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Research Paper

Screening of alternative nitrogen sources for sophorolipid production through submerged fermentation using *Starmerella bombicola*

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ABSTRACT

To explore a sustainable sophorolipid production, several hydrolysates from agricultural byproducts, such as wheat feed, rapeseed meal, coconut waste and palm waste were used as nitrogen sources. 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 ($R^2 = 0.90$ and 0.83, respectively). At 0.31 g/L total nitrogen, wheat feed hydrolysate achieved the highest production, yielding 72.20 ± 1.53 g/L of sophorolipid crude extract and 60.05 ± 0.56 g/L of diacetylated lactonic C18:1 at shake flask scale with productivities of 0.43 and 0.36 g/L/h, 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 sophorolipid production and their further application on industrial biotechnology.

1. Introduction

The microbial biosurfactants (BSs) global market was valued at USD 1.2 billion in 2022 and is expected to reach USD 1.9 billion by 2027 with an annual growth rate of 11.2 %. Notably, in 2021, sophorolipids (SL) emerged as the largest subtype in terms of value within the BSs market (Markets and Markets, 2022). However, despite increasing demand, economical large-scale BS production remains a challenge when compared on a manufacturing cost basis to chemical surfactants (approximately 34–40 USD per kilogram for BSs 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).

The use of purified substrates constitutes 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. These byproduct streams, obtained from industrial, agricultural, and food waste, do not compete with food crops, mitigating ethical and resource allocation concerns. Indeed, a large part of these byproducts are often burned or improperly discarded, leading to negative environmental impacts (Raza et al., 2021; Sundaram et al., 2024). Estimates demonstrated a rising trend in global 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). In this regard, their utilization provides a dual benefit by reducing residues and creating value-added bioproducts, thereby aligning the process with the principles of the circular bioeconomy (Domínguez Rivera et al., 2019; Dierickx et al., 2022).

As consequence of its notable surface activity and versatile biological properties, SLs have garnered attention from both industry and researchers with a wide range of applications including their use in

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detergents and cleaning products or in bioremediation processes (Roelants et al., 2019; Eras-Muñoz et al., 2022). SL production relies on the availability of a hydrophilic carbon source for the sophorose moiety structure, a hydrophobic carbon source for the fatty acid tail, and a nitrogen source for yeast growth. 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 the use of several alternative substrates for SL production such as 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), among others. 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.

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 solid-state fermentation (SSF) as a supporting material or as co-substrates (Jiménez-Peñalver et al., 2020; Rodríguez et al., 2021); or they can be pretreated to increase the availability of nutrients and enable their use in submerged fermentation (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 combination of various enzymes (Yang et al., 2011). Indeed, enzymatic hydrolysis has been applied successfully for SL production by authors such as Kaur et al. (2019) and Wongsirichot et al., (2022a) using food waste and agricultural residues, 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 SL 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 high-value bioproducts production, suggesting that a promising alternative is to integrate enzyme production as a byproduct within a biorefinery context.

From a circular economy perspective, the successful production of SL relies on the implementation of cost-effective processes and the utilization of low-cost substrates as raw materials to achieve zero-waste generation. The goal of this work is the assessment of enzymatic hydrolysates derived from agricultural byproducts wheat feed, rapeseed meal, coconut waste, and palm waste as nitrogen sources for SL production, and to select the best hydrolysate to subsequently scale-up the process. To the best of our knowledge, this is the first report of these agricultural biomass as nitrogen feedstocks for SL production.

2. Material and methods

2.1. Chemical reagents and agro-based byproducts

Analytical grade substrates such as glucose, ammonium sulphate and corn steep liquor (CSL) were provided by Sigma-Aldrich (St. Louis, MO, USA) while, rapeseed oil (Crisp ŃDry, UK) was used as the hydrophobic substrate in this study. Four different agro-industrial wastes were used as nitrogen sources after their pretreatment: 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). Standards of diacetylated lactonic C18:1 sophorolipid were acquired from Biosynth Carbosynth (England).

2.2. Biomass characterization and hydrolysis process

The agro-industrial biomass was physiochemically characterized. Parameters measured were pH, dry matter (DM), moisture content (MC) and organic matter (OM), according to the standard methods (Thompson et al., 2001). The enzymatic hydrolysis process was conducted based on Wang et al. (2010) study with some modifications. Briefly, for 1-L hydrolysate the solid loading was 1:10 (w/v) while the commercial protease from *Aspergillus oryzae* (Sigma-Aldrich, Merck, Germany) was used with a biomass/enzyme loading ratio of 20:1 (w/v). The operating conditions were 55 °C, 24 h and 200 rpm. Following enzymatic hydrolysis, the residual solids were separated through centrifugation at 7500 rpm for 20 min at 4 °C. The resulting supernatant was collected, characterized, and subsequently frozen at −20 °C until it was utilized in the submerged fermentation (SmF) process.

2.3. Microbial seed culture and strain maintenance

The yeast *Starmerella bombicola* ATCC 22214 was cryopreserved at −80 °C with glycerol 10 % (v/v) as described by Ingham et al. (2022). Seed culture flasks were prepared using 10 % (v/v) of the cryopreserved stock in 50 mL growth media containing: 4 g/L $(NH_4)_2SO_4$, 5 g/L corn steep liquor, 100 g/L glucose and 100 g/L rapeseed oil. Growing conditions were set up at 30 °C, 200 rpm at 24 h. Once the seed culture reached the exponential phase with an optical density (OD_{600}) around 12–15 units it was inoculated into the fermentation media.

2.4. Fermentation process

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. 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 glucose as the hydrophilic carbon source, 100 g/L rapeseed oil as the hydrophobic carbon source and a target 0.15 g/L 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 below and all production media were prepared under sterile conditions and inoculated with *S. bombicola* seed to a 10 % (v/v) ratio. After each fermentation samples were frozen at −20 °C prior to analysis.

2.4.1. Screening of biomass hydrolysates supplemented with glucose and rapeseed oil

To assess the feasibility of using hydrolysates as a nitrogen source, the CSL and $(NH_4)_2SO_4$ were substituted by WF, RM, CW, and PW hydrolysates. The TN concentration was standardized at 0.15 g/L while the concentration of rapeseed oil and total glucose were maintained at 100 g/L each, consistent with the control group. Firstly, media combinations were prepared and 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 the inoculum were added. Finally, production flasks were incubated in an orbital shaker (Infors HT, UK) at 30 °C, 168 h, and 200 rpm (Wongsirichot et al., 2022a; Ingham and Winterburn, 2022).

2.4.2. Evaluation of different nitrogen concentrations

The hydrolysates that showed the best outcomes (SL production) were used to assess a wider range of nitrogen concentrations. Initially, a TN concentration of 0.15 g/L was employed, with the impact on the SL producing fermentation evaluated at half (0.07 g/L), one- and a half-fold (0.23 g/L), and two-fold (0.31 g/L) concentrations. The maximum hydrolysates TN (1.53 g/L and 3.78 g/L) 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 the previously described conditions.

2.5. Batch bioreactor fermentation

Process scale-up was conducted using a 2 L bioreactor (BioStat® B, Sartorius, Germany) with a working volume of 1.8 L. The fermentation media consisted of 100 g/L glucose and 100 g/L 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. The fermentation process was developed following Wongsirichot et al., (2022a) methodology with modifications. Briefly, the operational parameters established were: temperature 30 °C, airflow rate 2 mL/min., 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) or H_2SO_4 (2 mM) as needed. An antifoam agent (Glanapon 2000 KONZ, Bussetti, Austria) was used when necessary to control foam formation. Finally, the fermentation time was 168 h and samples were aseptically withdrawn at regular 24 h intervals for subsequent post-fermentation analysis. Due to the utilization of a second batch of hydrolysate, the fermentation medium for the scale-up was also assessed in triplicate using 4-bottom baffled Erlenmeyer flasks (250 mL) at 30 °C, 200 rpm for 168 h.

2.6. Post-fermentation analysis

2.6.1. Diacetylated lactonic C18:1 quantification

Frozen samples were thawed at 60 °C for 15 min with the aim of dissolving 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. To prepare the supernatant for HPLC-UV analysis, it was filtered through 0.2 µm Nylon syringe filters. Subsequently, HPLC analysis was performed following the conditions described by Ingham et al., (2023), using a Macherey-Nagel™ Nucleosil™ 100 mm × 3 µm × 4.7 mm C18 EC column with a binary gradient of HPLC grade water (phase A) and acetonitrile (phase B) with 0.1 % formic acid. Finally, the SL peaks were detected at a wavelength of 198 nm and quantified against serial dilutions (2.5 to 20 g/L) of the standard diacetylated lactonic C18:1 SL prepared in pure ethanol.

2.6.2. Solvent extraction

Oil recovery and SL crude extract quantification were performed using n-hexane/ethyl acetate triple extractions following the method described by Wongsirichot et al., (2022a) with some modifications. The procedure involved combining equal volumes of fermentation broth and solvent. In brief, to quantify residual oil an equal volume of n-hexane was added to the sample, 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 2 min, after which the top layer was carefully transferred into a pre-dried aluminum tray. Once the n-hexane extraction was completed, the same procedure was repeated using ethyl acetate for SL quantification. Subsequently, the organic phase was placed on aluminum trays and air-dried overnight at room temperature. Gravimetric measurements were then conducted to determine the concentrations of oil and SL crude extract in the hexane and ethyl acetate extractions, respectively. Finally, the remaining aqueous phase was subjected to centrifugation at 7000 rpm for 15 min, and the resulting pellet was resuspended and placed on a pre-dried aluminum tray that was further dried at 105 °C for cell dry weight (CDW) analysis.

2.6.3. Routine methods

The hydrolysates and the fermentation samples were used for pH, sugar analysis, total carbon (TC) and TN. Initially, the supernatants underwent centrifugation at 7000 rpm for 15 min, followed by membrane filtration using a 0.2 µm membrane filter. 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). While sugar quantification was performed via HPLC using a CarboSep CHO 782 lead form 300 mm × 8 µm × 7.8 mm column (Concice Separations, USA), coupled to a refractive index detector (RI). Briefly, a constant flow rate of HPLC grade water at 0.6 mL/min was used for 30 min with a column temperature maintained at 70 °C. Subsequenlty, samples were compared with a calibration curve for nine different mono- and disaccharides.

2.7. Statistical analysis

All experiments were carried out in duplicate to estimate the biological error, except for the scale-up validation, which was conducted in triplicate. Statistical analyses were performed using JMP® 15 statistical software (JMP Statistical Discovery LLC, USA), with all tests conducted at a 95 % confidence interval ($\alpha = 0.05$).

3. Results and discussion

3.1. 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 SL production. Table 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 and 2.47 ± 0.51 g/L, respectively). Conversely, PW hydrolysate presented the lowest TN content (0.69 ± 0.09 g/L). 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. SL). 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 (see supplementary material Fig. S1). 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), followed by CW (1.48 ± 0.03 g/L) and PW (1.30 ± 0.02 g/L) 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. This 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 SL 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 SL production. (Nayak and Bhushan, 2019). As reported by De Castro and Sato (2014), the biochemical characterization of *A. oryzae* protease revealed that the

Table 1
Characterization of the alternative substrates and their corresponding hydrolysates used in this study.

Feedstock	Biomass characterization									Hydrolysate characterization				
	pH	Glucose (g/kg)	DM (%)	M (%)	OM (%, db)	C (%, db)	H (%, db)	N (%, db)	S (%, db)	C/N		pH	Glucose (g/L)	TC (g/L)	TN (g/L)
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; M, moisture; 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).

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.

3.2. 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 SL (Fig. 1). Despite having the same nutrient concentration (Section 2.4), Control 1 exhibited a higher dry cell weight (p-value = 0.02) and SL 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

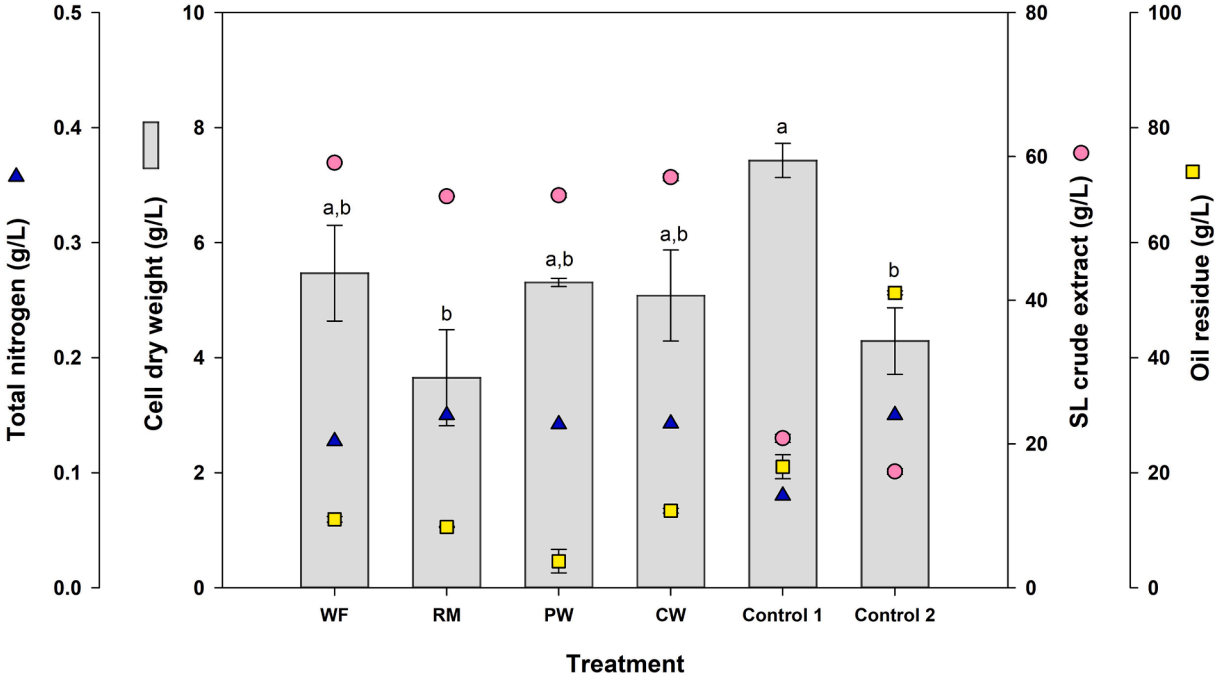

Fig. 1. Shake flask fermentations using agricultural byproducts hydrolysates as a nitrogen source at 168 h. Hydrolysate abbreviations: WF (wheat feed), RM (rapeseed meal), PW (palm waste), CW (coconut waste). Control 1 (ammonium sulphate + corn steep liquor) and Control 2 (ammonium sulphate). Error bars 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).

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, whereas Control 2 attained 4.29 ± 0.58 g/L. Concerning the hydrolysates, WF, RM, PW and CW yielded 5.47 ± 0.83 g/L; 3.65 ± 0.83 g/L; 5.31 ± 0.07 g/L and 5.08 ± 0.79 g/L, 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 SL 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 SL production. Concerning SL crude extract production, the tested hydrolysates gave a higher concentration compared to Control 1 (20.78 ± 0.57 g/L) 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), followed by CW (57.11 ± 0.49 g/L). Moreover, RM and PW hydrolysates do not exhibit significant differences (p-value 0.99) in SL production levels (54.44 ± 0.06; 54.59 ± 0.30 g/L, respectively). In a prior study conducted by Zhu et al. (2013), RM was used for BSs 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.

It is well known that SL 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 (Fig. 2) ranges from 34.90 to 47.40 g/L which represents a four-fold increase compared to Control 1 (11.64 ± 0.44 g/L). 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.

3.3. 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

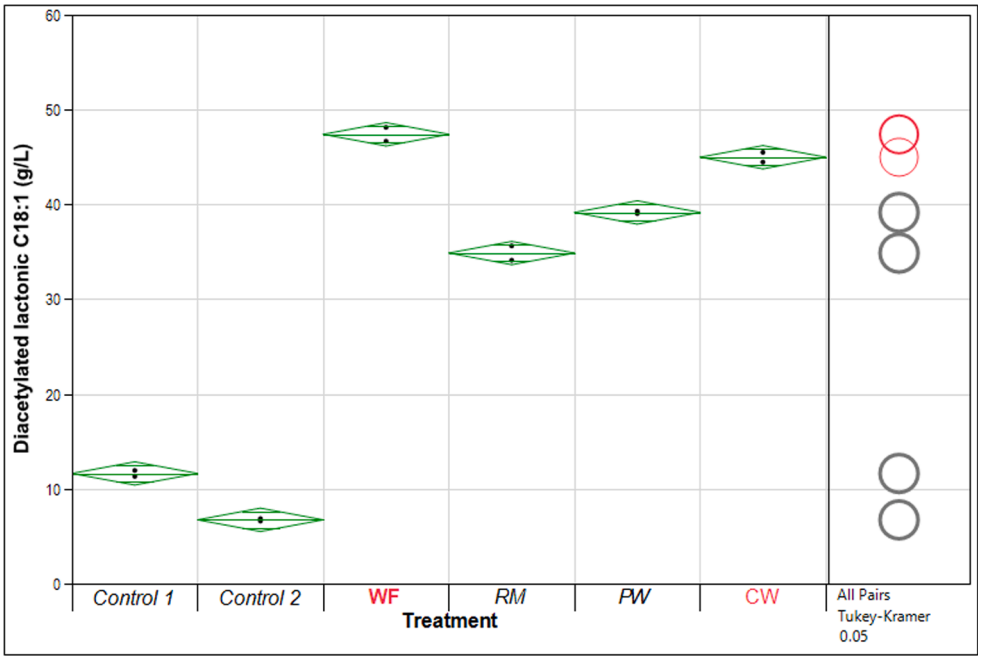

Fig. 2. Comparison of diacetylated lactonic C18:1 production at 168 h using agricultural byproducts hydrolysates as nitrogen . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Source. The box graph displays individual samples 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). Abbreviations: wheat feed (WF), rapeseed meal (RM), palm waste (PW), coconut waste (CW), Control 1 (ammonium sulphate + corn steep liquor) and Control 2 (ammonium sulphate)

C18:1 (section 3.2). The results indicated that the tested concentrations exhibited similar behavior for both hydrolysates (Fig. 3). The highest SL crude extract production using WF hydrolysate (72.20 ± 1.53 g/L) was achieved at 0.31 g/L TN. Conversely, the lowest (33.84 ± 0.17 g/L) was obtained at 0.07 g/L TN. Furthermore, CW hydrolysate displayed a similar production pattern, achieving the highest (72.40 ± 0.82 g/L) and the lowest (36.60 ± 1.56 g/L) SL crude extract production under the same TN concentrations. Moreover, it is noteworthy that both hydrolysates exhibited a SL crude extract volumetric productivity of 0.43 g/L/h at 0.31 g/L TN concentration. At nitrogen concentrations of 0.07 g/L, 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, respectively). Similarly, at 0.15 g/L TN, an insignificant difference (p-value 0.59) was found in the final SL concentrations (43.78 ± 0.27 and 43.54 ± 0.45 g/L, respectively). However, WF and CW showed significant differences at 0.22 g/L TN (51.94 ± 0.25 and 48.79 ± 0.54 g/L, respectively) and 0.31 g/L TN (60.05 ± 0.56 and 56.63 ± 0.37 g/L, 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

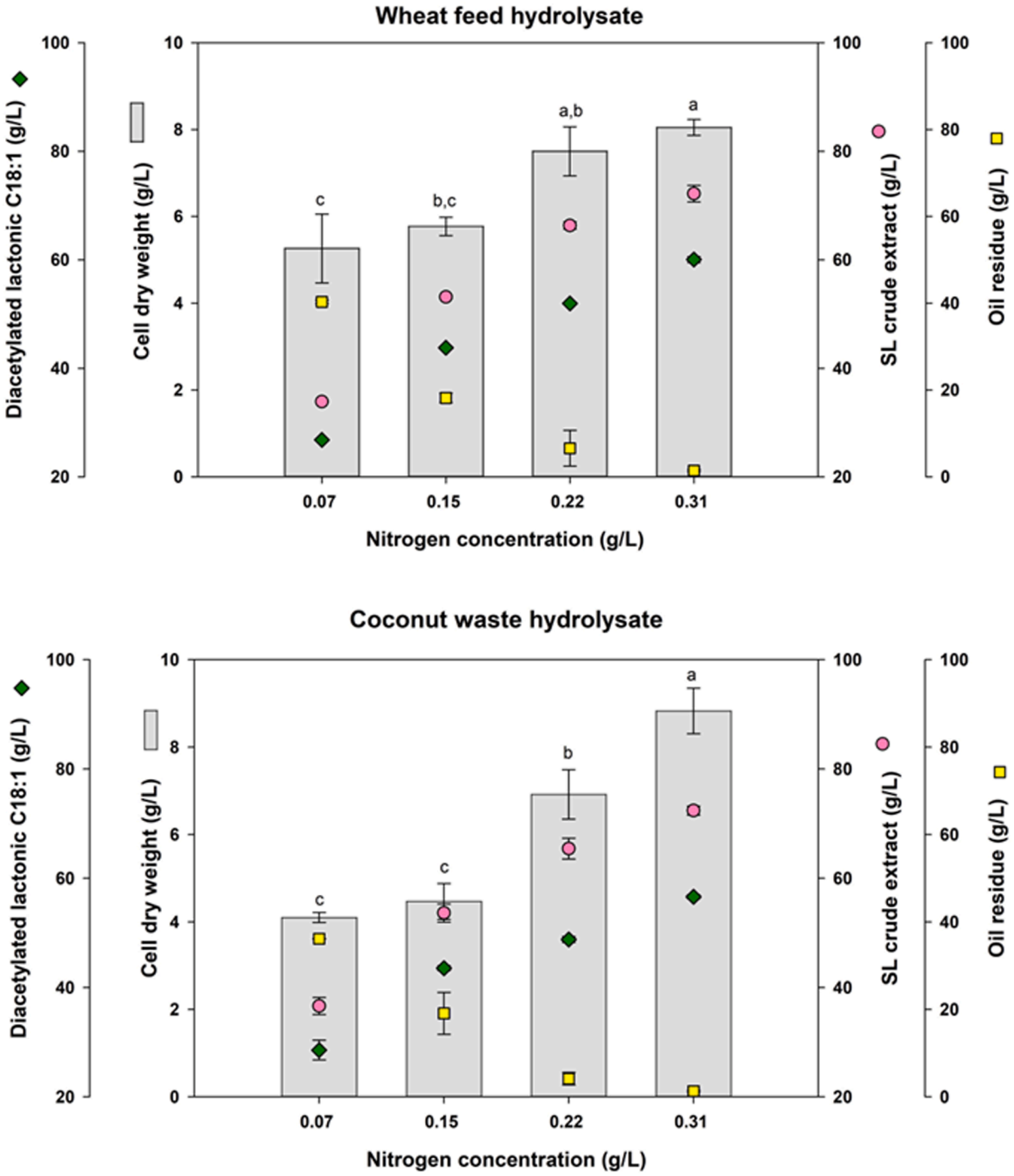

Fig. 3. Influence of total nitrogen concentration on sophorolipids production. Main fermentation outcomes using agricultural byproducts hydrolysates at total nitrogen concentrations range from 0.07 to 0.31 g/L. a) Wheat feed hydrolysate (WF). b) Coconut waste hydrolysate (CW). Error bars represent the standard deviation of biological replicates (n = 2). Cell dry weight means that do not share a letter are significantly different (p-value < 0.05).

potential amino acids within such byproducts or hydrolysates that could serve as supplementary nitrogen source and evaluate whether their presence positively impacts SL production.

Furthermore, as reported by Ingham and Winterburn (2022), a direct relationship between SL 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 was used in all the treatments. After 168 h of fermentation, oil concentrations neared depletion when 0.31 g/L TN for both types of hydrolysates. Residual oil quantities of 1.42 ± 0.23 g/L and 1.29 ± 0.21 g/L were observed for WF and CW hydrolysates, respectively. Importantly, these concentrations were concurrent with the highest SL reported production, as previously described.

Our findings validate a strong correlation between yeast growth and SL production at the tested concentrations in a 168-h fermentation. 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 SL production. In this context, TN was increased to 1.53 g/L and 3.78 g/L, the uppermost limits of CW and WF hydrolysates, and were evaluated with and without supplementation (Fig. 4).

Significant differences (p-value 0.01) in SL 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 3.3) and to the inorganic nitrogen source used to supplement CW to reach 3.78 g/L TN. Indeed, HPLC-UV analysis revealed that CW-3.78 mainly produces acidic SL congeners instead of lactonic ones (supplementary material Fig. S2). Additionally, CW-3.78 presented the highest oil residue content (70.96 ± 8.63 g/L) which is directly correlated with a low SL (16.46 ± 1.50 g/L) and diacetylated lactonic C18:1 production (9.99 ± 0.06 g/L). When comparing results in terms of glucose consumption, CW-1.53 (3.23 ± 0.58 g/L) and WF-1.53 (2.22 ± 0.10 g/L) 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, respectively). This discrepancy implies that the C/N ratio within the WF hydrolysate facilitated the highest glucose consumption after 168 h of fermentation, consequently increasing SL production.

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, respectively). In contrast, when supplemented with glucose, CW-3.78 achieved the highest cell dry weight (26.32 ± 0.31 g/L), significantly (p-value < 0.01) surpassing WF-3.78 (20.25 ± 0.21 g/L). Despite both combinations presenting higher yeast growth, there was a noteworthy decline in SL production and diacetylated lactonic C18:1 (Fig. 5). 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 SL production is not correlated with biomass concentration at higher nitrogen concentrations (>1.53 g/L). Similarly, Daverey and Pakshirajan (2010) reported that the optimal TN concentration for yeast growth was 10 g/L, while for SL production was 2 g/L 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

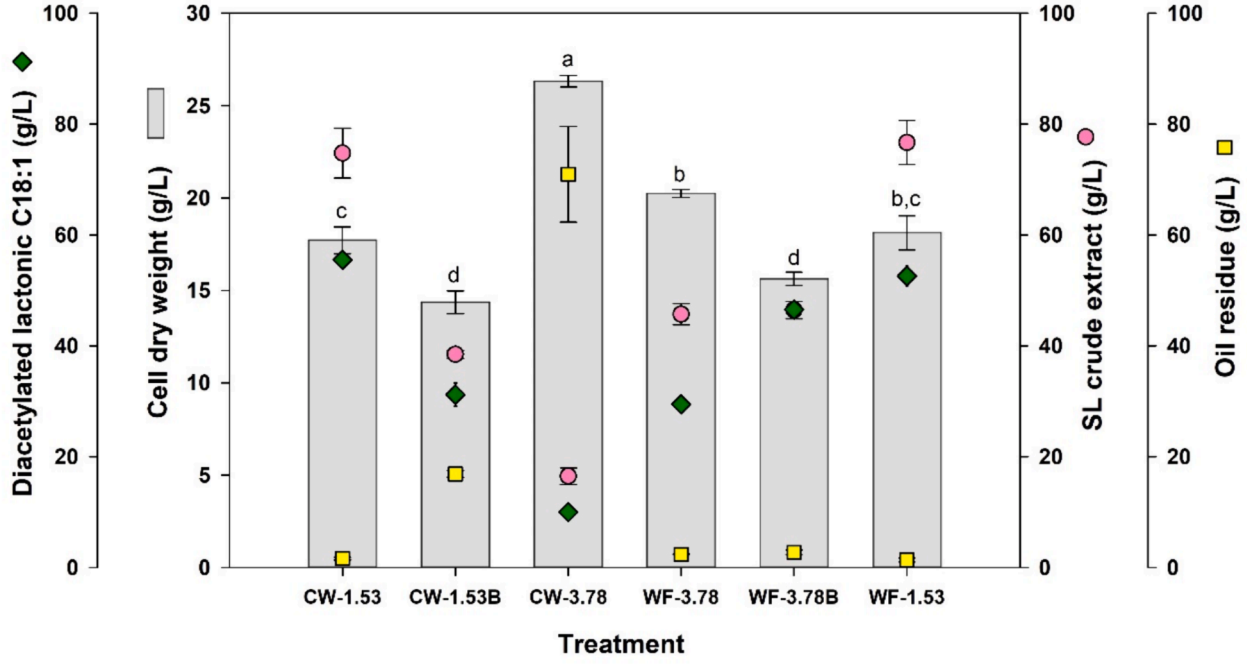

Fig. 4. Maximum hydrolysate total nitrogen (TN) evaluation with and without glucose supplementation. Error bars represent the standard deviation of biological replicates (n = 2). Cell dry weight means that do not share a letter are significantly different (p-value < 0.05). Abbreviations: CW-1.53, CW-3.78, 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 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 TN.

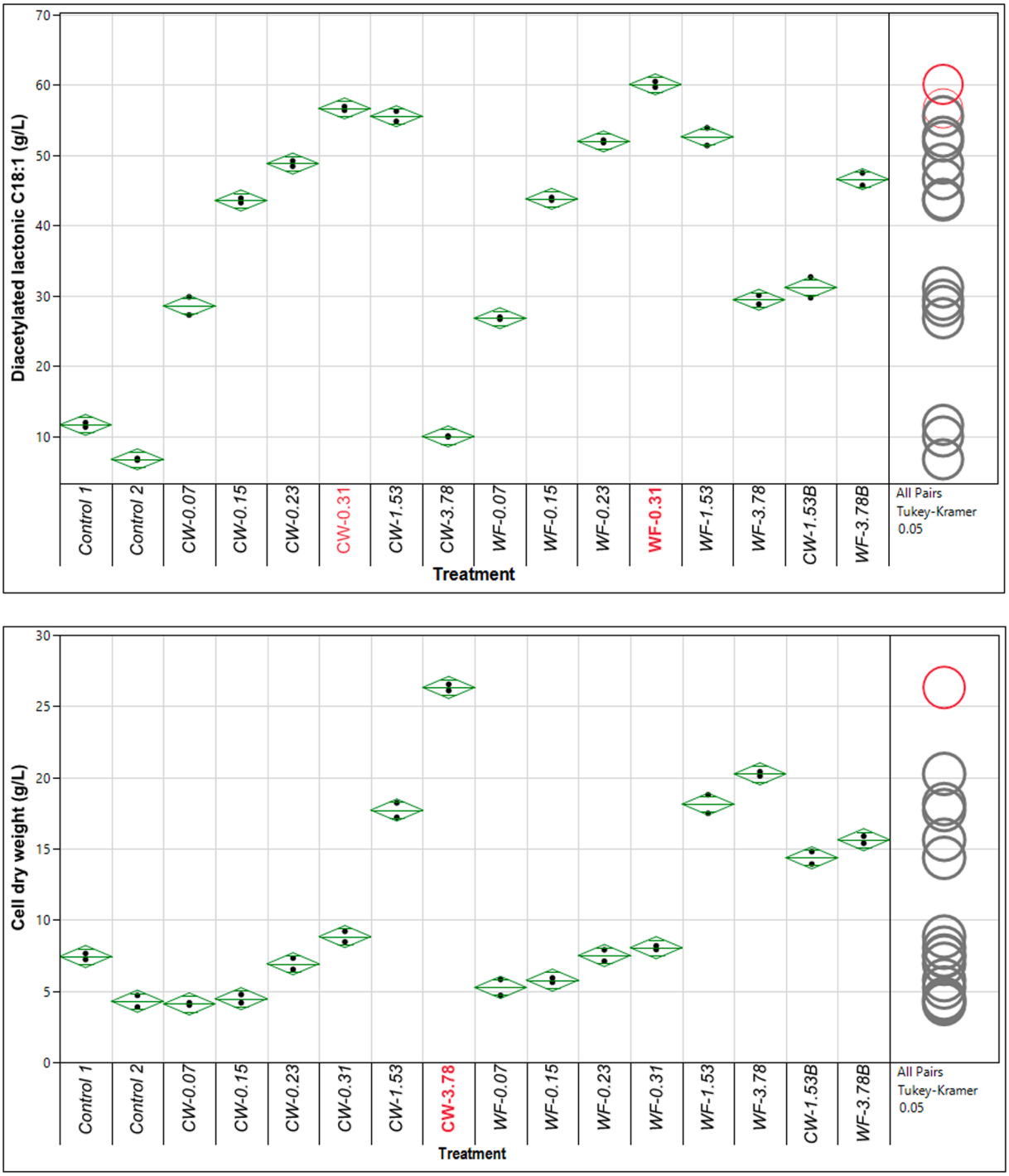

Fig. 5. Influence of total nitrogen concentration on diacetylated lactonic C18:1 production and cell dry weight. a) Diacetylated lactonic C18:1 production (g/L). b) Cell dry weight (g/L). The box graph displays individual samples as dark points (biological replicates for each treatment n = 2). Diamonds 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 + corn steep liquor) 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) and the letter B represents hydrolysates without glucose supplementation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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).

Crude SL 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 total SL crude extract

mix. 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 structure (supplementary Fig. S3). This result is directly associated with the achieved values of SL crude extract (46.46 ± 1.56 g/L) and diacetylated lactonic C18:1 (46.57 ± 1.26 g/L) 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 sophorolipids crystallization. HPLC-UV quantification revealed a higher ratio of diacetylated lactonic C18:1 production when employing WF-3.78B, comprising 88.5 % of the SL crude extract mix (supplementary Fig. S4). 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. 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 SL production, it was clear that WF hydrolysate also contains other sugars (see section 3.1) 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, SL production ranges up to 40.15 g/L which is in concordance with our findings.

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 SL 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 SL synthesis based only on glucose as the carbon source. Indeed, literature reported that an elevated C/N ratio facilitates the initiation of SL biosynthesis, while a lower ratio could result in unmetabolized SLs or lead to the metabolism of previously synthesized which consequently decreases SL 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 SL, 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.

3.4. Batch bioreactor fermentation

In the existing literature SL 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 2). The results of the batch cultivation of *S. bombicola* in a bioreactor are illustrated in Fig. 6 (see supplementary material Fig. S5 for operational control parameters). During the fermentation, a maximum cell growth of 6.23 ± 0.39 g/L was achieved at 96 h, followed by a decrease in cell concentration to 5.28 ± 0.04 g/L. This decrease in biomass indicates the transition from the growth phase to the stationary phase, which is favorable for SL production. Notably, significant SL levels were observed at 48 h (11.20 ± 0.21 g/L), and final (168 h) SL concentration was 51.70 ± 0.07 g/L, with a volumetric productivity of 0.31 g/L/h, and a diacetylated lactonic C18:1 concentration of 39.24 ± 0.29 g/L. These results closely resemble those outlined by Samad et al. (2017) who achieved a SL concentration of 52.1 g/L with a titer of 0.31 g/L/h using agricultural biomass hydrolysates. Furthermore, Kaur et al. (2019) achieved a SL concentration of 28.15 g/L with a volumetric productivity of 0.39 g/L/h at 72 h using food hydrolysates at batch scale. In contrast, in this study at the same fermentation time, a SL crude extract concentration of 21.88 ± 1.03 g/L with a productivity of 0.30 ± 0.01 g/L/h 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) 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 SL 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 glucose and 33.33 ± 0.25 g/L oil at the end of the fermentation process. HPLC-MS chromatograms (supplementary material Fig. S6) 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). 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).

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 and 34.24 ± 2.71 g/L for SL crude extract and diacetylated lactonic C18:1, respectively. In comparison with 2-L bioreactor outcomes, there were no significant differences observed for SL 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 SL highest productivity was 8.46 g/L/h using bagasse sweet sorghum and soybean oil (Samad et al., 2015) while at bioreactor scale it was 1.25 g/L/h using food waste and oleic acid (Kaur et al., 2019). These results highlight the potential of alternative feedstocks and the challenges associated with SL scaling-up process. In operational procedures, maintaining a certain amount of oil prevents foam formation, which commonly causes mass transfer limitation in this

Table 2
Summary of biomass hydrolysis for SL production from literature and present study.

Pre-treatment	Substrate	Hydrophobic substrate	Fermentation strategy	Microorganism	Time (h)	Yield (g/L)	Productivity (g/L/h)	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. (2013)
Enzymatic hydrolysis: protease, lipase and glucoamylase	Food waste	Oleic acid	Batch Fed-batch	*S. bombicola* ATCC 22214	72 92	28.15 115.2	0.39 1.25	Kaur et al. (2019)
Enzymatic hydrolysis: cellulase, α-amylase and glucoamylase	Potato byproduct	Rapeseed oil	Batch Fed-batch	*S. bombicola* ATCC 22214	312 216	60.5 77.8	0.19 0.68	Wongsirichot et al., (2022a)
	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.

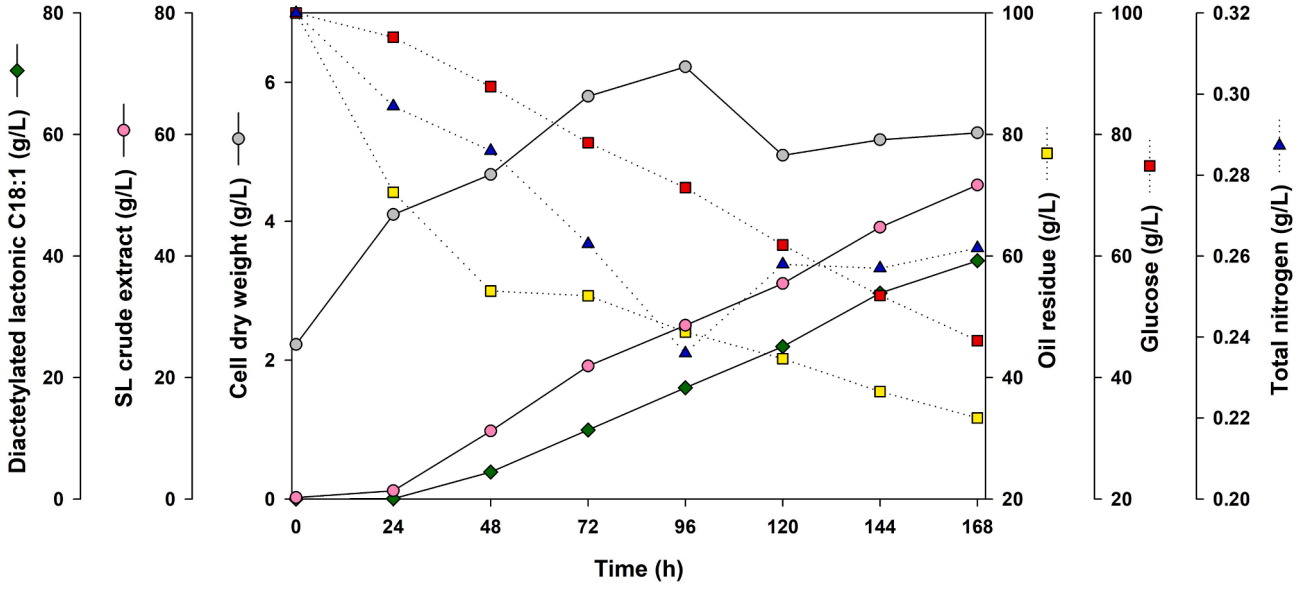

Fig. 6. Time course fermentation in a batch bioreactor by *S. bombicola* using wheat feed hydrolysate (WF) at a total nitrogen concentration of 0.31 g/L. Fermentation was carried out at 30 °C, 200 – 800 rpm, airflow at 2 mL/min with dissolved oxygen set to > 30 %.

kind of fermentation (Dolman et al., 2019).

Wongsirichot et al., (2022a) reported a SL titer for a batch bioreactor over 10 times higher than the shake flask scale with a SL production of 40 g/L using potato media hydrolysate at 168 h. In the current study, at the same fermentation time, a 15 % increase was observed in diacetylated lactonic C18:1 production using WF hydrolysate at 0.31 g/L 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 SL 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 SL 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 SL production due to their function in the BS pathway. In addition, literature on the topic reported that during the stationary phase, when SL production begins, the addition of a lipid substrate/hydrolysate in a stepwise or continuous manner is practiced for improving SL 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 SL fermentation.

4. Conclusion

In summary, this paper represents the first steps towards the use of sustainable nitrogen sources for SL 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 SL production for TN concentrations between 0.07–0.31 g/L. 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 SL production, future research should explore a broader range of biomass hydrolysates, investigate enzymatic cocktails, and assess alternative feedstocks without supplementation.

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CRediT authorship contribution statement

Estefanía Eras-Muñoz: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Phavit Wongsirichot:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Benjamin Ingham:** Writing – review & editing, Supervision, Methodology, Conceptualization. **James Winterburn:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Teresa Gea:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Xavier Font:** Writing – review & editing, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

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

Data availability

The raw data supporting this article is available at https://doi.org/10.34810/data1392.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2024.05.048.

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