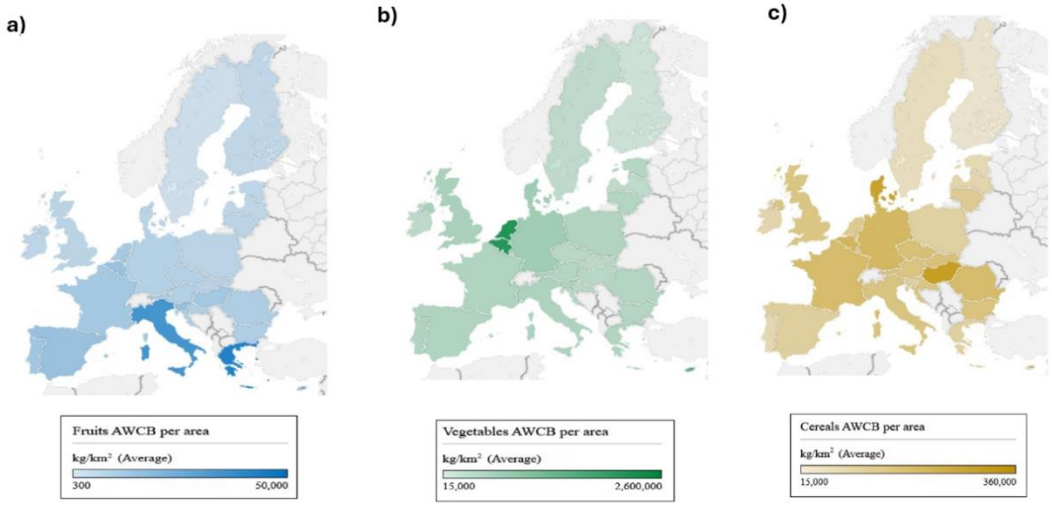

Figure 7.1 Distribution of agricultural, co-products, and byproducts waste (AWCB) around EU countries and UK. a) Fruits AWCB per area, b) Vegetable AWCB per area and c) Cereals AWCB per area. Adapted from Bedoic et al. (2019).

Therefore, several efforts have been developed to reduce the generated residues and mitigate their accumulation. In recent decades, sustainable processes have been proposed based on the use of residues and taking

advantage of biotechnological tools to obtain marketable products such as biopesticides (Sala et al., 2021); biostimulants (Ghoreishi et al., 2023), biosurfactants (Eras-Muñoz et al., 2024a) among others. Moreover, sustainable processes play an essential role in the development of the circular bioeconomy (Šelo et al.,2021).

Biosurfactant production has been widely reported using first-generation feedstocks however, their influence on production costs and their negative social or environmental impacts promotes the utilization of second-generation substrates derived from waste streams or byproducts (Gudiña et al., 2015; Bedoic et al. 2019; Wongsirichot et al., 2021). Even though biosurfactants sustainable process involves several factors that must be considered such as economic and environmental aspects (Figure 7.2) this thesis is focused on feedstock selection and supply chain identification where several challenges have been identified and are discussed below.

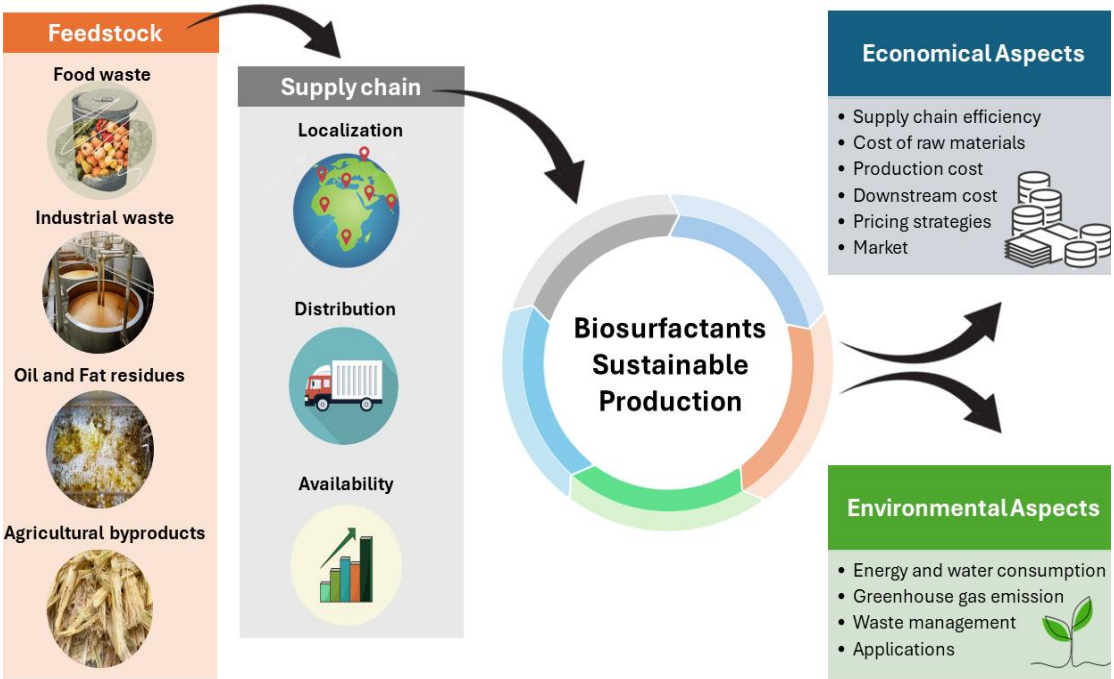

Figure 7.2 Sustainable biosurfactants production and considerations for feedstock selection.

One challenge in selecting residues is their variability, as the chemical composition can vary significantly based on the season, geographic location and agricultural practices and even between batches in the same facilities. This is evidenced by some residues utilized in this thesis. Winterization oil cake (WOC) is a residue obtained after industrial sunflower oil filtration and has been used as the main hydrophobic carbon source in biosurfactant production through SSF (Jiménez-Peñalver, 2017 Rodriguez, 2020). Over the years, it has been observed that different batches of WOC exhibit variability in fat content, ranging from 44% to 70% (Rodriguez et al., 2021a). Moreover, it was evidenced that depending on the season, it may have a high oleic content and/or come from the filtration of other types of oils such as corn. Even though it is an interesting potential candidate for biosurfactant production its characteristics play a key role in productivity and final product quality. Moreover, although it was initially a residue from the edible oil refineries, these have developed their own oil recovery process from WOC in recent years. Consequently, WOC is no longer produced as waste in some refineries. Another example is the difference observed between the two wheat feed hydrolysates used as nitrogen sources in Chapter 6, which presented different characteristics and led to different productivities in fermentations using the exact same protocol in shaking flasks.

Variability influence was also recognized when using rapeseed byproducts (Chapter 5 and Chapter 6). Rapeseed cake is co-produced with rapeseed oil after seed-pressing, while rapeseed meal is a coarse powdery material, produced from rapeseed cake after physical processes followed by solvent extraction of the remaining oil (Prime FL&K, 2024; Wongsirichot et al., 2022b). Despite being similar, the most striking difference between both is the fat content. In fact, their

characterization revealed notable differences in moisture and fat content. Rapeseed meal exhibited a higher moisture content of 14.82% and a lower fat content of 2.52% on a dry basis. In contrast, rapeseed oilseed cake had a lower moisture content of 3.55% and a higher fat content of 11.87% on a dry basis. The differences in their composition may be attributed to their origin and the specific characteristics required for their use in areas such as animal feed, industry, among others (Makkar et al., 2011). Rapeseed meal was obtained from the United Kingdom, while rapeseed oilseed cake originated in Spain. This observation was also reported by several authors when using food waste as alternative feedstock (Wongsirichot et al. 2024; Hu et al., 2021; Wang et al., 2020). Even though its use is reported for biosurfactants production, it can present different compositions based on its origin which represents a challenge for process reproducibility (Kaur et al., 2019; Wongsirichot et al., 2024).

These key points underscore the crucial importance of selecting an alternative feedstock that is abundant in a given region to fully realize its potential and ensure its consistent composition and availability. Additionally, choosing a feedstock based on its local abundance not only facilitates process reproducibility but also impacts cost-effectiveness at an industrial production scale, as transportation and storage costs must be considered. Since the production of biosurfactants requires hydrophilic carbon, hydrophobic carbon and nitrogen sources, more than one waste may be necessary and its availability in the given region should be balanced. Finally, measures should be taken to guarantee the supply of a given material by the producer, for example by negotiating long-term contracts among others.

7.2 Suitable substrates for sophorolipids production

As detailed throughout the thesis, several industrial waste, oilseed cakes and agricultural byproducts were used for SLs production. In Chapter 4, industrial waste such as winterization oil cake, sugar candy wastewater, cosmetic industry sludge and clean house product production sludge were able to produce SLs crude extract by SSF in a wide range (from 0.02 - 0.12 g $g^{-1}DM_i$) and were capable of being used as hydrophilic and nitrogen sources based on their characterization. To manage these industrial wastes, companies implement various strategies and practices to minimize environmental impact and comply with legal regulations. Among them, they promote recycling and reuse, the treatment of organic waste and source reduction, where they aim to implement improvements in production processes (Ahmad et al., 2023).

Regarding Chapter 5, oil cakes seem to be a potential hydrophobic source for sophorolipids production by SSF. Even though WOC is used as hydrophobic model residue, the tested oilseed cakes were selected due to their proximity and fat content (from 10.61-16.60 %, db). Moreover, in this chapter, the importance of fat content optimization is emphasized. Globally, approximately 80% of oilseed production comes from rapeseed (71.4 million tons), sunflower (49.7 million tons) and soybean crops (363 million tons) with a total output in 2020 that surpassed 600 million tons (European Commission, 2023). Indeed, it is reported that the EU is the world's leading producer of oilseeds where Spanish oilseed production represents 4% of this total and rapeseed is the most widely produced oilseed in the EU with almost 59% of the total (Mercasa, 2021).

According to The Spanish Ministry of Agriculture (2020), the area dedicated to the cultivation of rapeseed, soybean and sunflower totaled 772,000 hectares with a total production of 922,800 tons while for corn 98,151 hectares with a total production of 4,214 thousand tons. In addition, literature reports that cereals are the type of crops that generate huge amounts of agricultural waste during the harvesting period, generating straw accumulation (Bedoić et al., 2019). Even though straw is mainly used as thermal isolation material for stable animals, there is an excess production and a fraction ends up burnt in the fields. Besides straw, other lignocellulosic materials such as coco fiber, rise husk and pine bark have been demonstrated as suitable support materials in previous works (Rodriguez et al., 2021b) so a combination of available materials would ensure processing throughout the year.

As mentioned in Chapter 6, the versatility of agricultural byproducts and residues such as wheat feed, rapeseed meal, coconut waste and palm waste were demonstrated for SLs production through SmF. Interestingly, a high-purity final product was achieved when using hydrolysates without glucose or nitrogen supplementation. Global estimates demonstrate a rising trend for wheat feed production, estimated at 156 million metric tons per annum based on wheat husk data (Bledzki et al., 2010), approximately 40-50 million metric tons for rapeseed meal (George et al., 2021), around 9.5 million metric tons for palm kernel meal (Balandrán-Quintana et al., 2019) and 350,000 metric tons for coconut waste based on coconut coir data (Coirboard, 2014). Despite these byproducts and residues are utilized as animal feed, huge amounts are disposed of in landfills or incinerated (Šelo et al.,2021). So, the necessity of finding alternative

management options offers dual benefits such as minimizing residues and generating value-added bioproducts (Al-Kashif et al., 2023; Jadhav et al., 2019).

Table 7.1 presents a summary of the alternative substrates used for SLs production in this and the previous thesis developed in the research group. As was described in Chapter 1, *S. bombicola* requires a hydrophilic, hydrophobic carbon and nitrogen source to produce this kind of biosurfactant. In this way, the presence of, at least, one of these nutrients in the residues is crucial for utilizing them as feedstock. Moreover, the type of substrate that can be used by the yeast is determined by its metabolic pathways and its capacity to produce enzymes (Van Bogaert et al., 2011).

Even though glucose is the main hydrophilic source used, other saccharides present in residues have been reported for SLs synthesis such as wood sugars (Ingham et al., 2023), synthetic sweetwater (Wadekar et al., 2012c), soy molasses (Solaiman et al., 2007), deproteinized whey (Daverey and Pakshirajan, 2010), among others, in both SmF and SSF. From the studied alternative substrates in SSF, molasses which is a sucrose-rich byproduct of the sugar industry supplemented with WOC as hydrophobic achieved the highest SLs crude extract production at 168 h (0.19 g $g^{-1}DM_i$) and showed to be a potential substitute for pure hydrophilic carbon sources due to it can contain significant amounts of glucose and fructose. Indeed, residues with diverse sugar profiles or high contents of lactose, sucrose and fructose have been reported as potentially usable by yeast (Wongsirichot et al., 2021). This is due to the presence of enzymes such as saccharase, β-D-galactosidase and fructose transporter (Ffz1), which facilitate the conversion of complex sugars into simpler forms (Gonçalves et al., 2018; Zhou and Kosaric, 1993). It is important to note that when using

alternative substrates, the literature often reports only the glucose content and does not provide a complete sugar composition profile which can hinder the accurate interpretation of the data.

Regarding hydrophobic carbon sources, a variety of pure oils have been used for SLs production such as corn oil (Pekin et al., 2005), sunflower oil (Daverey and Pakshirajan 2009), olive oil (Konishi et al., 2015), palm oil (Poomtien et al., 2013) while framed into a sustainable process, waste fried oils have been used as the mainly alternative substrate (Li et al., 2018; Wadekar et al., 2012c). Indeed, authors such as Fleurackers (2006) report that when using waste frying oil SLs production increases by 40% in comparison to using pure oils. In this research, the highest SLs crude extract productivity was reached at 96 h where WOC achieved the highest yield (0.09 g $g^{-1}DM_i$) which is attributed to its composition, particularly its high oleic acid content which is the main fatty acid used by the yeast. However, corn oilseed cake, which is mainly composed of linoleic acid, also proved to be a viable pure oils substitute for SLs crude extract production (0.07 g $g^{-1}DM_i$) and emerged as a promising candidate for producing novel SLs congeners. Literature reported that higher SLs productivities are reached when C18 and C16 fatty acids are present in the hydrophobic source while a decrease is observed when using shorter ≤C16 or longer ≥C20 fatty acids (Felse et al., 2007). In this regard, it must be considered to report the residue's fatty acid profiles, due to comparisons between these alternative sources are often based on traditional quantities, which can vary due to the factors described in section 7.1.

Nitrogen source was also evaluated due to its importance for yeast growth and metabolism by applying both fermentation technologies SSF (Chapter 4) and

SmF (Chapter 6). Literature reported that using an organic nitrogen source increases SLs production in comparison with inorganic ones (Ma et al., 2011). In this way, the evaluated alternative nitrogen sources were mainly composed of organic nitrogen and our findings were aligned with this principle. The best candidates to substitute pure nitrogen sources mainly nitrogen salts were cosmetic industry sludge for SLs crude extract production by SSF (0.10 g $g^{-1}DM_i$) and wheat feed hydrolysate by SmF (59.12 g L^{-1}). Although not confirmed in this thesis, based on our experience, it is reasonable to hypothesize that if a nitrogen source is suitable for SLs production in SmF, it will also be suitable in SSF. The reverse is likely true as well, provided the source is water-soluble. Through the obtained results it was found that by SSF, growth and SLs production are not related while in SmF this correlation was observed at nitrogen concentrations above 1.5 g L^{-1}. Although solid waste or byproducts can be utilized in both fermentation strategies, their application involves implications specific to each process.

In SSF results could be influenced by the complex dynamics and the heterogeneous nature of solid residues that can lead to an unequal distribution of nutrients, which may affect the efficiency of microbial growth and product yield. Moreover, factors such as moisture, aeration and heat generation should be considered to ensure process efficiency. On the other hand, the use of solid residues in SmF process requires pre-treatment such as the use of protease enzymes to facilitate the breakdown of lignocellulosic residues and maximize the availability of nitrogen and nutrients present in the sample. However, substrate pretreatment is not exclusive of SmF, since SSF processes may require waste pretreatment in case the used microorganism does not have a suitable enzymatic

system to obtain the nutrients from that specific source. Both approaches require careful consideration of how the physical and chemical properties of the residues interact with the fermentation conditions, as well as how they influence the overall process efficiency and product quality.

Hydrolysates are used in several processes; however, they are successfully reported for SLs production due to their ability to provide essential nutrients for the yeast. In addition to the agricultural byproducts and residues hydrolysates used in this thesis, literature highlights the use of food waste hydrolysates as a very promising second-generation feedstock for SLs production (To et al., 2023; Wang et al., 2019; Wongsirichot et al., 2024). The chemical composition of common food waste includes lignin, cellulose, hemicellulose and protein however it could vary based on its origin (Šelo et al.,2021). It is estimated that one-third of the food produced globally is wasted annually, amounting to approximately 1.3 billion tons, making it a potentially profitable alternative substrate to pure ones. Nevertheless, the application of food waste and agricultural byproducts hydrolysates requires the use of high-water volumes and commercial enzymes. Molina-Peñate et al. (2024) reported that commercial enzymes represent up to 91% of the variable cost for high-value bioproduct production. Consequently, a promising alternative could be to integrate enzyme production into the process in a biorefinery context or apply genetic engineering to create recombinant and mutant strains capable of producing the necessary enzymes for utilizing complex substrates.

For sustainable SLs production, three main aspects are proposed: process optimization, the use of second-generation feedstocks and the genetic engineering of *S. bombicola* to maximize production (Mukherjee et al., 2006).

This thesis contributes to the first two aspects, while the last aspect was not covered but remains an open field of research. Even though genetic engineering efforts have focused on the synthesis of new SLs and SL structures modifications to obtain specific congeners (Van Bogaert et al., 2011; Van Bogaert et al., 2016), modifications to produce enzymes such as cellulases and amylases, among others, have not been reported and could represent a novel option within a sustainable process framework. For instance, if the yeast can produce these enzymes during the fermentation process it can hydrolyze complex substrates such as lignocellulosic residues and do not require a previous enzymatic pretreatment.

Table 7.1 Alternative substrates used in this and previous GICOM thesis, their characteristics and SLs crude extract production

				Composition (%)			Characterization (g/L or g/Kg)			Elemental analysis (%, db)				Production		Source
Substrate	**Origin**	**Strategy**	**pH**	**DM**	**~~M~~**	**OM**	**Glucose**	**TC**	**TN**	**Fat**	**C**	**N**	**C:N**	**SLs**	**Time** (h)	
Hydrophilic source																
Food waste hydrolysate	Spain	SSF	5.2	2.01	97.99	99.10	17.03	28.70	1.20	n.a	45.4	2.1	21.62	0.11 g $g^{-1}DM_i$	100	Chapter 4
Sugar candy wastewater	Spain	SSF	4.4	12.76	87.24	99.68	2.08	57.30	0.02	n.a	n.a	n.a	n.a	0.12 g $g^{-1}DM_i$	100	Chapter 4
Molasses	Spain	SSF	6.4	73.96	26.04	87.93	592	732	27	n.a	n.a	1.89	n.a	0.19 g $g^{-1}DM_i$	168	Rodríguez et al. (2021)
														0.18 g $g^{-1}DM_i$	240	Jiménez-Peñalver (2016)
Hydrophobic source																
Corn oilseed cake	Spain	SSF	6.5	94.33	5.67	93.31	2.68	75.94	4.58	16.6	46.78	2.54	18.43	0.07 g $g^{-1}DM_i$	96	Chapter 5
Rapeseed oilseed cake	Spain	SSF	6.0	96.45	3.55	93.43	0.88	93.92	8.23	14.29	48.8	4.11	11.87	0.06 g $g^{-1}DM_i$	96	Chapter 5

Soybean oilseed cake	Spain	SSF	6.4	93.57	6.43	93.41	0.83	106.38	7.38	10.61	47.87	7.43	6.44	0.01 g $g^{-1}DM_i$	96	Chapter 5
Sunflower oilseed cake	Spain	SSF	6.0	95.48	4.52	93.97	6.00	55.08	3.27	14.3	49.07	5.28	9.29	0.02 g $g^{-1}DM_i$	96	Chapter 5
Winterization oil cake	Spain	SSF	6.0	91.87	8.13	50.51	n.d	10.50	0.04	47.82	33.55	n.d	n.a	0.20 and 0.17 g $g^{-1}DM_i$	240	Chapter 4 and Chapter 5
Nitrogen source																
Cosmetic industry sludge	Spain	SSF	6.5	14.19	85.81	93.87	n.d	21.24	3.89	n.a	8.54	0.34	25.29	0.10 g $g^{-1}DM_i$	100	Chapter 4
Clean house products sludge	Spain	SSF	11.8	8.39	91.61	91.96	3.86	11.80	2.16	n.a	n.a	n.a	n.a	0.02 g $g^{-1}DM_i$	100	Chapter 4
Coconut waste hydrolysate	Thailand	SmF	5.5	91.72	8.28	95.44	0.73	10.46	0.18	10.01	44.57	2.57	17.34	57.11 g L^{-1}	96	Chapter 6
Corn steep liquor hydrolysate	UK	SmF	3.9	52.5	47.5	95.10	9.98	16.5	364	1.2	n.a	7.95	n.a	20.78 g L^{-1}	168	Chapter 6
Palm waste hydrolysate	Thailand	SmF	4.9	91.73	8.27	95.84	0.16	9.49	0.23	9.3	45.41	1.73	26.25	54.59 g L^{-1}	96	Chapter 6

Rapeseed meal hydrolysate	UK	SmF	5.7	85.18	14.82	92.55	n.d	10.87	0.11	2.52	44.8	6.17	7.26	54.44 g L^{-1}	96	Chapter 6
Wheet feed hydrolysate	UK	SmF	6.3	89.71	10.29	95.35	5.21	14.43	1.16	3.02	43.69	2.58	16.93	59.12 g L^{-1}	96	Chapter 6
Support																
Coconut fiber	Spain	SSF	5.6	91.3	8.7	98.6	n.a	n.a	n.a	0.3	5.8	0.2	29.2	0.12 g $g^{-1}DM_i$	168	Rodríguez et al. (2020)
Risk husk	Spain	SSF	7.2	87.3	12.7	83.4	n.a	n.a	n.a	0.9	5.2	0.5	10.4	0.15 g $g^{-1}DM_i$	168	Rodríguez et al. (2020)
Wheat straw	Spain	SSF	5.6	94.4	5.6	95.5	n.a	n.a	n.a	1.4	5.8	0.5	11.6	0.20 g $g^{-1}DM_i$	168	Rodríguez et al. (2020); This thesis.

TC and TN were characterized using liquid extractions when required. Elemental analysis was developed by using the solid residu and evaporations were developed when required. Abbreviation:* DM, dry matter; M, moisture; OM, organic matter; TC, total carbon; TN, total nitrogen. C, carbon; N, nitrogen; C:N, ratio carbon nitrogen; DM_i, initial dry matter; db, dry base; n.d, not detected; n.a, not analyzed.

7.3 Criteria for alternative substrates selection

Based on the discussion above, to use an alternative substrate for sophorolipids production, several considerations are detailed in Figure 7.3 as a sort of decision chart. Points to highlight include the availability and identification of the key substrate, its physical properties and the cost impact based on its pre-treatment.

First, for large-scale operations, high local volumes are required to ensure their similar chemical composition and avoid inducing variability in the process by using different batches. Secondly, identifying a key substrate target is crucial in this process where several types of substrates are needed. For SLs production, the chosen substrates must have a high content of carbon (mainly sugars), nitrogen, or fats (Rocha et al., 2023). Ideally, a versatile residue or byproduct should be used, serving as multiple sources as was evidenced with rapeseed byproducts or food wastes. Regarding the physical properties of the residue, a high-water content can increase transport costs and may require proper storage (Wongsirichot et al., 2021).

Finally, although the environmental impact and economic aspects of using residues or byproducts in the SLs production process are outside the experimental scope of this thesis, these considerations were included in the selection criteria. Indeed, this thesis provides data that are to be used in ongoing techno-economic and life cycle assessments framed around alternative substrates for sustainable SLs production.

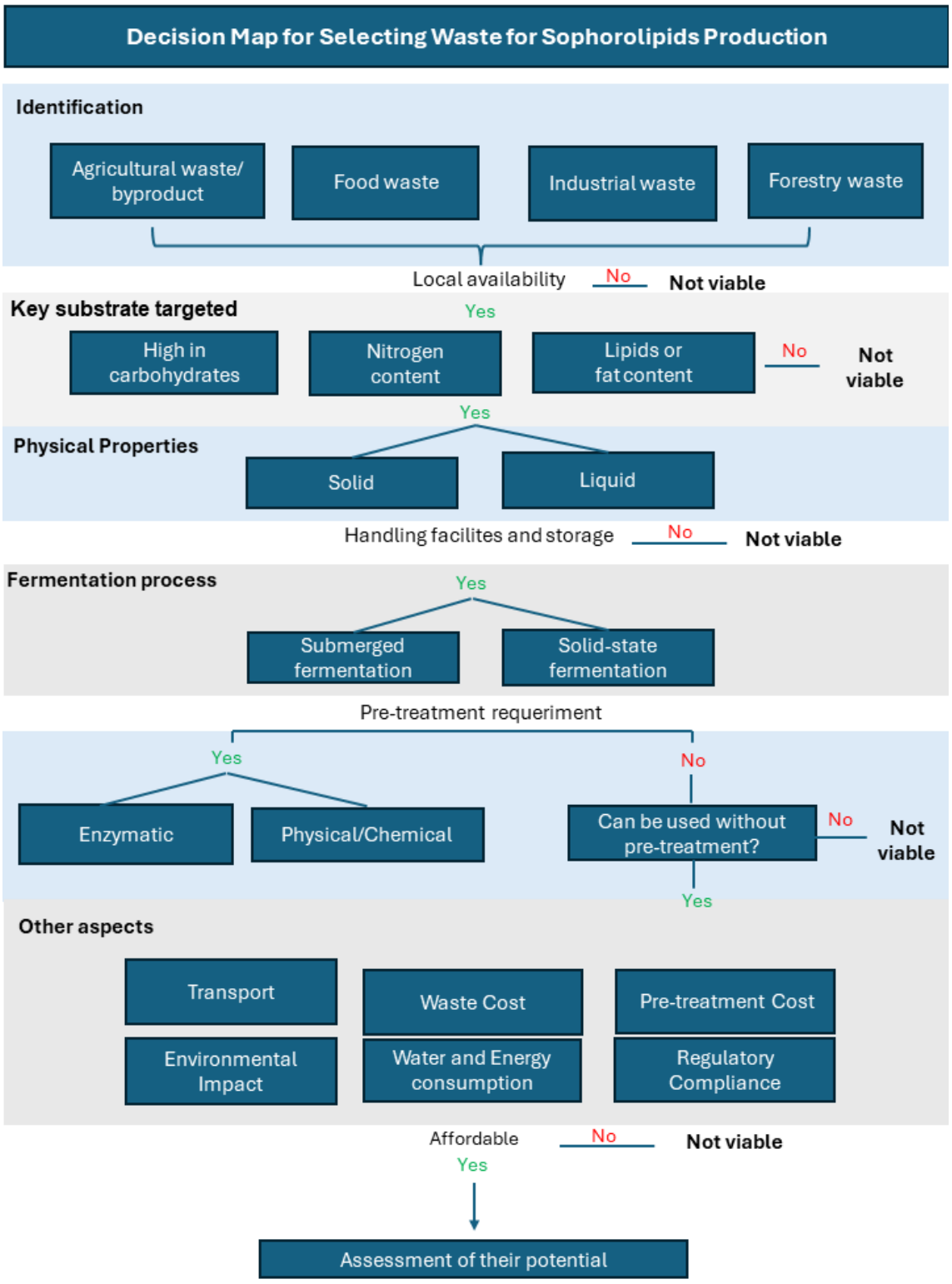

Figure 7.3 Selection criteria for waste, residue or byproduct for biosurfactant production.

7.4 SSF and SmF: Complementary tools for employing second-generation feedstocks

Even though different fermentation strategies were applied in this thesis, SSF was the most investigated due to its novelty around the use of solid byproducts and residues without pretreatments. A search using the keywords "biosurfactant" and "solid-state fermentation" reveals only about 147 related documents since 1989 (Elsevier B.V, Scopus, 2024). Due to the limited information, comparisons and discussions of results are primarily based on those obtained through SmF. In this context, it is common to compare these technologies and their associated yields. However, our proposal is that both technologies can be complementary when the goal is to maximize the utilization of a residue, particularly when the residue is versatile and produced in large quantities, such as the already described rapeseed byproducts.

Consequently, a starting point by applying the same residue in both SSF and SmF processes is provided. For 1 kg of rapeseed oilseed cake, there are two possible scenarios based on how oilseed cake is industrially pretreated and its composition. The first scenario involves the direct incorporation of rapeseed oilseed cake as hydrophobic carbon source. The second involves the solvent extraction of the oilseed cake to obtain rapeseed meal and proceed with an enzymatic hydrolysis to use it as nitrogen source (Chapter 5 and Chapter 6, respectively).

The first scenario involves the use of a mixture of rapeseed byproduct as hydrophobic source and the presented data is based on the results obtained at 22 L bioreactor scale (Chapter 5). In this case, rapeseed oilseed cake was used

as hydrophobic source (1 kg). It is necessary to consider nutrient supplementation to achieve the optimized hydrophilic carbon:nitrogen ratio (Chapter 4), so the solid matrix was supplemented with glucose (0.33 kg), urea (0.003 kg) and yeast extract (0.017 kg). Due to the availability and local production, the support material used for SSF was wheat straw (0.61 kg) a widely reported support for SLs production (Rodriguez, 2020). Through SSF, it is expected to obtain 66.24 g of SLs crude extract. The output of this scenario includes a post-extraction solid. Rodriguez et al. (2021a) spotlighted that this solid can be used in anaerobic digestion to produce a total methane yield of 225.84 L kg^{-1} SV. This value is comparable to those reported for the organic fraction of municipal solid waste, which is widely implemented at a commercial production level, a zero-waste process is proposed (Figure 7.4).

After obtaining the SLs crude extract, an enzymatic hydrolysis using commercial proteases can be developed to produce a nitrogen-rich hydrolysate from the post-extraction solid matrix. Then this hydrolysate can be fed back into the SSF as a nitrogen source, substituting (totally or partially) the pure substrates used in the process such as urea. When using an enzymatic cocktail including proteases, xylanases, etc, the hydrolysate could eventually serve as nitrogen and sugars source simultaneously.

This approach would contribute to future work using rapeseed oil cake as a hydrophobic carbon source in SSF that could be linked to the on substitution of pure substrates such as glucose, urea and yeast extract with hydrolysates or the residues that showed the best outcomes throughout the thesis. Due to the aim is to evaluate SLs production using only residues as feedstock, sugar candy wastewater can be used as a hydrophilic carbon source and if needed, it could

be supplemented with food waste hydrolysate while cosmetic industry sludge can be used as a nitrogen source.

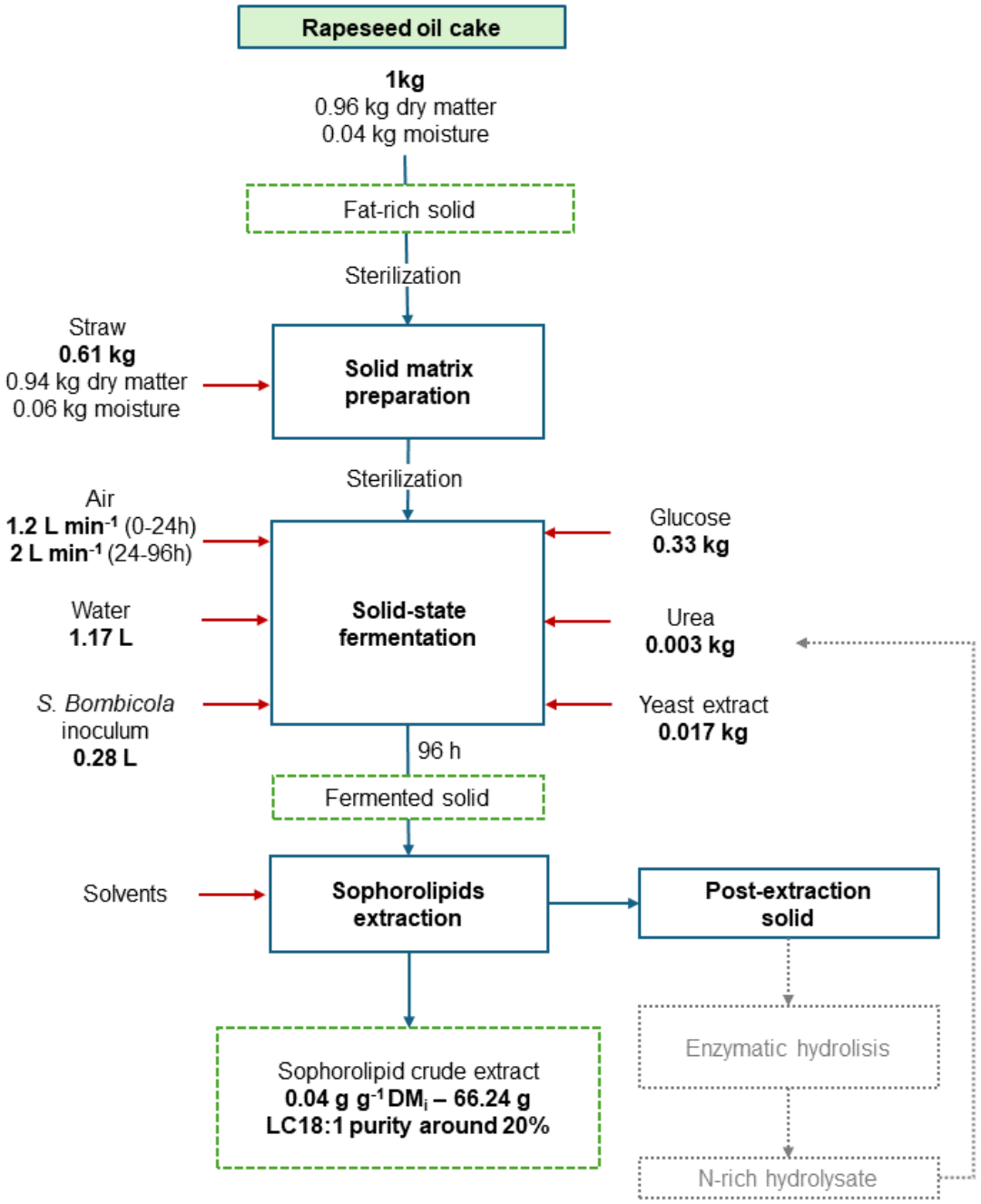

Figure 7.4 Zero waste approach for SLs production by solid-state fermentation using rapeseed oilseed cake as hydrophobic source.

The second scenario could be applied in an oil industry where large volumes of rapeseed oil cake are produced (Figure 7.5). In this case, the rapeseed oil cake usually undergoes solvent extraction, resulting in the production of rapeseed oil and rapeseed meal. Then, the rapeseed meal is hydrolyzed using 10 L of sterile water and 0.05 L of commercial protease from *Aspergillus oryzae* per each kg of rapeseed meal (data based on Chapter 6 findings). A total of 7.2 L of rapeseed hydrolysate is obtained while the remaining hydrolyzed solid could be used in consolidated processes such as anaerobic digestion or composting.

The rapeseed hydrolysate is primarily composed of carbon (18.46 g L^{-1}) and nitrogen (2.47 g L^{-1}). As reported by et al. (2014) and observed in Table 7.1 based on our characterization, rapeseed meal primarily consists of significant amounts of lignocellulosic material (~12% w w^{-1}) and high protein content (~35% w w^{-1}) which make it a good candidate to be used as nitrogen source. To reach the optimum nitrogen concentration achieved in Chapter 6, sterile water is required (51.12 L) in the process. Moreover, hydrophilic carbon source such as glucose (6.48 kg) and rapeseed oil (6.18 L) as hydrophobic carbon source were supplemented in the fermentation media. A total SLs crude extract production of 390 kg is estimated to be obtained. In the context of a circular economy, it is proposed to use the rapeseed oil obtained from the treatment of rapeseed oil cake as input for SmF, allowing for the development of an integrated process.

While only protease was used in this scenario, future research could focus on expanding the range of enzymes and testing an enzymatic cocktail to maximize residue versatility. Additionally, evaluating the potential of the hydrolysate as the sole source of hydrophilic carbon and nitrogen could be

considered for future work, based on the results obtained in Chapter 6 with wheat feed hydrolysate and with the OFMSW hydrolysate in Chapter 4.

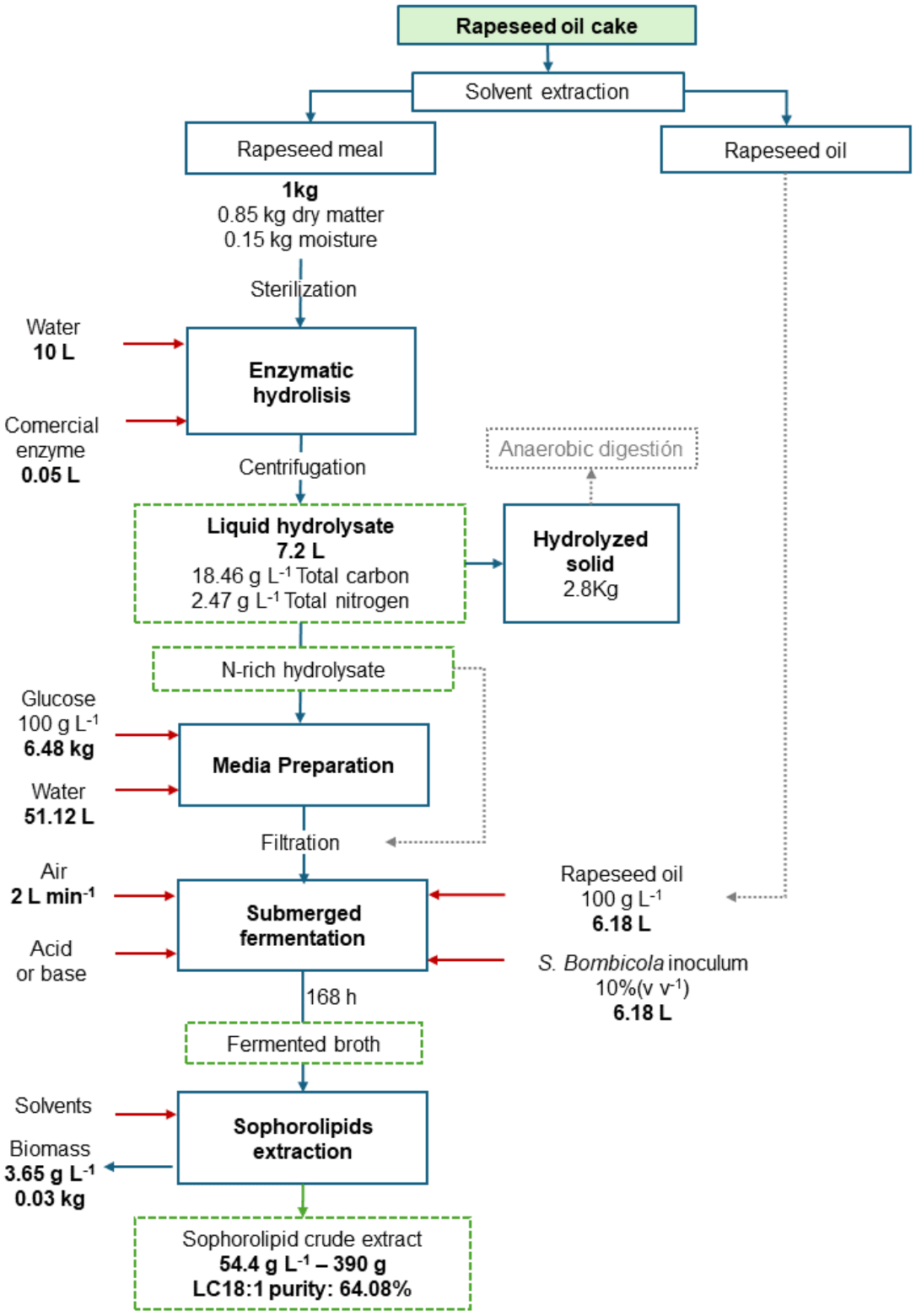

Figure 7.5 SLs production from rapeseed oil cake by submerged fermentation.

Although both processes have proven to be promising, the choice between one technology or the other depends on the production scale, the cost of the process, the available resources, the amount of byproduct generated and whether it can be applied to other types of residues predominant in the country of study. Specially, the economies of scale must be considered. When the production scale does not justify producing rapeseed meal, oil cake is obtained and can be used directly in SSF, which has been demonstrated to work well up to a 22 L scale (this thesis) and reasonably well up to 100 L (Rodríguez, 2020). However, when the production scale is very large and rapeseed meal is produced, SmF appears a better option.

Additionally, other factors, such as water consumption, should also be considered. As observed, SmF requires a significantly amount of water to carry out the process, with a consumption ratio of 156.21:1 (w w^{-1}) of water to produced SLs crude extract, compared to a ratio of 17.65:1 (w w^{-1}) in SSF. This difference does not imply competition between both technologies but highlights a key distinction between them, underscoring the importance of evaluating water consumption, product purity and overall yields to ensure sustainability at all stages of the process.

SSF and SmF are two complementary technologies in the development of new value chains framed in circular bioeconomy and herein they are specifically discussed for SLs production using *S. bombicola*. Other microorganisms capable of producing a wider range of hydrolytic enzymes could offer different possibilities. In addition, it should be emphasized that even both fermentation strategies allow the obtention of SLs crude extract, if the intended application requires high purity SLs such as in biomedicine or cosmetic industry, SmF would

be the best option while SSF final product could be applied in an environmental field (Ceresa et al., 2020; Al-Tamimi et al., 2018; Eras-Muñoz et al., 2022).

Research on SLs production typically focuses on yields, creating a gap in understanding the environmental and economic aspects of the process. Wang et al. (2020) conducted a techno-economic analysis using food waste in a biorefinery context to produce SLs by SmF. Based on operational costs, the net production cost for SLs crystals and SLs syrup was US$19.65 per kg and US$16.45 per kg, respectively. This is 51-75 times higher than the price of other byproducts generated in a biorefinery context such as succinic acid crystals, lactic acid and fructose syrup which is reasonably high for the market.

Conversely, Rodríguez et al. (2021) and Martínez et al. (2022) provided an initial inventory for economic and environmental assessment of SLs produced by SSF focusing on obtaining 1 kg of SLs crude extract. To complement this information, it is essential to establish a baseline where the process focuses on the use of alternative substrates (e.g. 1 kg of oilseed cake, agricultural byproducts among others) for evaluating and guiding decisions toward a sustainable process and offers an initial overview of the potential of alternative substrates for further economic study and life cycle assessment of the global process.

7.5 Challenges around sophorolipids downstream processing and quantification when using alternative substrates

After alternative substrate selection and fermentation process optimization, SLs extraction is a challenge mainly in processes such as SSF where a complex solid matrix is used. Although ethyl acetate/n-hexane solvent extraction was used throughout this thesis to obtain SL crude extracts, reviews and comparisons

frequently involve other analytical techniques, such as the anthrone method, ethanol extraction and HPLC-UV.

Even though solvent extraction is well-documented for SmF, it was found that when applied to SSF this should be complemented with HPLC-UV analysis due to SLs crude extract could contain a high level of impurities (Chapter 4). These impurities correspond mainly to free fatty acids which are not completely removed with n-hexane washes due to a possible “matrix effect” which hinders effective interaction between the crude extract and the solvent. This finding is in concordance with Ingham et al. (2024) who noted that solvent extraction and gravimetric SLs quantification often result in the co-extraction and presence of non-sophorolipid components in the final crude extract. Indeed, it was observed that the amount of fats in the initial solid matrix to be fermented also affects the purity of the SLs crude extract and thus the process should be optimized in terms of fats concentration.

As mentioned in Chapter 1, SLs are reported as a mixture of congeners with both lactonic and acidic natures where the lactonic/acidic balance seems to be influenced by fermentation conditions, oxygen supply and the provided hydrophobic substrate (Van Bogaert et al., 2011). Several authors have reported that the primary congener produced by *S. bombicola* is the diacetylated lactonic C18:1, as evidenced when a high oleic acid hydrophobic source was used. When feeding other fatty acids, such as stearic acid (Jiménez-Peñalver et al., 2018), the amount of C18:0 derived SLs increases dramatically although the yeast is yet able to significantly produce C18:1 SLs (either *de novo* or by saturating C18:0). In contrast, when the hydrophobic carbon source was substituted with other oilseed cakes, varying SL congener profiles were observed. Indeed, the

importance of the hydrophobic carbon source was emphasized due to fatty acids composition resulting in a different congeners profile (Chapter 5). This variation complicated the yield reporting due to the limited availability of pure standards to quantify them and the missing database with SLs congeners that allow their identification by HPLC-MS.

Moreover, a global challenge in SLs quantification is standards acquisition. In this thesis, the 1′,4″-sophorolactone 6′,6″-diacetate was used as standard with a purity ≥ 80% (Cayman Chemical, United States). However, it is important to highlight that this unspecific purity level can lead to either an overestimation or underestimation of the production. Nevertheless, due to the complexity and similarity of SL structures, obtaining a single congener is challenging. Oiza et al. (2024) reported achieving >99% purity in the purification of lactonic congeners from an SLs crude extract obtained via SSF. Thus, findings described in Chapter 6 support the use of agricultural byproducts hydrolysates to achieve a high-purity final crude extract (>89% diacetylated lactonic C18:1) that can be used as standard.

In this context, it is proposed to report not only the concentration of SLs crude extract and diacetylated lactonic C18:1 but also production data based on the SLs mixture area. This represents the sum of congener equivalent areas from a retention time of 10 to 28 minutes when the SLs crude extract is analyzed by HPLC. While this is not an exact measure, it can provide a general production overview when uncommon substrates are used. Moreover, it is important to note that, despite containing impurities, SLs crude extract has been successfully used for environmental (El-Shahed et al., 2022; Alsaqer et al., 2018) and commercial applications (e.g. surface cleaner product commercialized by Ecover, Belgium)

which underscores the importance of considering purification processes based on their further application.

Finally, some challenges related to SSF fermentations are highlighted, particularly concerning process yield and reproducibility. When using WOC as a hydrophobic source, a production ceiling of approximately 0.14-0.20 g of SLs crude extract per gram of initial dry matter is observed and cannot be surpassed. Consequently, as previously noted, the quantification of SLs congeners should be considered. Regarding process reproducibility, it is clear that fat concentration affects the process. However, reproducibility issues persist even when using the same batch of WOC and nutrient concentration, suggesting that factors beyond the composition of the solid matrix may be influencing the results. Variables such as the inoculum and the co-existence of different metabolic phases during the process are influencing in SLs production. Measures such as maintaining consistent inoculum optical density could potentially help mitigate these effects, though further research is needed to guide this approach.

Chapter 8

Conclusions and Future work

The general conclusions from this thesis are outlined below, with an emphasis on future work in this field.

- Solid-state fermentation (SSF) has proven to be a sustainable technology for producing sophorolipids (SLs) by *Starmerella bombicola* using second-generation feedstocks such as industrial waste and oilseed cakes. This study contributes valuable insights into new hydrophilic and hydrophobic carbon sources that can serve as substitutes for the conventional pure substrates used in this process.
- To facilitate the experimental research by performing a great number of assays, a scale-down of the SSF process was validated.
- Glucose as a hydrophilic carbon source and urea as a nitrogen source were successfully optimized by using scale-down SSF. Moreover, the optimized ratio of glucose:nitrogen (181.75:1.43 w w^{-1} initial dry weight or 127:1 w w^{-1} initial dry weight) at 0.5 L bioreactor showed an increase in production of 22% for SLs crude extract and 30% of diacetylated lactonic C18:1 when comparing with the reported in the literature (100:1 w w^{-1} initial dry weight).
- Based on the optimized ratio, sugar candy wastewater and cosmetic sludge have been identified as promising alternatives to pure substrates, serving as hydrophilic carbon and nitrogen sources, respectively, for producing SLs crude extract reaching yields of 0.117 ± 0.002 g g^{-1} DM_i and 0.085 ± 0.004 g g^{-1} DM_i, respectively.
- Corn oilseed cake and rapeseed oilseed cake have shown potential as hydrophobic alternative substrates. It is important to optimize the fat content of the hydrophobic substrate in the SSF due to higher fat levels have been shown to reduce the purity of the final crude extract, possibly due to a

"matrix effect" that hinders the removal of impurities during the n-hexane wash.

- It was demonstrated that SLs congener profile is highly influenced by the second-generation feedstock nutrient composition. Therefore, it is recommended to include both the SLs crude extract production and the SLs mixture area in reports to ensure more accurate results.
- Agricultural byproduct hydrolysates can serve as nitrogen sources and enable SLs production via SmF when hydrolyzed with commercial proteases. Moreover, hydrolysate versatility was demonstrated when using wheat feed hydrolysate serving as both, a hydrophilic carbon source and a nitrogen source, enabling the production of high-purity diacetylated lactonic C18:1 (~89% wt.)
- Despite the fermentation strategy used (SmF or SSF), it was confirmed that nitrogen concentration significantly influences yeast growth. However, a higher biomass concentration does not necessarily correlate with a higher SLs yield.
- This thesis addresses the research gaps concerning alternative nitrogen sources for SLs production in both SSF and SmF, as highlighted by several authors. It offers valuable insights into nitrogen concentrations and their impact on yeast growth, demonstrating how alternative nitrogen sources can be effectively utilized in these fermentation processes.
- The data obtained from the fermentation processes will provide insights for completing further inventories needed for economic studies (TEA) and life cycle assessment (LCA) using second-generation feedstocks, as well as for

developing a mathematical model, which will be developed by the other PhD students that belong to the project.

- The use of second-generation feedstocks was successfully assessed through this thesis. The data obtained from their characterization, fermentation process and yields provide information for creating the database proposed in the project and expanding the range of second-generation feedstocks reported by the research group.

Proposals for Future Work

1. *Evaluate the combined effect of alternative substrates in SSF*: It is important to assess the impact of using alternative substrates in SSF to replace pure substrates and to determine their influence on the SLs crude extract profile. Additionally, since fat concentration significantly affects the recovery of the final product, optimizing fat levels is crucial.
2. *Consider the fed-batch strategy*: The experiments in this thesis were conducted in batch mode. So, it is highly recommended to evaluate the fed-batch strategy due to literature reports that the supply of hydrophobic carbon sources during the stationary growth phase can enhance SLs production.
3. *Explore other reactor configurations*: Investigate the use of different reactor configurations, such as trickling reactors and tray reactors, which have been successfully applied in biopesticide production. This reactor configuration could also help counteract the temperature increases observed in 22-L batch reactors.

4. *Develop a partial SLs purification protocol*: A partial purification protocol for SLs should be tested using crude extracts from varying solid matrices. This will help to identify the implications of different substrates on downstream processes.
5. *Investigate oxygen limitation and nutrient influence*: Given that SLs are secondary metabolites produced under yeast stress, it is recommended to evaluate the effects of oxygen limitation. Additionally, the use of precursors such as citrate and other nutrients like phosphates should be considered due to their influence on SLs metabolic pathways.
6. *Hydrolysates and enzymes*: Future work should explore the hydrolysis of agricultural byproducts using a variety of enzymes due to their lignocellulosic composition. Moreover, hydrolysates evaluation without supplementation in the process is highly recommended.
7. *Conduct Toxicity Assays*: To complete the cycle, it is proposed to conduct toxicity assays. While the primary focus of this thesis was on production, it would be valuable to explore the potential applications of SLs crude extract by performing tests such as phytotoxicity, microbial toxicity and antibiofilm activity.
8. *TEA and LCA Studies*: This thesis should be complemented with Techno-Economic Analysis (TEA) and Life Cycle Assessment (LCA) studies to evaluate the feasibility of the process when using alternative substrates as the sole source of nutrients.

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Chapter 9

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Estefanía Nathaly Eras Muñoz was born in Quito, Ecuador, on January 27th, 1995. In 2012, she began her studies in Biotechnology Engineering at the *Universidad de las Américas* (UDLA) and graduated in 2017. During her bachelor's degree, she had the opportunity to be a teaching assistant and worked as an intern researcher at the *Instituto de Investigaciones Biomédicas* (IBB-UDLA). In 2017, she moved to Barcelona, Spain, to pursue her master's studies in Advanced Microbiology at the *Universitat de Barcelona* (UB) (2017-2018). Her master's thesis, entitled "Detection, isolation, and quantification of *Campylobacter* spp. and Microbiological Indicators in water samples from two drinking water treatment plants" was conducted in the *Departament de Genètica, Microbiologia i Estadística* at the UB.

At the beginning of 2019, Estefanía Nathaly worked as a Quality Technician in Microbiology at *Laboratorios Anabiol*, *S.L.U*. until October 2020, when she began her doctoral studies in Biotechnology at *Departament d'Enginyeria Química, Biològica i Ambiental* (*Universitat Autònoma de Barcelona*) in the *Grup de Recerca en Compostatge* (GICOM), supported by a grant from the *Generalitat de Catalunya* (2020 FISDU-00436). Her PhD research mainly focused on the production of biosurfactants within a circular bioeconomy framework under the guidance and supervision of Dr. Teresa Gea and Dr. Xavier Font. She also completed three-month research stay with Dr. James Winterburn's research group at The University of Manchester, UK, from September 2022 to December 2022.

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List of Communications

Eras-Muñoz, E., Font, X., Barrena, R., and Gea,T. (2022). Optimization of carbon and nitrogen sources for solid-state sophorolipid production. Poster presentation at the *Biosurfactants International Conference*, Stuttgart-Germany.

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