



# Characterization of Spent Yeast Streams from Precision Fermentations Driven by Genetically Engineered Yeasts: Valorization Potential for a Circular Bioeconomy

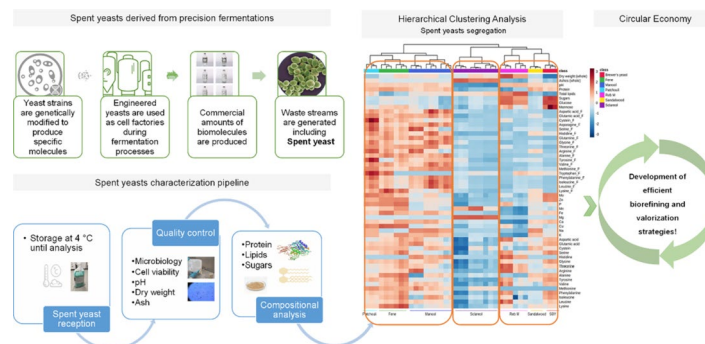
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## Abstract

Currently, valuable biomolecules are sustainably produced through fermentation using genetically modified microorganisms. However, this industry also generates several waste streams, such as spent yeast (SY). To close the sustainability loop in bioproduction, it is essential to define valorization strategies for such waste streams. Hence, this study aimed to investigate the compositional differences among these novel SYs, intending to pave the way for devising highly effective and beneficial valorization strategies for these streams. In this work, SYs derived from the production of beer (SBY) and six biomolecules [ $\beta$ -farnesene (Fene), rebaudioside M (Reb M), manool (MN), patchouli (PAT), sandalwood (SDW), and sclareol (SCL)] through fermentations driven by engineered yeasts were characterized. The results showed that SY from Fene, MN, and PAT production have similar concentrations of protein (35.9–44% dw), minerals, and amino acids, being compatible with bulking and use as nitrogen sources. Those derived from Reb M production showed significantly higher levels of lipids ( $21 \pm 2.6\%$  dw) compared to all the other SYs (1.7–10.8% dw), whereas the ones derived from SCL production showed significantly higher levels of inorganic matter (45%) compared with those found in the other SYs (1.2–11.3% dw). The results showed that assessing the extent of compositional variation among different SYs derived from precision fermentations represents a primary step toward developing efficient valorization or biorefining strategies.

## Graphical Abstract



**Keywords** Biomass composition · Biorefining · Fermentation · *Saccharomyces cerevisiae* · Spent yeast · Waste valorization

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## Statement of Novelty

Fermentation, such as in beer production, relies on *Saccharomyces* spp. Throughout the fermentative process, various waste streams are produced, and SY is the second major by-product. While the composition and valorization strategies

of SBY have been thoroughly examined, the characterization of its counterpart resulting from fermentations driven by genetically engineered yeast strains remains unknown. These engineered strains, designed for biomolecule synthesis spanning cosmetic, pharmaceutical, and agri-food sectors, pose an unresolved challenge. In the dynamic expanse of industrial biotechnology, the stream of SY accompanies exponential growth, and effective management strategies for these accumulating by-products require foundational insights into these waste streams. In this work, we present a pioneering investigation—a comparative analysis of the attributes of SY from beer fermentation juxtaposed with those derived from six distinct fermentations, each driven by unique genetically engineered *S. cerevisiae* strains. The results unequivocally reveal substantial disparities in the composition of these residual streams, amplifying the implications for strategies aimed at their valorization.

## Introduction

Current global concerns related to climate change and food security prompt the development of a sustainable bioeconomy where biobased products replace oil-based products and industrial wastes, and side streams are valorized using biotechnology-based processes to produce value-added products, contributing to the development of a sustainable circular bioeconomy. In this context, an industry based on synthetic biology and fermentation processes emerged and is currently under expansion. Microbial strains are genetically modified, and their metabolism engineered, to produce valuable biomolecules such as flavors, fragrances, or bioactive molecules with potential applications in sectors such as cosmetics, pharmaceuticals, or agri-foods. The most widely used yeast for biosynthesis of complex molecules is *Saccharomyces cerevisiae*. This yeast species is well known, it is easy to modify genetically, and although other yeast species such as *Yarrowia lipolytica* and *Kluyveromyces lactis* have been studied for the large-scale production of a myriad of molecules, yeasts derived from *Saccharomyces cerevisiae* are the most used [1]. Even though the synthetic biology industry creates a reliable supply of cost-effective molecules, being a sustainable alternative to the capturing and extractive industries, to close the loop towards a circular bioeconomy, side streams and wastes generated during the fermentation processes, including SY, bottom, vinasse, or sugarcane straw, should be utilized as biomaterials to produce other products. Improved waste management is required to reduce the environmental impacts of fermentation industries constituting an essential step in the transition toward a bioeconomy, in which biotechnology tools add value to the by-products resulting from industrial processes in biorefineries [2, 3].

SY is the second major by-product derived from fermentation processes, representing about 15–20% of the total residues generated [4]. Although most SY produced comes from the brewing industry, as the platforms of synthetic biotechnology evolve, the production of a myriad of molecules through fermentation increases, and consequently, the side streams generated in these processes are also foreseen to increase in the coming years. In 2013, it was estimated that the European Union market for biotechnology-derived products could develop from 28 billion euros in 2013 to 40 billion euros in 2020 and up to 50 billion euros in 2030 [5].

Spent brewer's yeast (SBY) is an inexpensive source of protein with a carbon content of approximately 45% dry weight. It is rich in minerals and B vitamins [6, 7], mannans, known to have a prebiotic effect in ruminants [8], and  $\beta$ -glucans, known to possess antimicrobial, anticancer, anti-diabetic, and anti-hypercholesteremic activities [9–11].

Due to its high nitrogen content, SBY is frequently used as a fertilizer or supplement for animal feeds after minimal processing [8].

Even though there are currently limited industrial uses for these waste streams, their potential for fostering new products and applications expands as the understanding of their composition and biological activity advances. Several SBY utilization strategies have been under investigation in recent years including its use as a biosorption agent for wastewater treatment [12–14], enzyme source, fermentation substrate for biofuel production in the form of methane, encapsulating material for bioactive ingredients or flavor enhancer, among others [15]. A review from Puligundla et al. [16] provides an overview of the potential applications of SBY.

Although the composition of SBY is known, and it is *Generally Recognized as Safe* (GRAS) by the Food and Drug Administration (FDA) the SYs derived from synthetic biology platforms are genetically modified, which impacts the whole yeast cell functioning and composition. Therefore, the nutrient composition and valorization potential of those SYs are mainly unknown. This study aimed to explore the compositional variability among novel SYs and its impact on potential valorization strategies. Understanding these differences is crucial for waste management and valorization. Notably, to the best of our knowledge, no previous study has detailed the composition of SYs from different industrial precision fermentations using genetically engineered yeast strains. This research highlights the need to understand how genetic modification affects microbial strain composition and underscores the importance of characterizing these SYs for future efficient valorization strategies, aligning with the transition towards a circular, biobased economy.

## Materials and Methods

### Spent Yeast Material

The composition of seven SYs was evaluated: SY derived from beer fermentation, driven by native *Saccharomyces cerevisiae*, and six SYs derived from the production of six biomolecules driven by six different genetically engineered *Saccharomyces cerevisiae* strains. The six biomolecules produced by the different strains through precision fermentations were: (i)  $\beta$ -farnesene (Fene, terpenoid like molecule, two strains, produced at industrial scale) with several applications including biofuels industry; (ii) rebaudioside M (Reb M, terpenoid based steviol glycoside, two strains, production at pilot scale), a sweetener destined to the food industry; and four fragrances used in cosmetics, and produced at pilot scale including: (iii) manool (MN, labdane diterpenoid, three strains), (iv) patchouli-type oil (PAT, sesquiterpene alcohol, one strain), (v) sclareol (SCL, diterpene alcohol, three strains) and (vi) sandalwood-type oil (SDW, sesquiterpene alcohol, one strain). Hereafter, the SYs derived from the production of the different biomolecules are referred to as Fene, Reb M, MN, PAT, SCL, and SDW, whereas the SY derived from beer fermentation is referred to as SBY.

The SBY was obtained as a by-product of beer production (heavy phase of the yeast slurry resulting from the fermentation) and it was kindly provided by UNICER (Porto, Portugal). The SYs derived from the production of the six biomolecules were provided by Amyris Inc. (Emeryville, California, USA). These by-products consisted in the heavy phase of the waste stream containing the SY, like a yeast slurry. For some of these waste streams, more than one batch of samples was analyzed, with each corresponding to slightly changed fermentation settings. All samples were collected between April and August 2019.

### Sample Preparation

All samples were refrigerated upon arrival at the laboratory and maintained at 4 °C. SYs quality was evaluated within 24–72 h using the samples as received without any pre-treatment. These analyses comprised of pH, dry weight, ash content, and minerals. In addition, two sets of whole samples were prepared and stored under different freezing conditions: one set was stored at – 20 °C, and the other at – 80 °C. The set stored at – 80 °C was then lyophilized for 48 h (Gamma 2–16 LSCplus, Martin Christ, Osterode am Harz, Germany). The freeze-dried samples were used to determine the protein, sugar, and lipid contents, and the total amino acid and sugar profiles. The free

amino acid profile was determined using the supernatant of the whole SY samples stored at – 20 °C, obtained after thawing and centrifugation at 4696×g (Thermo Scientific Heraeus for Multifuge X1R, Osterode am Harz, Germany) for 10 min at 4 °C.

### Spent Yeast Quality

#### Cell Viability, pH, Dry Weight, Ash Content

Cell viability was determined using a Neubauer chamber. The sample was diluted to  $10^{-3}$  and stained with methylene blue (0.05%). The viable and non-viable cells were counted using the microscope. The pH was determined using a pH meter (SevenExcellence pH meter S400, Mettler Toledo, USA). The dry weight and ash content were determined following the standard procedures of the Association of Official Analytical Chemists (AOAC) [17] and were performed in triplicate. For dry weight determination, 1 mL of fresh SY was placed in a pre-tared crucible, weighed, and placed in an oven at 105 °C overnight and then transferred to a desiccator to cool before weighing. For ash determination, the previously dried samples were placed in a muffle (Muffle furnace L3/11, Nabertherm GmbH, Lilienthal, Germany) with a temperature ramp set to 500 °C for 4 h. Then, the samples were cooled in the desiccator and weighed.

#### Minerals

For minerals determination, frozen SY was allowed to thaw at 4 °C, and 2 mL of sample was mixed with 5 mL of 65% HNO<sub>3</sub> plus 1 mL of 30% H<sub>2</sub>O<sub>2</sub> in a Teflon reaction vessel and digested in a microwave system (Speedwave XPERT, Berghof, Eningen, Germany). Digestion was conducted in five steps: 160 °C for 5 min; 190 °C for 5 min (twice); 100 °C for 2 min (twice). The resulting solutions were filtered using 0.45  $\mu$ m PTFE filters and brought up to 30 mL with ultrapure water for analysis. Mineral concentrations were analyzed by inductively coupled plasma argon spectrometry (ICP; ICP-OES Optima 7000 DV, PerkinElmer, Waltham, Massachusetts, USA). Minerals were quantified using calibration curves of a commercial mix standards for ICP analysis (Inorganic Ventures, Christiansburg, USA) (molybdenum, zinc, phosphorus, manganese, iron, magnesium, calcium, copper, sodium and potassium) from 0.05 to 10 mg/L. The analyses were conducted in triplicate, and the results were presented in the form of a heatmap after clustering analysis (Fig. 1). Mineral concentrations were expressed in mg/L fresh weight (Table S1 and S2).

## Protein

Protein content was determined as described by Dumas et al. [18] with a Dumatec™ 8000 system (Foss, Hilleroed, Denmark). Helium and oxygen were used for analysis at flow rates of 195 and 300 mL/min, respectively, at 1100 mbar. Lyophilized SY samples (50 mg) were weighed in tin foils and placed in crucibles for analysis. Total nitrogen was determined using a calibration curve performed using 10 to 150 mg of ethylenediaminetetraacetic acid (EDTA) calibration standard (Foss, Hilleroed, Denmark). Protein content was determined from the total nitrogen content, multiplied by a conversion factor of 5.8 [19].

## Amino Acids Profile

For the analysis of free amino acids, the supernatant of the whole SY was separated by centrifugation for 10 min at  $4696\times g$  at 4 °C, collected, diluted in 0.1 M HCl, and filtered through 0.45 µm filters. The dilution factor was adjusted for each sample and varied from 20 to 80.

For the determination of the total amino acid profile, the whole freeze-dried samples were subjected to acidic hydrolysis before analysis. For the hydrolysis, 3 mL of 6 M HCl were added to 10 mg of sample, vortexed, and then in a closed flask with a septum, bubbled with nitrogen for 3 min. Nitrogen was injected through an inlet needle to substitute sample atmosphere and another needle was used as a gas outlet. The sealed sample was then incubated at 115 °C for 20 h, after which, 4 mL of deionized water were added. The pH was adjusted to 3.2 with 10 M NaOH, and deionized water was added up to 10 mL. The sample was then filtered with a 0.45 µm PTFE filter for HPLC analysis [20].

The amino acids profile was analyzed by iodoacetic acid derivatization and o-phthaldialdehyde methodology, using reverse phase high performance liquid chromatography with a Chromolith® Performance RP18 (4.6 × 100 mm) column (Merck KGaA, Darmstadt, Germany) for separation, and coupled to a high resolution fluorescence detector (Agilent Technologies, Inc., California, USA) [21]. For quantification of free amino acids, 20 µL of the supernatant previously diluted in HCl 0.1 M was derivatized and 10 µL was injected [22]. The analysis was conducted in triplicate and the amino acids quantified using calibration curves of pure standards, aspartic acid (Asp), glutamic acid (Glu), cysteine (Cys), asparagine (Asn), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys)) (Sigma-Aldrich, Inc., St. Louis, USA) from 1 to 30 mg/L, using norvaline (Sigma-Aldrich, Inc., St. Louis, USA) as internal standard.

## Lipids

The total lipids were determined by the method of Bligh and Dyer [23] adapted as described by Breil et al. [24] with slight modifications. In brief, to 0.5 g of lyophilized and ground SY sample, 3 mL of ethyl acetate: ethanol (2:1) and 100 µL of distilled water were added. The mixture was thoroughly mixed for 1 min in the vortex and then 2.25 mL of ethyl acetate, 500 µL of ethanol, and 4.15 mL of distilled water were added. The mixture was homogenized in the vortex for 1 min and centrifuged at  $1046\times g$  for 1 min for phase separation. After complete separation and clarification, the volume of the organic phase was registered, and a 3 mL aliquot was placed in a pre-tared tube. For the determination of total lipids, the aliquot was evaporated in a rotational vacuum concentrator Christ RVC 2–18 CDplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) at 50 °C for 30 min, plus 1 h at 100 °C in an oven or until constant weight.

## Neutral Sugars

The neutral sugar content and sugar profile were determined by gas chromatography-flame ionization detection (GC-FID) as alditol acetates [25, 26], modified as described by Bastos et al. [27] with slight adaptations. Two mg of freeze-dried SY were homogenized with 200 µL 58% v/v H<sub>2</sub>SO<sub>4</sub> at room temperature for 3 h (stirring 2–3 times). After the hydrolysis with 1 M H<sub>2</sub>SO<sub>4</sub> at 100 °C for 2.5 h, the monosaccharides were reduced with 15% (w/v) NaBH<sub>4</sub> in 3 M NH<sub>3</sub> and then acetylated acetic anhydride in the presence of 1-methylimidazole. After some stirring, the tubes were kept at 30 °C in a heating block for 30 min. The organic phase was washed twice with 3.0 mL of distilled water and 2.5 mL of dichloromethane and then again twice with 3 mL of distilled water. The organic phase was evaporated in the rotational vacuum concentrator (Christ RVC 2–18 CDplus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and 1 mL of anhydrous acetone was added to the dried sugars and then evaporated. This procedure was repeated, and the tubes were stored in a desiccator until analysis. For the analysis, the dried sugars were suspended in 50–100 µL of anhydrous acetone and the analytes were separated and detected by GC-FID (Agilent Technologies, 7890B model, DE, USA) using a DB-225 (J&W Scientific, Folsom, CA) capillary column (30 m length, 0.25 mm diameter, 0.15 µm thickness) in the following conditions: 2 µL injection volume, split 1/60; 2 min at 200 °C, 7 min at 220 °C and 5 min at 230 °C. For quantification of neutral sugars, 200 µL of 2-deoxyglucose at 2 mg/mL was used as internal standard.



## Statistical Analysis

The analysis of variance was conducted using GraphPad Prism version 8.0 (San Diego, California, USA). Significant differences among the SYs regarding each compositional parameter were determined by one-way ANOVA followed by multiple comparisons using Tukey's test at  $p < 0.5$ . Principal Component Analysis (PCA) was conducted using all the parameters evaluated (59 variables, 7 groups of SYs). Before the analysis, data was auto scaled and subjected to square root transformation. Hierarchical clustering analysis (HCA) was conducted using Euclidean similarity measure and the average algorithm. The PCA and HCA were conducted using the MetaboAnalyst version 5.0.

## Results and Discussion

### Initial Quality: Cell Viability, pH, Dry Weight, and Ash Content

The cell viability of all modified SYs was higher than the detected in SBY, which showed a cell viability of 8.5%. The highest viability was found in the SDW ( $78 \pm 2.3\%$ ), followed by SCL ( $70 \pm 7.0\%$ ) (Table 1). The cell viability of Fene and Reb M was between 16 and 36%. The differences found may be related to the specificities of the fermentation processes. While the yeast used in beer production is re-utilized in consecutive production cycles, the yeast used in the fermentation processes aimed to produce the specific biomolecules herein referred to, are not reused, being inactivated after their first use. In addition, the method used to obtain the molecule may also impact cell viability. While in some fermentative processes, the yeast excretes the molecule of interest to the fermentation medium, in others, the yeast accumulates the molecule of interest inside its cells, implying a subsequent extractive

process with a consequent impact on yeast cell viability. Considering that the spent yeasts originate from genetic modification, yeast inactivation becomes crucial for their subsequent use and valorization.

The pH of most SYs was between 5.0 and 5.8. The exception was the SCL, which had the lowest pH, ranging from 2.3 to 2.6 (Table 1). Beer fermentation usually starts at pH 5.0–5.2 and falls to a final pH of 3.8–4.0 [28]. The lower pH of SCL suggests that such yeasts may have been exposed to higher stress during fermentation, but its high cell viability suggests that the genetic modification may have made them more resilient and able to multiply under such acidic conditions. Low pH has been previously shown not to significantly affect *Saccharomyces* growth and most yeasts can grow at pH as low as 3.0 [29]. The low pH may have implications when considering the extraction of value-added compounds from such streams. To ensure an efficient extraction of such components through methods such as autolysis, raising the pH of these streams might be necessary to ensure optimal efficiency [30].

The dry weight of all modified yeast strains was significantly higher when compared with the dry weight of SBY ( $4.8 \pm 0.1\%$ ) (Table 1). The highest difference was about Reb M, which presented a five-fold higher dry weight compared to SBY, followed by Fene (3.2-fold), MN and SCL (3.0-fold), SDW (2.4-fold), and PAT (2.0-fold) (Table 1). The differences found in the dry weight of these samples may be related to differences in fermentation. For instance, for Reb M production, the yeast accumulates the molecule of interest inside the cell instead of excreting it to the medium, which may imply a higher yeast biomass in the final residue. The moisture content of SBY has been shown to correspond to 86% [31]. In our study, considering the dry weight, the moisture content was 95.2%, indicating that, in our SY, the fraction of medium collected from the fermentation tank was higher.

Variations in the dry weight of these streams could impact their valorization, especially when used on a dry mass basis. Higher moisture content increases the expenses related to drying methods like spray drying.

Regarding the ash content, SCL showed the highest level, more than 21.4-fold higher levels compared to SBY. In contrast, Reb M had the lowest concentration ( $1.2 \pm 0.8\%$  dw) with 2-fold lower levels than those found in SBY and about 5-fold lower than the levels found in Fene, MN, PAT, and SDW (Table 1). Ash content of SBY extracts has been previously reported to be between 7 and 14% [6, 15, 32, 33], whereas in the whole SY it has been reported to be between 1.8 and 5.9% [31, 34]. In our study, considering the SBY, ash content was estimated at  $2.1 \pm 0.2\%$ , falling within the reported range. The most discrepant result for ash content was found in SCL, whereas Reb M showed an ash content proximate from that of SBY.

**Table 1** Cell viability, pH, dry weight, and ash content of different spent yeast biomass

	Cell viability (%)	pH	Dry weight (%)	Ash (% dw)
Fene	$33.7 \pm 3.3^a$	$5.3 \pm 0.1^{ad}$	$15.6 \pm 2.0^{ac}$	$9.6 \pm 1.9^a$
Reb M	$24.2 \pm 11.5^a$	$5.7 \pm 0.1^b$	$25.0 \pm 3.7^b$	$1.2 \pm 0.8^b$
Manool	$60.3 \pm 37.9^{ab}$	$5.2 \pm 0.2^{ad}$	$14.7 \pm 1.8^a$	$10.2 \pm 1.4^a$
Sclareol	$70.5 \pm 7.0^b$	$2.5 \pm 0.1^c$	$15.5 \pm 1.8^a$	$45.0 \pm 1.9^c$
Patchouli	$36.1 \pm 2.1^a$	$5.4 \pm 0.1^{ab}$	$9.7 \pm 0.1^{cd}$	$10.3 \pm 1.0^a$
Sandalwood	$78.8 \pm 2.3^b$	$5.2 \pm 0.0^{ad}$	$11.5 \pm 0.1^{ac}$	$11.3 \pm 1.7^a$
SBY	$8.5 \pm 1.1^c$	$5.0 \pm 0.0^d$	$4.8 \pm 0.1^d$	$2.1 \pm 0.2^b$

The results represent the mean of three biological replicates  $\pm$  SD. Means followed by the same letter in the columns are not significantly different by the Tukey test ( $p < 0.05$ )

## Mineral Profile

In general, mineral concentrations influence yeast growth and have an impact on yeast metabolic processes during fermentation since they are involved in fundamental physiological and biochemical processes responsible for cell viability and stress tolerance [35]. Therefore, the levels of yeast minerals change during the fermentation process.

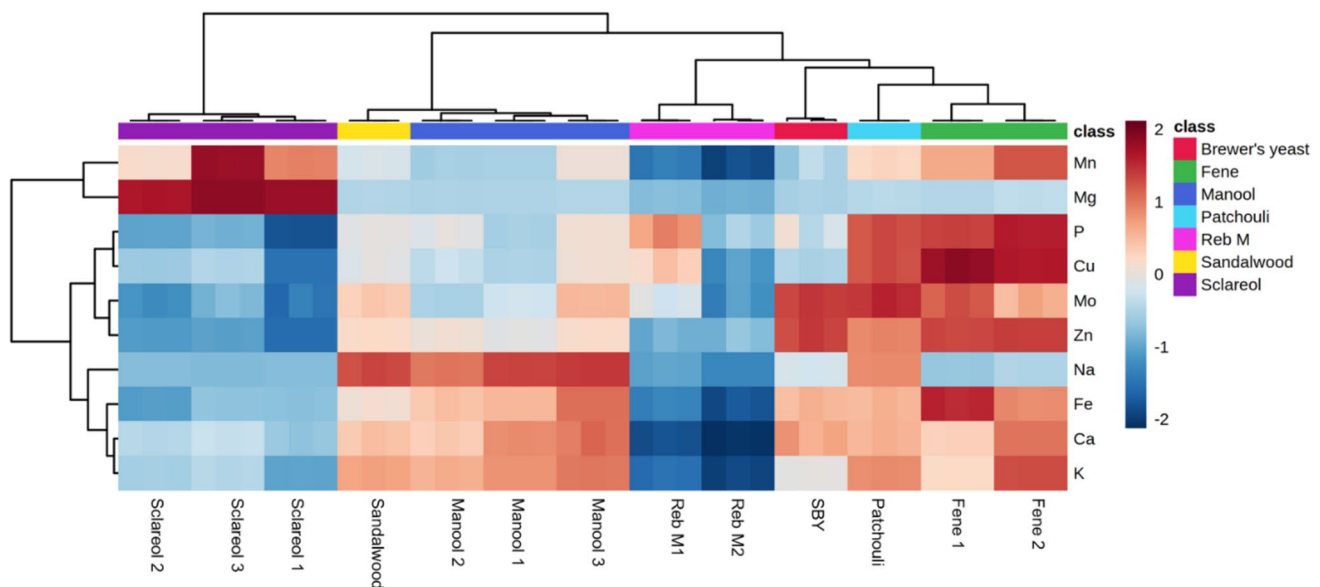
The mineral profiles of the SYs analyzed were significantly different.

The results of the HCA presented in Fig. 1 showed that Fene and PAT had similar mineral profiles with higher levels of P (Fene,  $2600 \pm 139$  mg/L; PAT,  $2379 \pm 56$  mg/L) and Cu (Fene,  $2.16 \pm 0.08$  mg/L; PAT,  $1.81 \pm 0.04$  mg/L) than the levels found in all the other SYs (Fig. 1, Table S1). Cu is involved in yeast respiration, and P is involved in many metabolic processes, being structurally important, and may accumulate in yeast in the form of polyphosphates working as an energy reserve [35, 36]. Therefore, higher P levels may indicate a better optimization of feedstock availability or that yeasts were younger at harvesting. Despite the similarities, Fene and PAT significantly differed in the Na levels, with PAT showing 6.5-fold higher levels than Fene ( $51.3 \pm 10.9$  mg/L). Although Na is usually present at high concentrations in yeast cells and fermentation medium, it does not seem to play an essential role in yeast growth and maintenance. For instance, it may become toxic to yeast antagonizing K-dependent functions [37]. Fene and PAT, together with SBY also showed higher levels of Mo (Fene,  $1.8 \pm 0.3$  mg/L; PAT,  $2.3 \pm 0.1$  mg/L. SBY,  $2.22 \pm 0.06$  mg/L) and Zn (Fene,  $19.03 \pm 0.33$  mg/L; PAT,  $15.09 \pm 0.17$  mg/L, SBY,

$19.00 \pm 0.71$  mg/L). Zn is essential for yeast growth, acting as a cofactor in several important enzymes, particularly aldehyde dehydrogenase, a crucial enzyme in alcoholic fermentation. In addition, Zn contributes to protein accumulation in yeast cells [36, 38].

It was also observed that Fene, MN, PAT, and SDW had similar levels of K and Ca, with these samples showing significantly higher levels of these minerals when compared to Reb M and SCL (Fig. 1). The SBY also had levels of Ca similar to those found in Fene, MN, PAT, and SDW but not K levels, which were significantly lower than those in the MN (Fig. 1, Table S1). Despite its crucial role in cell division and growth, as well as being a secondary messenger, the Ca requirement for fermentation is very low. In contrast, K is the most abundant cation in yeast cytoplasm and plays a key role in osmoregulation, cation uptake, and charge balancing, also acting as a cofactor for enzymes involved in protein biosynthesis, oxidative phosphorylation, and carbohydrate catabolism [36, 37, 39]. Therefore, higher levels of K may benefit yeast performance.

The highest difference in the mineral profile of the different SYs was regarding the levels of Mg. SCL showed the highest Mg concentration ( $16.09 \pm 0.86$  g/L), whereas Reb M showed the lowest ( $0.15 \pm 0.08$  g/L). The remaining samples showed levels between 0.65 and 0.95 g/L (Table S1). Mg is essential for yeast growth and an unreplaceable asset in yeast cell division and DNA and ATP synthesis [36]. It also acts as a cofactor of more than 300 enzymes and alleviates the toxic effects of heavy metals such as aluminum [40] and cobalt [41]. Indeed, the Mg/Ca ratio was approximately one for most samples, except Reb M, SCL, and SBY. In



**Fig. 1** Heatmap resulting from hierarchical clustering analysis of minerals composition of the different spent yeasts derived from the production of  $\beta$ -farnesene (Fene), Reb M, manool, sclareol, patchouli, sandalwood, and beer (SBY)

Reb M and SCL, the ratio favored Mg, whereas in SBY it favored Ca. It is known that Ca can antagonize Mg uptake, blocking crucial Mg-dependent metabolic processes [42–44] and that a higher Mg: Ca ratio benefits yeast physiology and fermentation processes [45, 46]. Therefore, the difference in this ratio may partially explain the highest biomass of Reb M and the lowest biomass of SBY. In the case of SCL, the higher Mg: Ca ratio did not highly correlate with increased SY biomass, possibly due to exposure to low pH (Table 1, S1).

Considering the total mineral content, Reb M showed the lowest content, and SCL showed the highest. Despite the difference in absolute mineral content, these two SYs showed approximated mineral profiles (Fig. 1). The relatively high mineral content in SCL was mainly due to the very high Mg concentration of this SY (Table S1). Concerning all the other analyzed minerals, SCL showed similar or lower concentrations than those found in Reb M (Fig. 1, Table S1 and S2). These results support those of ash content (Table 1) and show that the SY derived from SCL production may have potential as a source of Mg, considering the consistently high Mg amounts.

### Protein Content

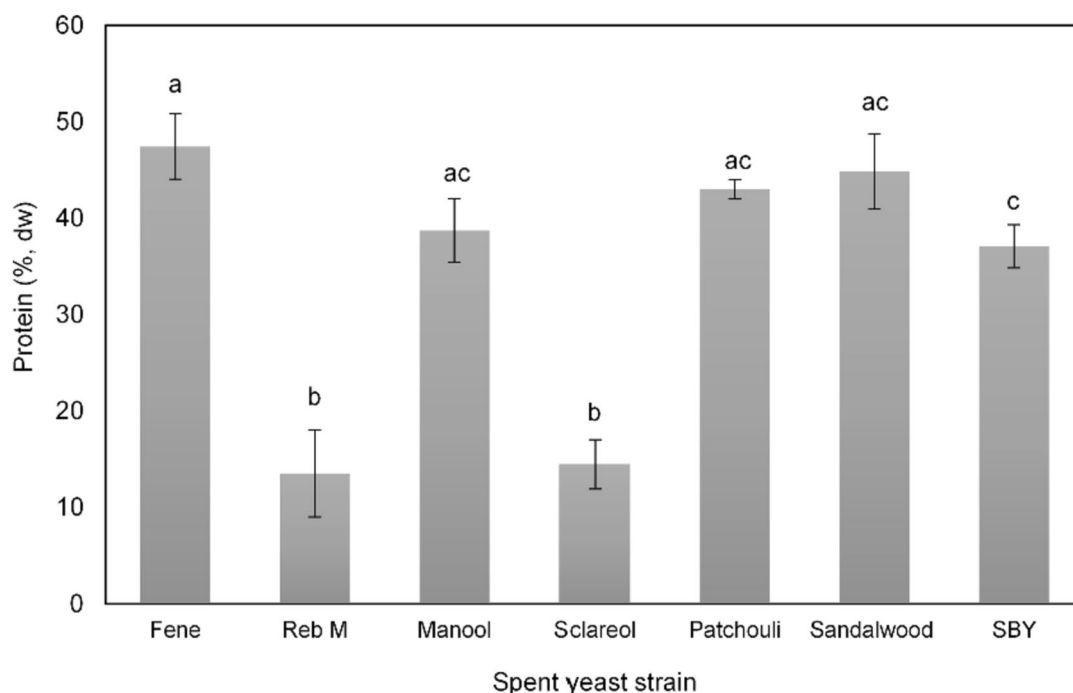
Different SYs showed significantly different protein contents. Fene, PAT, and SDW showed the highest protein content, with percentages ranging from  $39.9 \pm 0.9\%$  in PAT and up to  $44.0 \pm 3.2\%$  in Fene. The protein content of

SBY ( $34.4 \pm 2.1\%$ ) was almost similar to the levels found in these samples, except for Fene, which showed significantly higher levels ( $p < 0.05$ ). Reb M showed the lowest levels ( $12.5 \pm 4.2\%$ ), followed by SCL ( $13.4 \pm 2.4\%$ ), and these levels were significantly lower than those found in all the other samples ( $p < 0.05$ ) (Fig. 2). The protein content of extracts from SBY has shown to be between 39 and 74% [32, 33, 47], whereas that reported for whole SBY was between 34.2 and 47.2% [31, 34, 48]. Yamada and Sgarbieri (2005) [49] also reported showed a value of 39.6% protein in whole SY cells. The protein content of the SBY found in our study was following those.

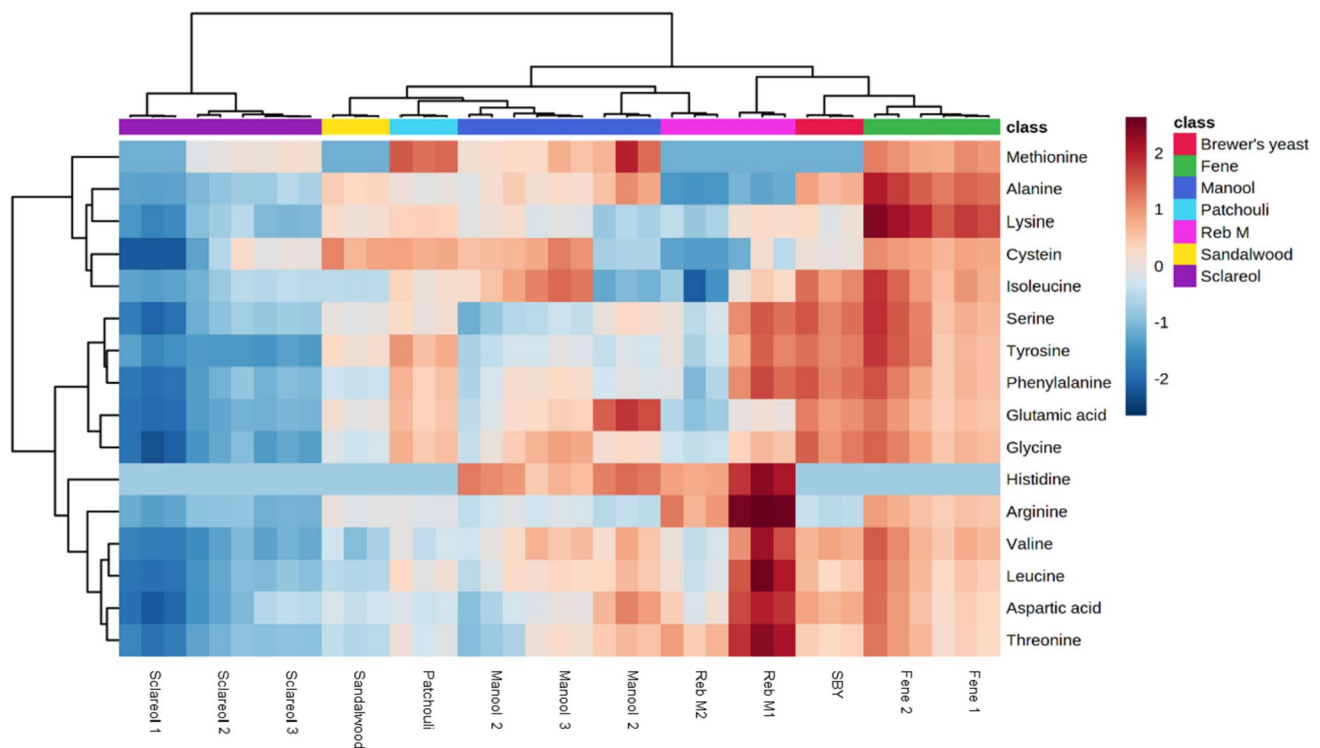
From a valorization perspective, SCL and Reb M may not be of interest as protein sources, considering the significantly higher levels of protein found in all the other SYs. Thus, combining these SYs with those derived from the other fermentation processes may contribute to decreased bulk protein content.

### Total and Free Amino Acids Profile

The total amino acid profiles, including the soluble and insoluble fractions, differed among the SYs analyzed. SCL and SDW showed almost similar profiles, whereas Fene, MN, Reb M, and SBY profiles were more closely related. PAT showed an intermediate profile with averaging levels of amino acids when considering all samples analyzed (Fig. 3). In general, Fene, SBY, Reb M, and MN showed higher concentrations of amino acids than the SCL and SDW. SBY and



**Fig. 2** Protein content of different spent yeasts. The same letters above bars indicate no significant differences at  $p < 0.05$



**Fig. 3** Heatmap resulting from the hierarchical clustering analysis of total amino acids in the different SYs including the data from each analyzed batch and the three analytical replicates conducted for each batch

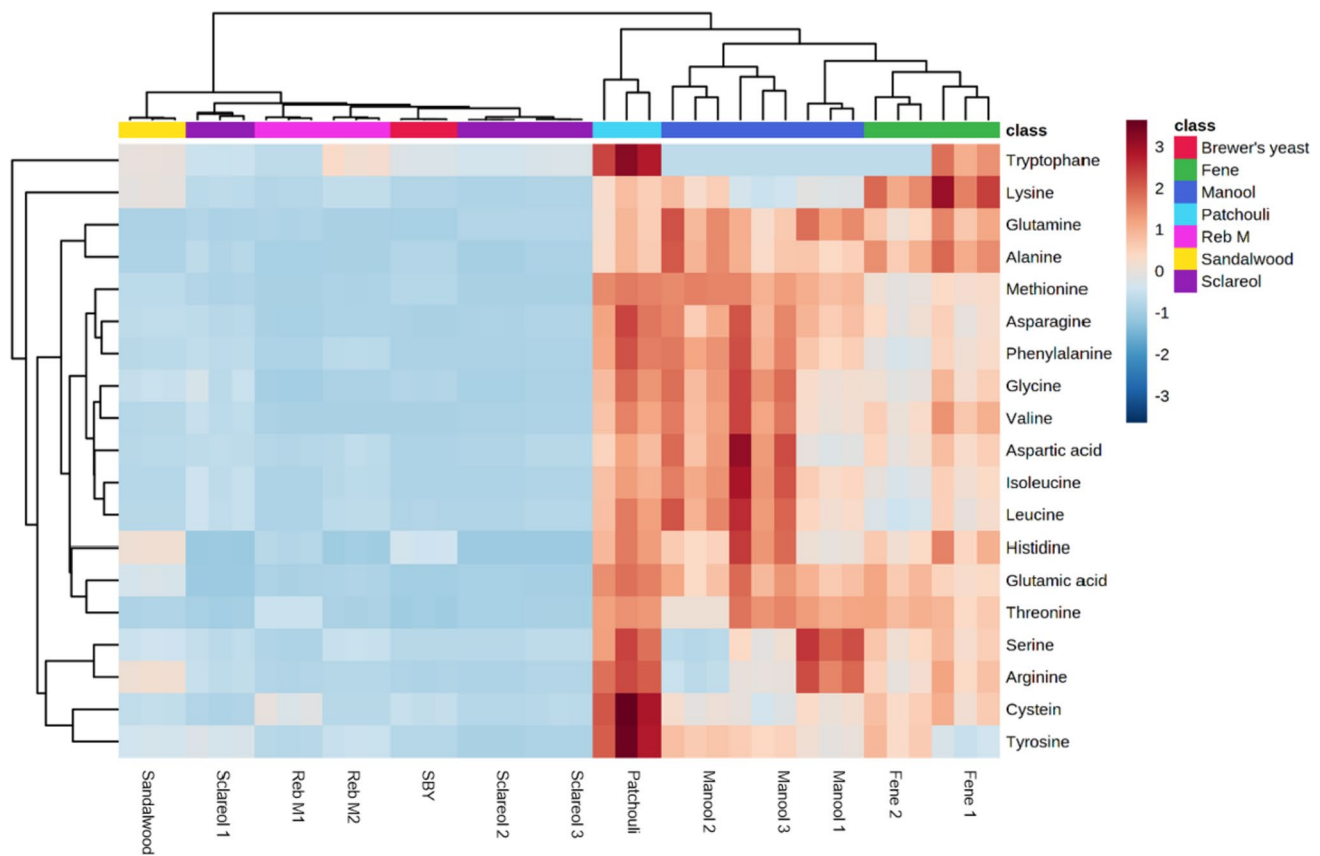
Fene showed the highest amino acid concentrations with Reb M, MN, and PAT showing only slightly lower levels. SDW and SCL showed the lowest levels (Table S3). The elevated levels found in Reb M and the lower levels of SDW are in contrast with the lower protein content of Reb M and higher protein content of SDW, respectively (Fig. 2). The method used to hydrolyze the protein involved acidic hydrolysis, during which some amino acids suffer transformations; tryptophan degrades, and asparagine and glutamine suffer deamination reactions converting into aspartic and glutamic acid. Serine and threonine are also partially damaged, and cysteine can deteriorate, while methionine can oxidize with reported losses of 5–15% [50]. Perhaps the levels of less stable amino acids were lower in Reb M, compared with those in the other SYs, and vice versa for SDW. In addition, differences related to protein structure resulting from yeast metabolic and genetic engineering may have determined a different sensitivity of Reb M and SDW SY to acidic hydrolysis. In the case of SCL, the lower levels of total amino acids correlated with its low protein level (Fig. 2, Table S3).

The most abundant amino acid in all samples was glutamic acid, with levels ranging from 15 mg/g in SCL and up to 51.3 mg/g in SBY (Fig. 3, Table S3). The elevated levels of glutamate in SY extracts have been reported in some studies. According to Jaeger et al. [15], glutamic acid is the most abundant amino acid in SY (~15%), as evidenced

in some studies [33, 47, 51]. Besides glutamate, in the SYs herein analyzed, other amino acids found in significant quantities included aspartate, threonine, arginine, alanine, leucine, and lysine. The elevated levels of glutamate, alanine, and aspartate confer a pleasant ‘umami’ taste to SYs, which is an advantage of these waste streams when applied to animal feeding since they tend to be more accepted by animals [52]. Methionine was undetected in any sample and cysteine was present at very low levels. Sulfur amino acids such as methionine and cysteine were reported to be present in lower concentrations in SY [47–49], and our results were in accordance with these findings.

Considering the free amino acid profile, there were two very distinctive groups. The profiles of free amino acids in the SDW, SCL, Reb M, and SBY were similar, whereas the profile of Fene was highly proximate from that of MN and PAT. Fene, together with MN and PAT showed, in general, higher levels of free amino acids. The most consistent results were for glutamine, alanine, and glutamic acid, which were significantly higher in Fene, MN, and PAT than in the others (Fig. 4, Table S4). The levels of methionine and cysteine found in SBY were very low, but SCL and Reb M showed even lower methionine levels. In previous works, it has been shown that the most prevalent free amino acids in SY were glutamic acid, lysine, leucine, valine, threonine, and phenylalanine, whereas the sulfur amino acids such as





**Fig. 4** Heatmap resulting from the hierarchical clustering analysis of the free amino acids in the different SYs including the data from each analyzed batch and the three analytical replicates conducted for each batch

methionine and cysteine were the less abundant [6, 33, 48, 49, 53]. Cysteine is involved in cell resistance to oxidative stress [54], and its lower levels in the modified strains, when compared with SBY, may indicate further cell exposure to oxidative stress.

## Lipids

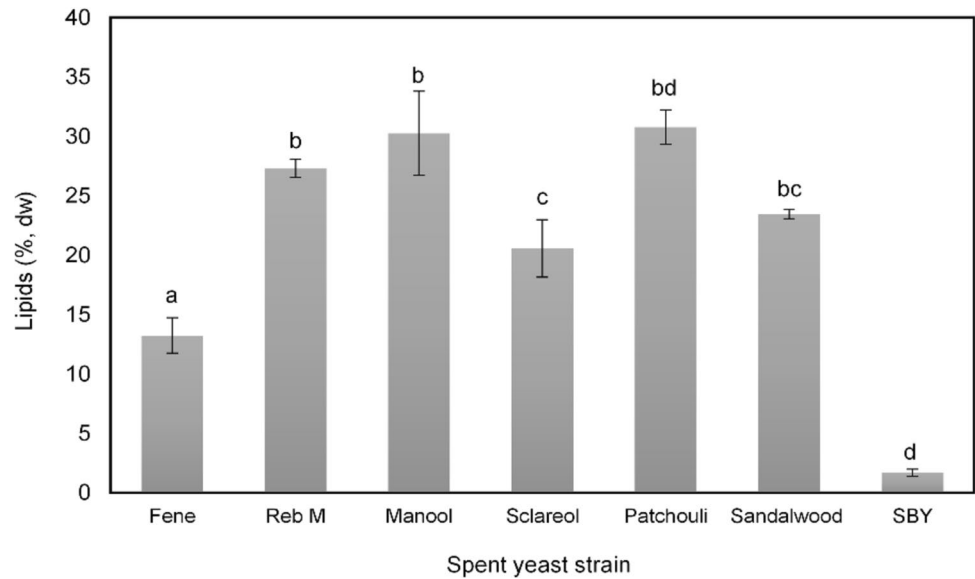
The lipid content varied among SYs. Reb M showed the highest lipid content ( $21.0 \pm 2.6\%$ ), and it was significantly higher than that found in all the other SYs ( $p < 0.05$ ). Reb M lipid content was about two-fold higher than the lipid content of MN, SCL, and PAT, six-fold higher than Fene and SDW, and 12-fold higher than SBY. SBY showed the lowest lipid content ( $1.7 \pm 0.2\%$ ), and it was significantly lower than the lipid content of MN. SCL PAT and Reb M but not in comparison to SDW and Fene (Fig. 5). The lipid content of extracts derived from SBY is reported to be between 0.67 and 1.32 [33, 51]. Pinto et al. [34] also reported a content of 2.3–3.2% dw in the whole SY, whereas Caballero-Córdoba et al. [48] reported a value of 3.53% dw, and Thammakiti et al. [55], a value of 1.2%. The results found in our study for SBY are in accordance with those reported results. From a

valorization perspective, considering the low protein levels of Reb M and SCL and the superior levels of lipids, it may be interesting to combine SCL and Reb M to get a bulk of SYs rich in lipids, being valorized through the extraction of alternative oils. Combining Reb M and SCL with MN and PAT would also be feasible but considering the high protein content of MN and PAT, those SYs have higher potential as nitrogen sources rather than lipid sources.

## Sugar Content and Profile

The polysaccharide content of SBY consists mainly of  $\beta$ -glucans, glycogen ( $\alpha$ -glucans), and mannoproteins [26]. In SY most of the carbohydrates come from yeast cell wall and glycogen ( $\alpha$ -glucan) and trehalose stored in the yeast cell. The yeast cell wall represents 15–30% of the yeast cell dry weight [56] and more than 90% of its composition corresponds to polysaccharides divided into three main groups: (i) polymers of mannose (mannans and mannoproteins), representing ca. 40% of the cell wall dry mass, (ii) polymers of glucose, namely  $\beta$ -glucans which consist of ca. 60% of the cell wall dry mass and (iii) polymers of N-acetylglucosamine (chitin), corresponding to ca. 2% cell wall dry mass [57, 58].

**Fig. 5** Lipid content of different spent yeasts. The same letters above bars indicate no significant differences at  $p < 0.05$



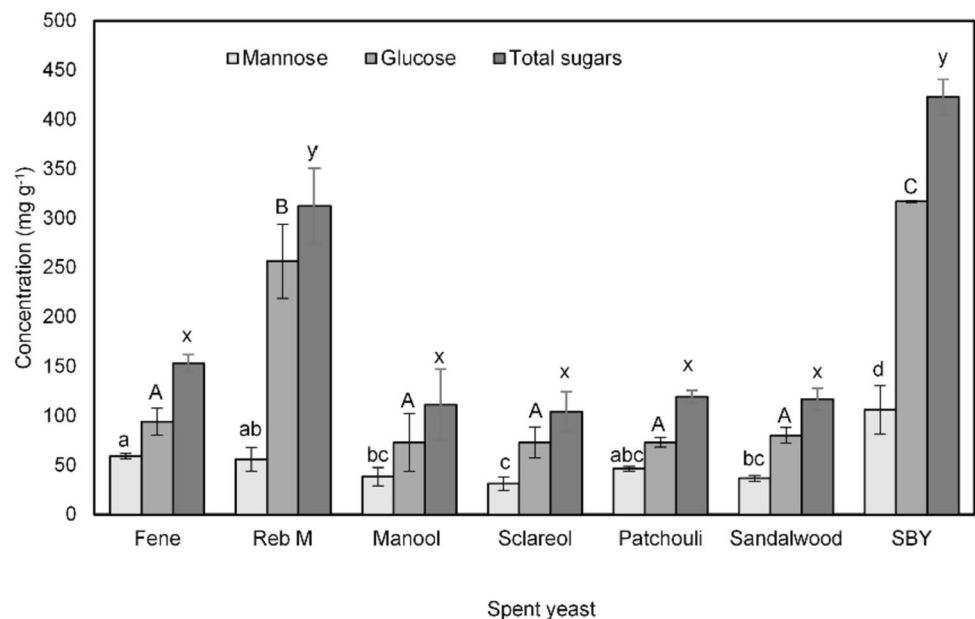
In the SY, mannose mainly derives from yeast cell walls, whereas glucose derives from yeast cell walls, fermentation medium, and yeast glycogen and trehalose reserves representing 1–23% [59].

In the analyzed SYs, the total sugar content of most of them was not significantly different. Only Reb M and SBY showed significantly higher levels when compared with all the others and did not show significant differences between them. SBY showed the highest sugar content ( $39.8 \pm 1.8\%$ ) followed by Reb M ( $30.8 \pm 3.8\%$ ) (Fig. 6). In MN, SCL, PAT, and SDW, the sugar content was almost similar, with values ranging from  $10.4 \pm 2.0\%$  (SCL) to  $11.9 \pm 0.6\%$  (PAT). The levels in Fene were slightly higher ( $15.3 \pm 0.9$ ) but not significantly different from those of MN, SCL, PAT, and

SDW (Fig. 6). In previous works, the carbohydrate content of SY extracts has been reported to range from 12.9% [33] up to 50.7% [6]. In whole SY cells, sugar content is between 21.52% [48] and 59.6% [55]. The levels found in SBY are in accordance with the results reported in the literature, and among the SYs derived from precision fermentations, Reb M showed a sugar profile almost similar to the observed in SBY. This result indicates that Reb M is a potential source of glucans and mannans, which are known to possess bioactivities including antioxidant, anti-tumoral, or prebiotic [26].

All the analyzed SYs had higher levels of glucose than mannose. SBY showed the highest levels of glucose ( $317.0 \pm 1.0$  mg/g) and mannose ( $105.9 \pm 24.7$  mg/g) compared with all the other SYs. Reb M showed levels of glucose

**Fig. 6** Concentration of mannose, glucose and total sugars of SYs derived from the production of beer (SBY) and six biomolecules ( $\beta$ -farnesene, Fene; Reb M; Manool, Sclareol, Patchouli, and Sandalwood). The results represent the mean of the samples from the same group of strains measured in triplicate  $\pm$  SD. The same letters above the bars indicate no significant differences. Lowercase letters (a, b, c, d) compare mannose and cap letters (A, B, C), glucose levels and “x” and “y” compare total sugars at  $p < 0.05$



close to the measured in SBY, being significantly higher than the observed in the other SYs ( $256.0 \pm 37.6$  mg/g) (Fig. 6). The higher levels of glucose found in these, particularly the higher levels of both sugars in SBY indicates that during the fermentation process, these yeasts were not exposed to significant growth limitations. In the other strains, the values for glucose ranged from 73 to 94 mg/g and mannose from 31 to 59 mg/g. SCL, PAT, and SDW showed the lowest levels of both sugars, suggesting that these strains may have been exposed to stress conditions during the fermentation process which may have limited their growth.

The elevated glucose levels of SBY and Reb M suggest that they may be potential sources of  $\beta$ -glucans, which have antibacterial effects besides benefiting the intestinal environment, stimulating immunity, preventing cancer, and reducing blood sugar and lipids in animals [8, 60]. SBY, having higher mannose concentrations, may also be used as a source of mannans and mannoproteins. Mannans are known to possess prebiotic activity in animals, and some studies showed that they may also have immunomodulatory, antitumor, antimetastatic, and antioxidant effects [8, 61].

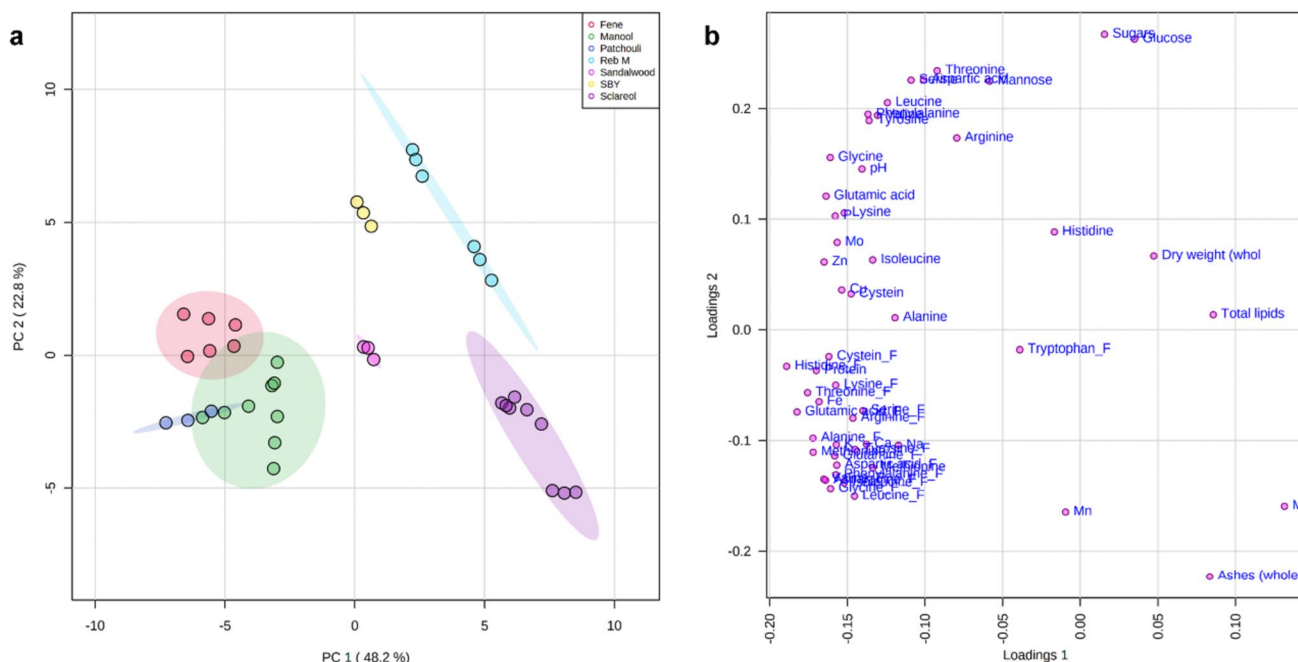
## Multivariate Data Analysis and Integrated Data Overview

The result of the PCA shows that, based on the analyzed parameters, PAT, Fene, and MN have a similar compositional pattern, and therefore, may be bulked to be used as a source of protein or specific amino acids to produce

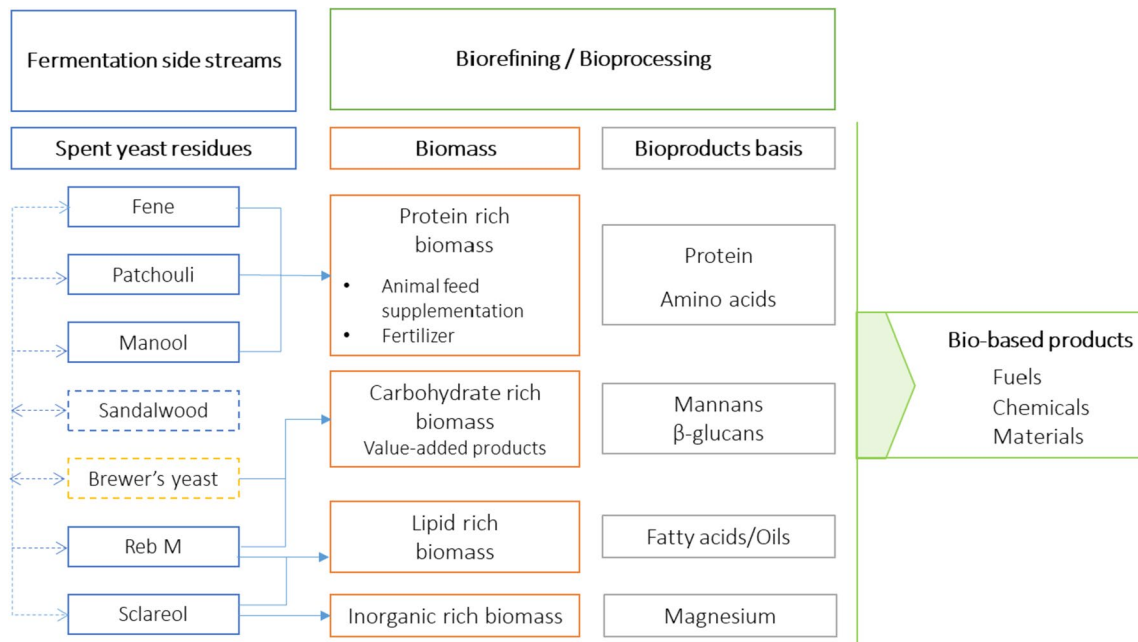
value-added products. SCL, in contrast, is significantly different from all the other SYs mainly due to its high Mg and ash content (Fig. 7). From a valorization standpoint, the fact that 45% of SCL biomass comprises ash, mainly composed of minerals, significantly influences its potential use. For instance, it could be well-suited for developing fertilizers or Mg-zeolites but may not be suitable for creating animal feed supplements due to its low protein content.

Reb M and SBY showed a distinctive higher sugar content. In contrast, SBY and SDW were not significantly different from each other, presenting an intermediate composition. This intermediate compositional pattern allows the combination of these SYs with all the others to form a single biomass stream for subsequent processing in biorefineries directed to biowastes valorization. Reb M also showed elevated levels of lipids and combined with SCL, which also showed reasonable amounts of lipids, may constitute a lipids source.

From the perspective of biorefining, considering the results of this study herein presented, it would be feasible to bring together PAT, Fene, MN, SBY, and SDW to create a stream of SY biomass rich in nitrogen for subsequent processing. It would also be of interest to combine Reb M with SBY to generate a carbohydrate-rich stream for subsequent valorization. Less feasible but still reasonable would be, joining Reb M, SCL, SBY, and SDW to be used as a lipid-rich biomass. However, combining PAT, Fene, and MN with Reb M and SCL to serve as a source of value-added products would not be beneficial since this combination would lead to



**Fig. 7** Results of principal component analysis (PCA) including the **a** scores plot of selected PCs and **b** the loadings plot. The name followed by “\_F” refers to free amino acids. For the analysis, all the parameters were used, including quality and compositional data)



**Fig. 8** Schematic representation of how to manage different spent yeast for biorefining and valorization

a decreased concentration of protein and sugar in the bulked stream (Fig. 8).

Although the compositional traits of some of these streams seem compatible, one must consider the sensorial and toxicological characteristics before proceeding to the bulking of different SY side streams. In the case of the streams herein presented, the ones derived from fragrance production such as PAT, MN, and SDW, have a very pronounced aroma, and this factor may limit the possibility of bulking such streams or can imply additional processing steps to reduce their scents. Moreover, analyzing the soluble and insoluble fractions separately would provide valuable insights into the potential valorization of each fraction. Nevertheless, additional efforts in characterization are essential to delve deeper into these streams, including exploring their biological activities and assessing cytotoxicity. Such data would significantly augment the potential for valorization of these streams.

## Conclusion

In a modern biobased economy, it is essential to develop alternative business models, combining the production of sustainable products with sustainable post-production strategies, including waste and side streams valorization, minimization of energy consumption, and improvement of environmental protection. SY, one of the main by-products derived from fermentation possesses valuable features with enormous potential for valorization. From the different

biotechnologically derived SYs evaluated in this study, three showed a similar compositional pattern, whereas the other three showed significantly different patterns. This result indicates that the knowledge gathered through characterization efforts may allow improving their potential for valorization. On one hand, the combination of SYs may contribute to ensuring a sustained supply of residue with uniform composition for subsequent processing. On the other hand, SYs with different beneficial features can be combined to obtain value-added products. In addition, the characterization of the SYs generated in the different fermentation processes may also provide clues to improve fermentation performance, considering the historical data of these streams and associating information on performance. The composition of the SY may be a valuable source of information regarding the physiological status of the yeast cell during the fermentation process and can bring additional information for the adjustment of fermentation processes. As observed in the results of this study, slight differences in the fermentation settings may lead to significant differences in yeast composition by the end of the fermentation process, as evidenced in the case of free amino acids of SCL obtained in different batches (Fig. 4).

Although further characterization efforts are required to improve the perspective of valorization, particularly regarding the potential bioactivities of these streams, this study showed that SYs derived from different fermentation processes may be significantly different but also that bulking some of these side streams may potentiate further processing and valorization, possibly in integrated biorefineries. The

characterization of these side streams will ultimately support decision-making toward the development of the best valorization strategies.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s12649-023-02394-z>.

**Author Contributions** All authors contributed to the study's conception and design. Material preparation, data collection, and analysis were performed by TD, JF and DR. The first draft of the manuscript was written by TD and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data Availability** All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Declarations

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

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