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Structural differences on cell wall polysaccharides of brewer's spent *Saccharomyces* and microarray binding profiles with immune receptors

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ABSTRACT

Brewing practice uses the same yeast to inoculate the following fermentation (repitching). *Saccharomyces pastorianus*, used to produce *Lager* beer, is widely reused, not changing its fermentation performance. However, *S. cerevisiae*, used to produce *Ale* beer, is partial or not even reused, due to its poor performance. It is hypothesized that cells modulate their wall polysaccharides to increase the cell-wall strength. In this work industrial *S. cerevisiae* and *S. pastorianus* brewer's spent yeasts with different repitching numbers were studied. Glucans were the main component of *S. cerevisiae* whereas mannoproteins were abundant in *S. pastorianus*. The major changes were noticed on glucans of both species, β1,3-glucans decrease more pronounced in *S. cerevisiae*. The increase of α1,4-Glc, related with osmotolerance, was higher in *S. cerevisiae* while β1,4-Glc, related with cell-wall strength, had a small increase. In addition, these structural details showed different binding profiles to immune receptors, important to develop tailored bioactive applications.

1. Introduction

Brewer's spent yeast (BSY) is the second major by-product from brewing industry (Marson et al., 2020) that has been receiving increasing attention due to its value-added constituents, namely the cell-wall polysaccharides, which are used in food and biomedical applications (Bastos et al., 2022; Ferreira et al., 2010; Yana Liu et al., 2021; Puligundla et al., 2020). The yeasts often used by brewing industry are from the genus *Saccharomyces*, mainly due to their fast growth, good capacity to produce ethanol and a great tolerance against environmental stress, including high ethanol concentration and low oxygen levels (Ferreira et al., 2010). The most widely used species are *S. cerevisiae*, *S. bayanus*, and *S. pastorianus*, being the latter a natural allotetraploid hybrid from the first ones (Dequin & Casaregola, 2011; Rainieri et al., 2006; Tamai et al., 1998). Brewing industry classifies yeasts in two types: top-fermenting and bottom-fermenting, and according to the yeast used, two broad categories of beer are classified, Ale and Lager, respectively (Ferreira et al., 2010). *S. cerevisiae* is used to produce Ale beers, where fermentation occurs typically at 16–25 °C resulting on the flocculation of yeast cells at the top. *S. pastorianus* is often used to produce Lager beers, with fermentation running at cool temperatures (8–15 °C) and flocculation of yeast cells occurring at the bottom. The different mechanisms of flocculation are not well understood, but it is established that the yeasts have a different surface chemical composition, hydrophobicity and electrical properties. The top-fermenting yeasts are less negatively charged at pH 4, with higher concentration of protein and hydrocarbon moieties and more hydrophobic at the surface than bottom-fermenting yeasts. These properties explain the attachment of these cells to CO_2 bubbles and the rise to the top of the fermented suspensions (Dengis et al., 1995). Structural analysis showed that both species have smooth and homogeneous cell surfaces, with circular bud scars. The cell-wall elasticity is lower in *S. cerevisiae* due to the stiffness of its bud scars, which is related to the accumulation of chitin (Alsteens et al., 2008). The polysaccharides are homogeneously distributed at the surface in both species. In *S. cerevisiae* mannoproteins seem to be more exposed and/or available for interaction (Alsteens

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et al., 2008).

The yeasts are used several times, taken from one fermentation to start the next, named as repitching process. They are used 4–6 times until degeneration or contamination of the yeast occurs. Due to brewing economical and resource saving reasons, reuses of up to 20 times of *S. pastorianus* are being studied, since Lager beers are responsible for 90 % of beer market (Bühligen et al., 2013, 2014). In addition, *S. pastorianus* is known for its high fermentative reproducibility upon repitching, not changing its fermentation performance. On the contrary, *S. cerevisiae* is usually considered in less extent for repitching due to its poor fermentative performance upon repitching or due to alterations of beer volatile profile (Martins et al., 2016).

Saccharomyces cell-wall is composed of up to 90 % polysaccharides, representing 15–25 % of the yeast cell dry weight (Bastos et al., 2022; Fu et al., 2022). The polysaccharides present are mainly β-glucans linked through (β1 → 3)- and (β1 → 6)-D-Glc linkages, representing 55–65 % of yeast cell-wall polysaccharides, followed by mannoproteins (35–40 %), glycogen (1–29 %), and chitin (1–2 %). Mannoproteins can be *N*- or *O*-glycosylated (Bastos et al., 2022). The *N*-glycosylated mannoproteins are composed of 90 % carbohydrates and 10 % protein, 50–200 residues of (α1 → 6)-Man linkages, highly branched by (α1 → 2)-Man and (α1 → 3)-Man terminals, linked to a *N*-acetylglucosamine (GlcNAc) residue, which in turn is linked to the protein through the amide group of asparagine (Asn) of the peptide backbone (Lesage & Bussey, 2006). The *O*-glycosylated mannoproteins are composed of 50 % carbohydrates and 50 % protein, 1–5 residues of (α1 → 2)-Man and (α1 → 3)-Man linked to the protein through the oxygen in the hydroxyl group of serine or threonine of the peptide backbone. Glycogen is a α-glucan linked by (α1 → 4)-D-Glc and highly branched by (α1 → 6)-D-Glc linkages, and chitin is a linear polymer of (β1 → 4)-linked GlcNAc (Arvindekar & Patil, 2002; Klis et al., 2002). More recently, the presence of (β1 → 4)-D-Glc linkages was also reported, but it is not clear how these are connected to the other polysaccharide structures (Bastos et al., 2015; Pinto et al., 2015). However, it is well known that BSY cell-wall polysaccharide composition and structure are influenced by environmental conditions during the brewing process. For example, when comparing the composition of the residues left after a sequential alkali treatment of *S. pastorianus* after 3–7 fermentations with the *S. pastorianus* inoculum yeast, a higher content of (α1 → 4)- and (β1 → 4)-D-Glc linkages and lower content of (β1 → 3)-D-Glc linkages was observed (Bastos et al., 2015). After a 2 h sequential up to 8 M alkaline extraction, which removes the majority of mannoproteins and glycogen, the residue left is composed of 60 % polysaccharides, namely (α1 → 4)-, (β1 → 4)-, and (β1 → 3)-glucans (92–98 %) (Coelho et al., 2015; Pinto et al., 2015). The three-dimensional structure of the yeast is still preserved in these conditions. After oxidation with chlorite solutions, which removed proteins and part of the glycogen, the destruction of the cell wall three-dimensional structure was observed (Coelho et al., 2015), which indicates the importance of these glucans network to the rigidity and shape of the cell-wall. There are several glucan purification processes described in literature (Avramia & Amariei, 2021). However, the majority of those are focused in the obtention of high purity β-glucan isolates instead of disclosing the polysaccharide BSY structural features established between β-glucans, mannoproteins, and glycogen in the cell wall. A detailed description of BSY polysaccharides' structural features is provided in our latest review (Bastos et al., 2022).

Despite the knowledge that structural, physical and chemical differences occur among *Saccharomyces* species, few reports are available comparing these and, to our knowledge, none focused on the chemical composition of the cell-wall. We hypothesise that the brewing process will also differentially affect the yeast species cell-wall, and the comparative results may help to deep the knowledge on the structural features of *Saccharomyces* cell-wall polysaccharides. Although the influence of the brewing process has been studied for *S. pastorianus* cell-wall polysaccharides, no information is available for *S. cerevisiae*. Based on the previous knowledge on the repitching effect on *S. pastorianus* (Bastos et al., 2015), we hypothesized that polysaccharide modifications could be transversal to other species and strains. In this work we look for the occurrence of modifications on the polysaccharide profile in other industrial BSY strains. It is also hypothesized that cells modulate their wall polysaccharides to increase the cell-wall strength. Thus, this work, aims to establish the structural features of cell-wall polysaccharides from brewer's spent *S. cerevisiae* and *S. pastorianus* yeasts towards a different number of industrial batches brewed. The solubilised material, as well as the residue left, after extraction by subcritical-water with microwave-assisted extraction (MAE), were analysed applying different techniques including sugar and glycosidic-linkage analysis, analytical enzymatic hydrolysis, scanning electron microscopy, FTIR, and interaction studies using carbohydrate microarrays. Based on the identified structural features, the soluble polysaccharides obtained from the different BSY used to produce the main types of beer were discussed to tailor different bioactive applications.

2. Material and methods

2.1. Samples

Four BSY from different beer productions were kindly supplied by Super Bock Group SA (Porto, Portugal). Two *Saccharomyces cerevisiae* yeasts: one which ferments Weiss beer and has not been reused (one fermentation) and other used in Ale beer, reused twice (three fermentations). Two *Saccharomyces pastorianus* yeasts: one used in Pale Larger beer production (Carlsberg) reused once (two fermentations) and the other used in the Lager beer fermentation, reused five times (six fermentations).

The yeasts were propagated from 100 mL to 15 L before going to the reactor, and then placed in the reactor with 1300 L of wort where successive fermentations occurred. One beer fermentation corresponds to two fermentations (1300 L) in propagation. The BSY were collected from the tank before being filtered in 1 L flasks, transported under refrigeration, and in the laboratory, these were centrifuged at 24,700 ×*g* (15 min, 4 °C), washed once with distilled water, collected and frozen.

2.2. Extraction of yeast polysaccharides from BSY

2.2.1. Thermal autolysis

Previously to the autolysis procedure a BSY washing step was performed three times with distilled water, centrifuged at 4696 ×*g*, for 10 min at 0 °C. The BSY washed pellet (250 g, wet basis) was suspended in water (1 L). To perform the autolysis, each BSY suspension was placed in a 60 °C bath and stirred for 2 h. After that, the temperature was increased to 80 °C for 10 min to inactivate hydrolytic enzymes. The sample was then centrifuged (24,700 ×*g*, 4 °C, 15 min), the precipitate with cell walls was washed three times with distilled water (resuspended and centrifuged at 24,700 ×*g*, 4 °C, 15 min), and the supernatant with the cytoplasmatic content was collected, concentrated using a rotary-evaporator, frozen, and freeze-dried. The efficiency of the autolysis was followed by the disappearance of ribose and deoxyribose in the residue and their recovery in the supernatant, determined as alditol acetates (Section 2.3).

2.2.2. Hot water extraction

The freeze-dried autolysis precipitate, in a ratio of 1:16 (mg/mL H_2O), was stirred in a hotplate (15 min, 100 °C), cooled, centrifuged (24,652 ×*g*, 4 °C, 20 min) and the residue and supernatant were collected. The supernatant was dialysed against distilled water at 4 °C with a 1 kDa cut-off cellulose membrane and then the dialysed supernatant and the residue were frozen and freeze-dried.

2.2.3. Subcritical water by microwave-assisted extraction (MAE)

The residue that results after extraction with hot water was resuspended in distilled water in a ratio of 1:6 (m/v) and placed in a Teflon

reactor for 2 min at 180 °C, under stirring in a microwave EthosSYNTH-Labstation. After extraction and depressurization, the sample was collected and centrifuged (24,700 ×*g*, 4 °C, 10 min). The precipitate and the supernatant were collected, frozen and freeze-dried. The polymers that compose the surface layer of yeast cell walls are expected to be extracted, namely mannoproteins and some soluble glucans (mainly glycogen), preserving the cell wall three-dimensional structure.

2.3. Sugar analysis

Neutral sugars were released from the polysaccharides by acid hydrolysis and analysed as their alditol acetates by gas-chromatography (Coimbra et al., 1996; Reis et al., 2020) using a Perkin Elmer-Clarus400 chromatograph with a split injector and a FID detector. A DB-225 column (30 m × 0.25 mm × 0.15 μm) was used. Samples hydrolysis was done in triplicate, and 2-deoxyglucose was added as internal standard for sugars quantification. The chromatograph analysis was done one per sample (the instrumental replicates have <1 % RSD).

2.4. Methylation analysis

Glycosidic-linkage composition was determined by gas-chromatography coupled to quadrupole mass spectrometry (GC-qMS) of the partially methylated alditol acetates (PMAA) (Ciucanu & Kerek, 1984; Reis et al., 2020). Briefly, polysaccharides were methylated with CH_3I under stirring and hydrolysed with 2 M trifluoroacetic acid (1 h, 120 °C). Permethylated monosaccharides were reduced with $NaBD_4$ and acetylated with acetic anhydride with 1-methylimidazole as catalyst. The PMAA were analysed by GC-qMS on a Shimadzu GCMS-QP2010-Ultra gas-chromatograph, equipped with a 30 m × 0.25 mm × 0.1 μm DB-1 fused-silica capillary column. The MS was operated in the electron-impact mode (EI) at 70 eV scanning from 50 to 700 *m/z*. Identification of the glycosidic linkages was achieved by comparing the obtained data with the standard mass spectra, CCRC spectral database and laboratory made database.

2.5. Carbohydrate microarray construction and analysis

Details on the polysaccharide samples, microarray construction, imaging and data analysis are compliant with the Minimum Information Required for A Glycomics Experiment (MIRAGE) guidelines for reporting glycan microarray based data (Liu et al., 2017).

2.5.1. Polysaccharides and microarray construction

Four polysaccharide rich fractions obtained after subcritical water treatment by MAE ($SnH_2O_{MAE\_180°C}$) from *S. cerevisiae*_Weiss, *S. cerevisiae*_Ale, *S. pastorianus*_Carls and *S. pastorianus*_Lager, were analysed in a carbohydrate microarray format. Eight glucans and mannans were included to increase carbohydrate structural diversity and as controls, for array validation and comparison purposes. The list of polysaccharides, their sources and main composition is given in Supplementary Table S1. The glucans (Palma et al., 2015) and mannans (Pinto et al., 2015) have been described previously. For microarray construction, the polysaccharides were dissolved (0.5 mg/mL) in MilliQ-water with 0.02 % (w/v) NaN_3 and immobilised by non-covalent means onto 2-pad nitrocellulose coated glass slides using a MicroCaster™ 8-Pin System. Each polysaccharide was printed in triplicate (20–70 nL *per* spot with 500 μm diameter each) at room-temperature.

2.5.2. Microarray binding analysis

The microarray was probed with six carbohydrate binding proteins with reported specificities including 3 mammalian immune receptors, 1 plant lectin and 2 monoclonal antibodies. Information on analysis conditions and their reported carbohydrate specificity is detailed in Supplementary Table S2. The microarray binding analyses were performed essentially as described (Liu et al., 2012). Briefly, for the biotinylated plant lectin Concanavalin A (ConA), nitrocellulose nonspecific binding sites were blocked (60 min) with 3 % (w/v) bovine serum albumin (BSA) in 10 mM HEPES pH 7.3, 150 mM NaCl (referred to as HBS) supplemented with 5 mM $CaCl_2$, followed by the overlay (90 min) with the protein diluted in the blocking solution at 5 μg/mL (Supplementary Table S2). For the mammalian lectins and the monoclonal antibodies, slides were blocked with 1 % (w/v) BSA, 0.02 % (v/v) casein in HBS, 5 mM $CaCl_2$, the microarrays were overlaid (90 min) with the protein solutions Supplementary Table S2, followed by incubation (60 min) with the corresponding detection reagents in specified blocking solutions. The mouse-Dectin-2 was analysed after pre-complexation with the detection reagents. The protein-antibody complex was prepared by pre-incubating the mouse monoclonal anti-poly-Histidine (1st detection reagent, Ab1) and biotinylated anti-mouse IgG (2nd detection reagent, Ab2) for 15 min, followed by addition of the His-tagged protein mDectin-2 (15 min), the ratio of the His-tagged protein: Ab1:Ab2 being 1:3:3 (w/w) (Supplementary Table S2). After diluting in the blocking solution, the mDectin-2 complex was overlaid (5 μg/mL, 90 min). The detection reagents in the absence of the proteins were also analysed to detect any nonspecific binding. For all the microarray analyses, the Alexa-Fluor647 labelled streptavidin (1 μg/mL), diluted in the corresponding blocking solutions (Supplementary Table S2), was added to each pad, incubated (30 min) and used as fluorescence reagent for readout, using a GenePix®4300A fluorescence scanner. The parameters for recording the fluorescence images were selected considering the S/N ratio, and saturation of the spot signals. Microarray data quantitation was carried out using the GenePix®Pro Software.

2.6. Total protein analysis

Protein content was determined as total nitrogen content (N × 5.99) by elemental analysis using a Truspec 630-200-200 equipment, combustion furnace temperature at 1075 °C, after burner temperature at 850 °C, using thermal conductivity detection.

2.7. Amino acids analysis

Amino acids determination was performed by acid hydrolysis (Zumwalt et al., 1987) followed by derivatization of amino acids (MacKenzie & Tenaschuk, 1974) as described elsewhere (Coimbra et al., 2011). Separation and identification were carried out on a Shimadzu GCMS-QP2010-Ultra gas-chromatograph, equipped with a 30 m × 0.25 mm × 0.1 μm DB-1 fused-silica capillary column. The MS was operated in EI at 70 eV scanning from 50 to 700 *m/z*. Identification was achieved by comparing the results with the standard mass spectra, the quantification was performed using internal standard L-norleucine. The calibration curves were determined for 11 amino acids (Supplementary Table S3). This methodology does not allow the distinction between amide and carboxylic acid groups, thus Asn and Asp, as well as Gln and Glu, are usually quantified together. To solve this, an enzymatic hydrolysis, followed by a derivatization with ethyl chloroformate (Ferreira et al., 2020; Ribeiro et al., 2015) was performed in the same samples and Gln was absent in all samples. A correlation between Asn and Asp was obtained for each sample.

2.8. Enzymatic hydrolysis

The residues left after subcritical water extraction (Final Residues) were treated with α-amylase and cellulase as described (Bastos et al., 2015) in order to determine the anomeric configuration of the (1 → 4)-Glc-linkages. The hydrolysis of (α1 → 4)-Glc-linkages was performed with a 62 U/mg α-amylase from *Bacillus subtilis* (EC 3.2.1.1) using 10 mg of sample, suspended in 15 mL of 50 mM phosphate buffer pH 7.0, during 2 h at 25 °C with continuous stirring. The hydrolysis of (β1 → 4)-Glc-linkages was carried out with a 6.9 U/mg cellulase from *Trichoderma viride* (EC 3.2.1.4) using 10 mg of sample, suspended in 15 mL of 20 mM

acetate buffer pH 5.0, during 13 h at 37 °C, with continuous stirring. The enzymatic reactions were stopped by boiling the samples for 10 min, which were then dialyzed, frozen, and freeze-dried. Estimated values of β1,4-Glc were accessed after the release of 1,4-Glc by α-amylase hydrolysis, while estimated values of α1,4-Glc were obtained after the release of 1,4-Glc by cellulase hydrolysis.

2.9. FTIR analysis

Final Residues (FR) were analysed on a Bruker Tensor-27 FTIR spectroscope coupled with a Specac Golden-Gate-Diamond ATR. Spectra were recorded on absorbance mode in the 4000–400 cm^{-1} range with a resolution of 4 cm^{-1}, co-adding 256 scans before Fourier transformation.

2.10. Scanning electron microscopy (SEM) analysis

SEM images were acquired using a field emission gun-SEM HitachiSU70 microscope operated at 15 kV to analyse the three-dimensional structure of each yeast before and after enzymatic treatment. Freeze-dried samples (FR) were resuspended in distilled water for 24 h and deposited (one drop) on a sample holder, kept in a desiccator overnight, and coated with carbon, as described (Sousa et al., 2014).

3. Results and discussion

3.1. Chemical composition and structural analysis of BSY solubilised material

The procedure adopted in this work for the extraction of yeast polysaccharides started with a yeast cell lysis, denominated as thermal autolysis, which aimed to remove the yeast intracellular material. The autolysis was followed by a water extraction at 100 °C, to remove the remaining intracellular material, and a microwave-assisted extraction (MAE), expected to extract, with subcritical water, the mannoproteins and glucans from the yeast cell-wall, in less time (2 min), but in the same extent of the sequential alkaline extractions reported in previous works. The autolysis process allowed to remove from 19 to 48 % of the yeast intracellular material in the following order: *S. cerevisiae* Ale (19 %) < *S. cerevisiae* Weiss (37 %) < *S. pastorianus* Lager (40 %) < *S. pastorianus* Carls (48 %). The hot water extraction of the residue left after the autolysis process yielded 2–3 %, probably of yeast intracellular material and/or cell-wall material corresponding to 1–2 % of yeast carbohydrates (Table 1). For all the strains, the solubilised material ($snH_2O_{100°C}$) was composed of 18–40 % carbohydrates. For BSY from *S. cerevisiae* species, 39 and 40 % of the carbohydrates were composed mostly of Glc residues, accounting for 69 % and 80 % in Weiss and Ale, respectively. In BSY from *S. pastorianus* species, the amount of carbohydrates of the solubilised material was lower than that obtained for the BSY from *S. cerevisiae* species, accounting for 29 % and 18 %, for the strains Lager and Carls, respectively. The content in Man residues was higher than in *S. cerevisiae* species, corresponding to 73 % in both Carls and Lager strains. According to the linkage composition (Table 2), the solubilised material in hot water was similar in all species, composed mainly of t-Man, (2 → 6)-Man, and (1 → 2)-Man linkages, that together with the low content of total carbohydrates points to the presence of yeast mannoproteins (Coelho et al., 2011). The solubilised material in hot water was also composed of Glc-linkages, the most representative being the (1 → 4)-Glc, t-Glc and (1 → 4,6)-Glc (Table 2), suggesting the occurrence of soluble glycogen from cytosol (Deshpande et al., 2011). The main difference between the BSY from the two species is that the content of glucose-linkages in the solubilised material from *S. cerevisiae* was higher than that observed in the solubilised material from *S. pastorianus*, which in turn contains a higher content of mannose-linkages (Table 2). This is in accordance with previous studies that showed that the mannoproteins are more exposed and/or available for interaction with other biomolecules on *S. cerevisiae* yeasts (Alsteens et al., 2008) than on *S. pastorianus*, and these are removed during the brewing process and decreased in BSY with repitching. According to our results, the shorter and less branched mannoproteins are present (Table 2), the solubilised material became richer in glycogen. It is expected that the residue left contained the cell wall polysaccharides deprived from cytosolic

Table 1
Yields and carbohydrate composition of fractions obtained after extraction with hot water and subcritical water using MAE, preceded by a thermal autolysis, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

Fraction	Yield (%) (w/w)[a]	Yield (%) (carbohydrates)	Carbohydrates (mol %)		Total carbohydrates (mg/g)
			Man	Glc	
S. cerevisiae Weiss					
$BSY_{after\ autolysis}$	63.1	–	31	69	532
sn $H_2O_{100°C}$	3.1	2.2	31	69	386
sn $H_2O_{MAE\ 180°C}$	37.6	40.5	23	77	573
Final residue	33.8	14.7	8	92	232
S. cerevisiae Ale					
$BSY_{after\ autolysis}$	81.2	–	17	83	668
sn $H_2O_{100°C}$	2.2	1.3	20	80	402
sn $H_2O_{MAE\ 180°C}$	34.1	27.8	18	82	545
Final residue	51.1	40.3	6	94	528
S. pastorianus Carls					
$BSY_{after\ autolysis}$	52.2	–	43	57	491
sn $H_2O_{100°C}$	3.3	1.9	73	27	285
sn $H_2O_{MAE\ 180°C}$	38.5	22.5	48	52	287
Final residue	34.7	25.2	13	87	357
S. pastorianus Lager					
$BSY_{after\ autolysis}$	59.6	–	45	55	516
sn $H_2O_{100°C}$	2.3	0.8	73	27	181
sn $H_2O_{MAE\ 180°C}$	33.6	21.2	65	35	325
Final residue	44.6	40.5	21	79	468

[a] Yield calculated from BSY after autolysis. The yield of BSY residue after autolysis was calculated from BSY. Abbreviations: BSY, brewer's spent yeast; Glc, glucose; Man, mannose; sn, supernatant.

Table 2
Glycosidic linkage composition (mol %) of fractions obtained after extraction with hot water and subcritical water using MAE, preceded by a thermal autolysis, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

Glycosidic linkage	*S. cerevisiae* Weiss			*S. cerevisiae* Ale			*S. pastorianus* Carls			*S. pastorianus* Lager		
	sn H_2O 100 °C	sn H_2O MAE 180 °C	Final residue	sn H_2O 100 °C	sn H_2O MAE 180 °C	Final residue	sn H_2O 100 °C	sn H_2O MAE 180 °C	Final residue	sn H_2O 100 °C	sn H_2O MAE 180 °C	Final residue
t-Ara	0.4	0.3	0.2	0.3	0.2	0.5	0.5	0.4	0.4	0.2	0.2	0.1
Total	**0.4**	**0.3**	**0.2**	**0.3**	**0.2**	**0.5**	**0.5**	**0.4**	**0.4**	**0.2**	**0.2**	**0.1**
t-Man	18.5	10.6	2.8	12.1	8.5	1.6	30.6	20.8	4.9	25.8	22.1	7.3
2-Man	13.0	5.7	1.8	6.9	7.9	1.2	17.7	16.3	3.6	19.0	17.5	5.8
3-Man	—	1.2	0.6	—	0.2	0.1	tr	0.6	0.1	tr	0.3	0.8
4-Man	—	0.2	0.1	—	—	0.1	0.2	—	0.2	0.4	—	0.1
6-Man	1.4	1.2	0.3	1.1	1.1	0.1	2.7	1.7	0.5	2.4	1.8	0.6
2,3-Man	0.1	0.2	1.3	—	0.1	0.3	0.2	—	1.5	0.4	0.2	1.6
2,6-Man	18.3	13.3	1.7	10.1	10.1	0.6	20.5	23.3	2.7	23.1	23.8	4.6
3,4-Man	0.2	0.4	0.5	0.3	0.5	0.1	—	—	0.3	0.1	0.1	0.3
3,6-Man	—	0.4	0.2	—	0.4	0.1	0.8	0.8	0.4	—	0.9	0.5
2,3,6-Man	0.4	—	—	0.3	—	—	—	—	—	1.7	1.2	—
Total	**51.8**	**33.3**	**9.4**	**30.8**	**28.7**	**4.3**	**72.7**	**63.5**	**14.2**	**72.9**	**68.0**	**21.7**
t-Glc	4.0	6.8	7.1	8.0	7.2	11.1	1.9	3.0	6.9	1.4	1.6	7.2
3-Glc	—	24.1	64.9	—	1.8	11.8	0.8	5.8	49.2	1.0	1.4	34.3
4-Glc	38.4	26.3	2.4	52.5	51.0	60.8	22.0	21.9	14.2	20.1	24.2	21.4
6-Glc	0.7	5.1	3.7	0.8	3.2	2.9	0.5	2.4	7.4	0.7	1.4	8.0
3,6-Glc	—	—	4.4	—	—	1.1	—	—	3.5	—	—	3.0
4,6-Glc	3.6	3.1	0.3	6.5	6.6	6.1	0.8	1.5	1.4	1.7	1.3	2.2
Total	**46.6**	**65.4**	**82.8**	**67.8**	**69.8**	**93.9**	**26.0**	**34.5**	**82.6**	**24.9**	**29.9**	**76.0**
4-GlcNAc	0.5	0.3	6.6	0.2	0.4	1.1	0.5	1.3	1.1	0.7	0.4	0.9
Total	**0.5**	**0.3**	**6.6**	**0.2**	**0.4**	**1.1**	**0.5**	**1.3**	**1.1**	**0.7**	**0.4**	**0.9**

tr, traces < 0.1 mol%. The shadow lines highlight the linkages where the main differences can be observed.

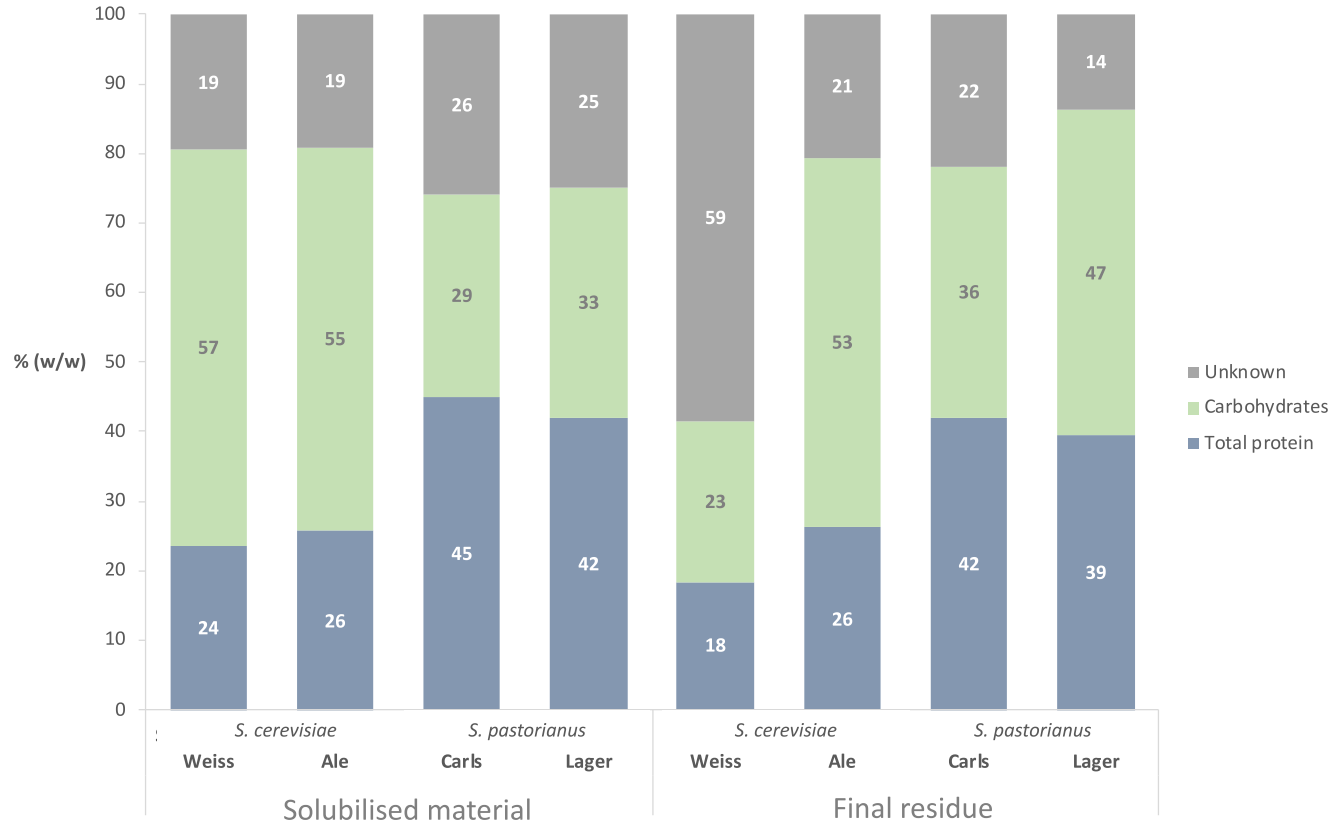

Fig. 1. Chemical composition % (w/w) of solubilised material and final residues obtained after extraction with subcritical water using MAE of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations). The subcritical water extraction was performed in the BSY samples preceded by a thermal autolysis and a hot water extraction.

glycogen. However, the presence of intracellular proteins cannot be excluded, as it is reported that mitochondrial and ribosomal proteins are not removed without mechanical disruption and Triton addition (Yammine et al., 2022).

The subcritical water extraction using MAE of the residue left after the hot water extraction, preceded by autolysis, allowed the extraction of 34–39 % of the yeast cell-wall material corresponding to 21–41 % of yeast carbohydrates (Table 1). This solubilised material was composed of 33–57 % carbohydrates, being the *S. cerevisiae* species the richest (57 % and 55 % in Weiss and Ale strains, respectively). These were composed of a higher content of glucans than solubilised material of BSY from *S. pastorianus* species, which in turn presented an increased amount of solubilised mannoproteins. Indeed, when analysing protein composition of this solubilised material, in contrast with BSY from *S. cerevisiae* species, *S. pastorianus* were composed of almost the double of protein: 24 % in Weiss, 26 % in Ale, 45 % in Carls and 42 % in Lager strains (Fig. 1). The amino acids content was also higher in *S. pastorianus* species (Table 3); however, the double content was only observed for Ala, Gly, Val, and Leu residues. The Thr content was similar for all species, Thr is known to be the linkage between Man residues and the *O*-linked mannoproteins (Bastos et al., 2022). Threonine was present in higher amounts than Asn, which is usually linked to GlcNAc and Man residues linked at (1 → 6)-Man and branched at (1 → 2,6)-Man, known as *N*-linked mannoproteins (Bastos et al., 2022). The Asn content was higher in BSY from *S. pastorianus* species than in *S. cerevisiae*, which is in accordance with the high content of mannoproteins in *S. pastorianus* species. Thr content was higher than Asn, suggesting that despite *N*-linked mannoproteins are responsible for the major weight of the total mannoproteins due to the quantity of Man residues (50–200 Man), the *O*-linkage repeats more often through the cell-wall. Isoleucine was also higher in BSY from *S. pastorianus* species than in *S. cerevisiae* but not the double. Phenylalanine, the second major amino acid residue seems to increase within the species with the number of fermentations, as well as lysine but in this case the species has no influence, only the number of fermentations. In contrast, glutamate, the major amino acid residue, decreases with the increase in the number of fermentations performed by the yeasts. Aspartate is the only amino acid residue which was higher in *S. cerevisiae* than in *S. pastorianus* species.

Solubilised mannoproteins by subcritical water using MAE have a similar glycosidic linkages composition and branching (2,6-Man/total Man) as the ones solubilised by hot water extraction from all BSY. Nevertheless, solubilised glucans presented a different composition among and within the species. It seems that (1 → 3)-Glc decreases with the number of fermentations. In Weiss (1 fermentation), the amount of (1 → 3)-Glc (24 %) and (1 → 4)-Glc (26 %) linkages showed to be similar, and in Carls (2 fermentations), the (1 → 4)-Glc (22 %) linkage was much higher than (1 → 3)-Glc (6 %). In Ale (3 fermentations) and Lager (6 fermentations) the amount of (1 → 3)-Glc linkage was 2 and 1 %, respectively (Table 2).

Aiming to further elucidate the structural composition of the different BSY polysaccharide samples and their recognition by proteins, a focused carbohydrate microarray was constructed containing the BSY solubilised material ($snH_2O_{180°MAE}$) from *S. cerevisiae*_Weiss, *S. cerevisiae*_Ale, *S. pastorianus*_Carls, and *S. pastorianus*_Lager obtained after MAE extraction with subcritical water. Glucans and mannans isolated from other sources were also included (Supplementary Table S1). The microarray was probed for recognition with the mammalian immune receptors: human-Dectin-1 (hDectin-1-Fc), human-DC-SIGN (hDC-SIGN-FC), and mouse-Dectin-2 (mDectin-2-His). Other carbohydrate-binding proteins with known carbohydrate binding specificity were also analysed and included: anti-(β1 → 3)-glucan monoclonal antibody 400–2, anti-mixed-link-(β1 → 3)/(β1 → 4)-glucan monoclonal antibody 400-3, and α-mannose specific plant lectin ConA (Supplementary Table S2). Microarray analysis showed that all the BSY extracted fractions were recognised by human-Dectin-1, particularly those from *S. cerevisiae*_Ale, which elicited a stronger binding signal (Fig. 2). This immune receptor is highly specific for (β1 → 3)-gluco-oligosaccharide sequences, requiring a minimum chain length of decasaccharide, and is likely recognizing an helical conformational epitope on the BSY fractions (Palma et al., 2015), Supplementary Table S2. The presence of (β1 → 3)-glucan structures was corroborated with the (β1 → 3)-glucan specific monoclonal antibody (mAb) 400-2, in agreement with the methylation analysis (Table 2). No binding was detected with mAb 400-3, suggesting the absence of mixed-link-(β1 → 3)/(β1 → 4)-Glc residues in these fractions (Fig. 2). The microarray analysis with the plant lectin ConA, specific for α-mannosylated glycans, confirmed the presence of these structures in the BSY extracted fractions of all studied *Saccharomyces* species (Supplementary Table S2, and Fig. 2). These were recognised by the immune receptor Dectin-2, particularly the fractions from *S. cerevisiae* Ale that elicited a stronger binding signal (Fig. 2). The binding of Dectin-2 was shown to be highly restricted to the core (α1 → 2)-linked *N*-mannan triantennary structure of fungal cell-wall (Vendele et al., 2020). In contrast, the immune receptor DC-SIGN exhibits a broader binding profile to mannans and glucans (Vendele et al., 2020), which explains the similar binding signals elicited with all of the BSY extracted fractions. Together, the microarray results corroborate the occurrence of (β1 → 3)-glucans and α-mannan structures pointed by the structural analyses and showed that the BSY subcritical water extracted fractions can be differentially recognised by lectin receptors of the immune system.

Table 3
Amino acids composition (mg/g) of solubilised material (sn H_2O $_{180°MAE}$) and final residues after extraction with subcritical water using MAE, preceded by a thermal autolysis and a hot water extraction, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

Amino acid	*S. cerevisiae* Weiss		*S. cerevisiae* Ale		*S. pastorianus* Carls		*S. pastorianus* Lager	
	sn H_2O MAE 180 °C	Final residue	sn H_2O MAE 180 °C	Final residue	sn H_2O MAE 180 °C	Final residue	sn H_2O MAE 180 °C	Final residue
Ala	3.1 ± 0.4	4.0 ± 0.2	2.5 ± 0.5	2.7 ± 0.0	5.4 ± 0.6	5.8 ± 0.7	5.2 ± 0.7	4.1 ± 0.4
Gly	3.5 ± 0.3	3.1 ± 0.1	3.8 ± 0.2	3.3 ± 0.1	7.6 ± 1.2	7.5 ± 0.5	8.2 ± 0.3	6.8 ± 0.6
Val	5.0 ± 0.4	5.2 ± 0.1	4.1 ± 0.7	4.4 ± 0.1	7.7 ± 0.7	10.6 ± 0.0	8.4 ± 1.0	9.5 ± 1.5
Thr	10.5 ± 2.4	<LOQ	10.4 ± 2.0	5.6 ± 0.5	11.7 ± 1.2	<LOQ	14.3 ± 0.6	8.7 ± 0.1
Leu	11.1 ± 0.8	9.8 ± 0.1	11.2 ± 0.6	13.0 ± 0.1	19.2 ± 2.1	25.0 ± 1.0	18.9 ± 0.7	20.0 ± 1.7
Ile	10.4 ± 0.7	9.7 ± 0.0	9.0 ± 1.1	10.5 ± 0.3	16.0 ± 1.8	21.7 ± 0.3	15.5 ± 4.1	16.8 ± 2.6
Pro	16.1 ± 1.4	7.8 ± 0.7	11.5 ± 2.8	10.7 ± 0.1	17.0 ± 2.6	16.1 ± 0.5	15.3 ± 1.0	11.1 ± 1.4
Phe	21.2 ± 2.5	10.0 ± 0.7	33.5 ± 1.8	26.7 ± 1.6	40.4 ± 3.7	29.6 ± 1.7	42.2 ± 1.5	39.9 ± 4.7
Asn	1.7 ± 0.1	—	—	11.5 ± 0.7	5.9 ± 0.7	11.7 ± 0.3	5.7 ± 1.1	9.0 ± 0.4
Asp	8.9 ± 0.4	8.9 ± 0.8	12.2 ± 2.0	5.3 ± 0.3	6.4 ± 0.8	6.4 ± 0.2	5.2 ± 1.0	4.4 ± 0.2
Glu	60.7 ± 3.3	25.8 ± 1.5	45.1 ± 2.9	36.9 ± 4.3	59.0 ± 6.5	47.0 ± 5.5	47.7 ± 6.8	42.9 ± 1.9
Lys	13.6 ± 1.8	5.9 ± 0.4	21.2 ± 1.9	21.2 ± 3.7	27.9 ± 4.3	21.2 ± 1.4	32.8 ± 4.9	34.1 ± 0.1
Total	***165.6***	***93.6***	***164.4***	***151.6***	***224.0***	***203.6***	***219.2***	***207.2***

*Final residue; <LOQ below the limit of quantification. The shadow lines highlight the amino acids where the main differences can be observed.

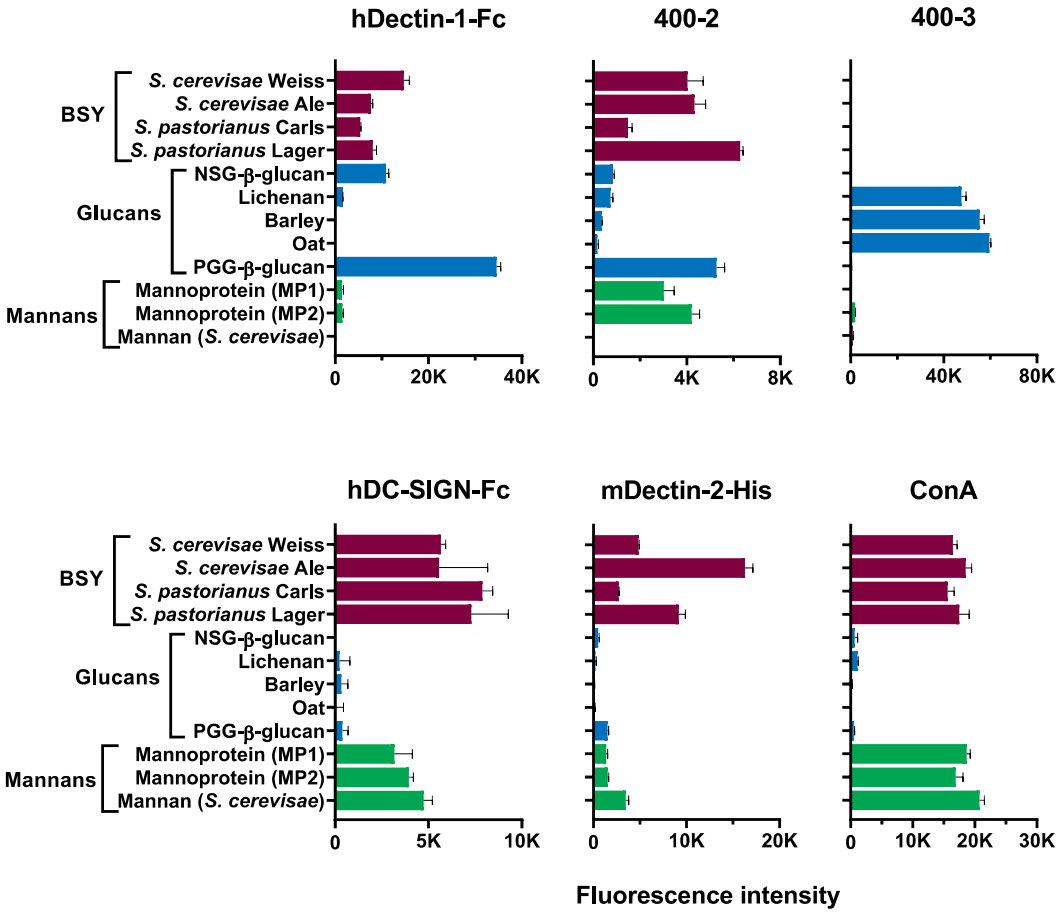

Fig. 2. Carbohydrate microarray analyses of the binding to solubilised BSY fractions by carbohydrate sequence-specific proteins. The BSY fractions were obtained after extraction with subcritical water using MAE, preceded by a thermal autolysis and a hot water extraction (sn $H_2O_{180°MAE}$): *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentation), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations) (wine bars). The binding to glucans (blue bars) and mannans (green bars) isolated from other sources is included as a control for the binding. Detailed information on the polysaccharide samples and proteins analysed is in Supplementary Tables S1 and S2, respectively. The scales for the fluorescence binding intensities are depicted at the bottom for each protein; these are means of fluorescence intensities of triplicate spots arrayed (with error bars) at 0.5 mg/mL per spot. Mammalian immune receptors: hDectin-1-Fc – Human Dectin-1; hDC-SIGN-Fc – Human DC-SIGN; mDectin-2-His – Mouse Dectin-2. Plant lectin: ConA – Concanavalin A. Monoclonal antibodies: 400-2 (anti-(β1,3)-glucan); 400–3 (anti-mixed link (β1,4)/(β1,3)-glucan).

Summing up, the extraction process here used allowed a maximum extracted yeast material in *S. cerevisiae*_Weiss (66 %) and *S. pastorianus*_Carls (65 %), the ones with less repitching. Also, these are the ones with high content of solubilised carbohydrates, 85 % and 75 %, respectively. However, from these solubilised carbohydrates from *S. cerevisiae*_Weiss it was recovered 43 % of carbohydrates mainly composed of glucans (74 %) with the highest content of (1 → 3)-Glc linkage, while from *S. pastorianus*_Carls only 24 % of carbohydrates were recovered and mainly composed of mannoproteins (60 %). From *S. cerevisiae*_Ale and *S. pastorianus*_Lager the amount of extracted material (49 % and 55 %, respectively) and carbohydrates (60 % in both) from yeast was similar between them, as well as the carbohydrates solubilised (29 % and 22 %, respectively), however, as seen before, the solubilised material from *S. cerevisiae* was mainly composed of glucans (81 %) and from *S. pastorianus* mainly composed of mannoproteins (68 %). Despite the amount of extracted material and carbohydrates were higher in the yeasts not reused or reused once than the yeasts with 3 or more repitchings, the carbohydrates solubilised were higher in the last ones.

Comparing the results with the ones reported when using sequential alkaline extractions with no previous autolysis, of *S. pastorianus*_Lager with 3–7 fermentations (Bastos et al., 2015; Pinto et al., 2015), the material extracted from BSY was higher (77–79 %) than the one obtained for the same yeast strain (Lager) in this work. The carbohydrates extracted were similar (66–76 %) and the polysaccharides solubilised were higher (41–55 %) composed by similar quantities of glucans (43–51 %) and mannoproteins (40–41 %) (Bastos et al., 2015; Pinto et al., 2015). This suggests that subcritical water using MAE is very effective in the removal of glucans from the yeast.

3.2. Chemical composition and structural analysis of final residues

The resultant final residues (FR) were different between and within species. The ones obtained from *S. cerevisiae* represented 34 % (Weiss) and 51 % (Ale) of yeast weight, still composed of 15 % (Weiss) and 40 % (Ale) of yeast carbohydrates (Table 1). The composition of carbohydrates was similar in both Weiss and Ale, mainly composed of glucans, 92 and 94 mol% of Glc, respectively, and a residual amount of mannoproteins, 8 and 6 mol% of Man (Table 1). However, the final residue (FR) from Weiss was poorer in carbohydrates (23 %) than the FR obtained from Ale (53 %), yet Weiss presented a considerable amount of unknown material (Fig. 1) which inferred by the FTIR analysis may suggest the presence of other components due to the presence of specific bands that are missing or higher than in the other BSY FR (Fig. 3). The FTIR spectra profile is similar within species containing expected polysaccharide and protein bands in all samples (1200–900 cm^{-1}, C—O, C—C stretching and C—O—H, C—O—C deformation; 1543–1531 cm^{-1}, CONH bending (Amide II); 1531–1614 cm^{-1}, C═O stretching (Amide I))

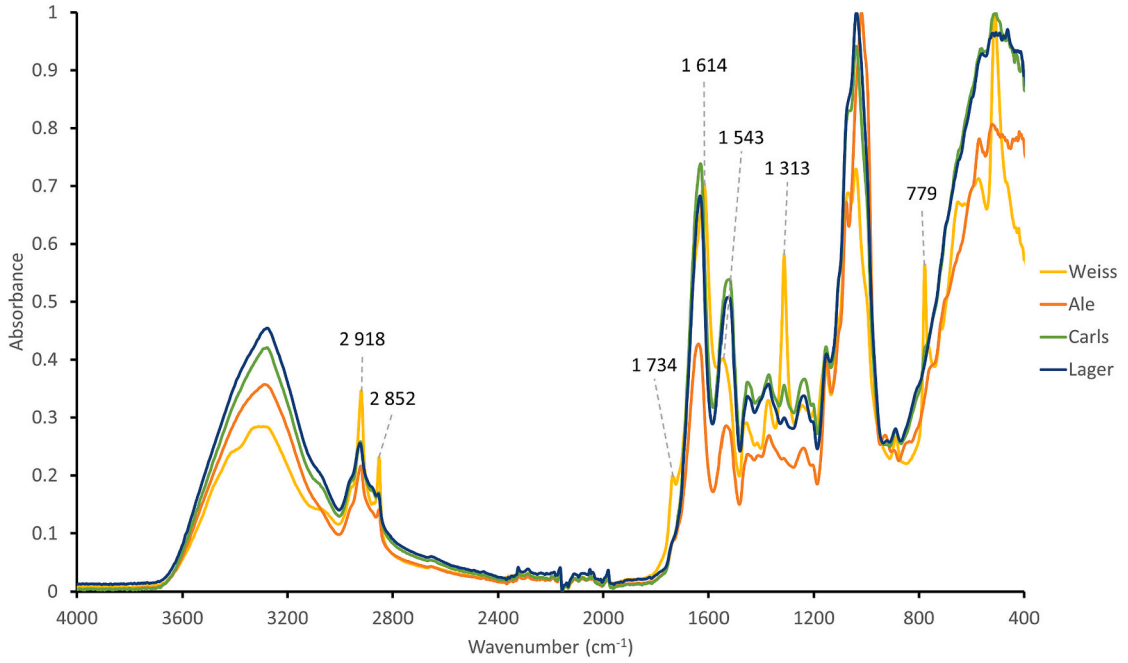

Fig. 3. Normalized FTIR spectra of final residues obtained after extraction with subcritical water using MAE, preceded by a thermal autolysis and a hot water extraction, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

except for Weiss which also revealed unique bands such as an ester C═O stretching band (1734 cm^{-1}), CH_3 bending (1313 cm^{-1}) and a CH_2 deformation (780 cm^{-1}), also sharp bands on C—H stretching bands (3000–2800 cm^{-1}), these bands could be related with lipid compounds (Shapaval et al., 2019; Wiercigroch et al., 2017). Despite *S. cerevisiae* is not usually described as oleaginous, under stress conditions this accumulation could occur, as it was already reported that in nitrogen-limited conditions genes associated with fatty acid biosynthesis are up-regulated, thus enhancing lipid storage during nitrogen deprivation (He et al., 2018). Glycosidic linkage analysis showed that the glucans present in the final residue resultant from Weiss were mainly composed of (1 → 3)-Glc (65 %) and in residual amount (2 %) of (1 → 4)-Glc linkages (Table 2). In contrast, the FR obtained from Ale was mainly composed of (1 → 4)-Glc linkages (61 %). These results suggest that the cell-wall composition of *S. cerevisiae* modifies with repitching verifying the results reported for *S. pastorianus*_Lager, when analysing the inoculum yeast and the yeast after 6 repitchings it was observed an increase of (1 → 4)-Glc linkage and a decrease on the (1 → 3)-Glc linkage being hypothesized an adaptation of *S. pastorianus* to fermentative stress (Bastos et al., 2015).

The FR from *S. pastorianus* represented 35 % (Carls) and 45 % (Lager) of yeast weight, composed of 25 % (Carls) and 41 % (Lager) of yeast carbohydrates (Table 1). These results were similar with *S. cerevisiae*, although, the composition of carbohydrates was different since *S. pastorianus* species presented a high amount of mannoproteins 13 and 21 % for Carls and Lager, respectively (Table 1). The protein content was similar between these two (Fig. 1), and the most representative amino acids were the same in both (glutamate and phenylalanine, the two major), yet Lager presented less content in most amino acids except for Phe, Lys and Thr (Table 3). The FR were composed of 36 % (Carls) and 47 % (Lager) of carbohydrates, mostly glucans (87 %, Carls; 79 % Lager) of (1 → 3)-Glc (49 %, Carls; 34 % Lager) and (1 → 4)-Glc (14 %, Carls; 21 % Lager) linkages. Despite (1 → 4)-Glc linkage was not in higher amount than (1 → 3)-Glc in both *S. pastorianus* (Table 2), it's obvious the decrease in the ratio of (1 → 3)-Glc/(1 → 4)-Glc with the increase of the number of batches brewed (pitchs) as seen previously with *S. cerevisiae*. Nevertheless, the linkages ratio of *S. cerevisiae*_Ale is the lowest of all the species studied, with only 2 repitchings. Even lower when compared with reported results of 6 repitchings (Bastos et al., 2015) of *S. pastorianus*, which suggests that *S. cerevisiae* is more susceptible to fermentative stress showing an adaptation/modification of yeast cell-wall in few generations.

3.3. Anomeric configuration of final residues (1 → 4)-Glc linkages

The FR were treated enzymatically with α-amylase and cellulase to identify the anomeric configuration of 1,4-linked Glc. Both treatments induced a loss of 1,4-Glc in a different extent for each enzyme used and each specie involved, for α-amylase treatment hydrolysis varied between 18 and 73 %, while for the cellulase treatment between 15 and 54 %. These results were transformed to mol% and the proportion of α, β, and unknown configuration of 1,4-Glc is also represented in Fig. 4. *S. cerevisiae*_Weiss presented the lowest percentages of hydrolysis, 18 and 15 % for α-amylase and cellulase, respectively. After transforming these percentages into mol%, most of the 1,4-Glc linkages configuration remain unexplained, α- and β-configuration was only 0.4 % each of the 2.4 % total. It seems that enzymes could not reach all the 1,4-Glc linkages, and since the amount of 1,4-Glc is too low when comparing to 1,3-Glc it's hypothesized that 1,3-Glc chains are more accessible and are hiding the 1,4-Glc chains, or even, the 1,4-linked glucose residues are inserted within the 1,3-Glc chains and being few, become difficult to be reached by the enzymes or the stereoselectivity of enzymes did not recognise them. However, this last hypothesis is not supported by the solubilised material which showed no binding (Fig. 2) with the monoclonal antibody 400-3 that recognises mixed-link-β1,3/1,4-glucose oligosaccharide sequences (DP < 7; 1:4 β1,3/1,4), however the ratio of intercalation could be different from cereal β1,3/1,4-glucans. In *S. cerevisiae*_Ale the amount of 1,4-Glc is higher than 1,3-Glc, with the α-configuration as the major configuration, 4-fold higher than the β-configuration, and only 8 % of 1,4-Glc configuration remains unknown, which indicates that *S. cerevisiae* with fermentative stress preferentially increases the content of glycogen, crucial to the yeast osmotolerance (Lillie & Pringle, 1980). Nevertheless, β1,4-Glc also increase with fermentative stress which may be related with the increase

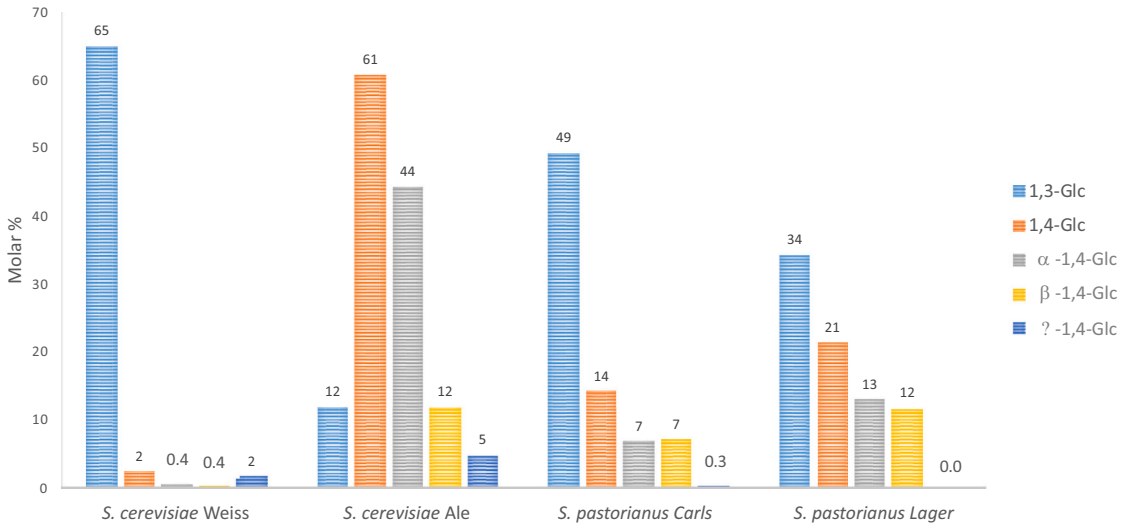

Fig. 4. Molar % of (1 → 3)-Glc and respective (1 → 4)-Glc linkages of final residues, after extraction with hot water and subcritical water using MAE, preceded by a thermal autolysis, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

in the cell-wall rigidity and strength necessary to prevent dissolution of the cell-wall.

In the case of *S. pastorianus* specie the 1,4-Glc linkages increased with the fermentative stress always maintaining the same proportion of α- and β-configurations. It seems also, that in *S. pastorianus* these 1,4-Glc linkages are more accessible to the enzymes than *S. cerevisiae*, since only a residual amount or even any 1,4-Glc linkages configuration remains unexplained. Moreover, the sum of α- and β-configurations is over 100 % in the case of *S. pastorianus* Lager which again indicates that these linkages may be interspersed with each other. However, looking to the binding result of solubilised material to 400-3 specific binding protein (Fig. 2) another hypothesis should be stated based on mixed-linkages probably with β1,3/β1,4/α1,4-Glc.

These results seem to indicate that *S. cerevisiae* modifies the cell-wall composition during the fermentative stress looking to the yeast osmotolerance (α1,4-Glc increase) over the cell-wall strength (β1,4-Glc increase) while for *S. pastorianus* both modifications are balanced.

3.4. Scanning electron microscopy of final residues

SEM images of FR suspensions at 3000× magnification showed in both species ellipsoid cells (Fig. 5), but in *S. cerevisiae* they seem to be glued to each other by an adhesive substance while in *S. pastorianus* they seem to be more isolated. The presence of more extractable glycogen from *S. cerevisiae* BSY acting as glue or more resistant *S. pastorianus* BSY due to the cell-wall strength conferred by a high ratio of β1,4-Glc/α1,4-Glc are possible hypotheses for the different appearance observed. Comparing the FR before and after treatment with α-amylase and cellulase (Fig. 6). In the case of *S. cerevisiae* Weiss, the cells seem to be very similar as the treatments did not have any influence on the cells,

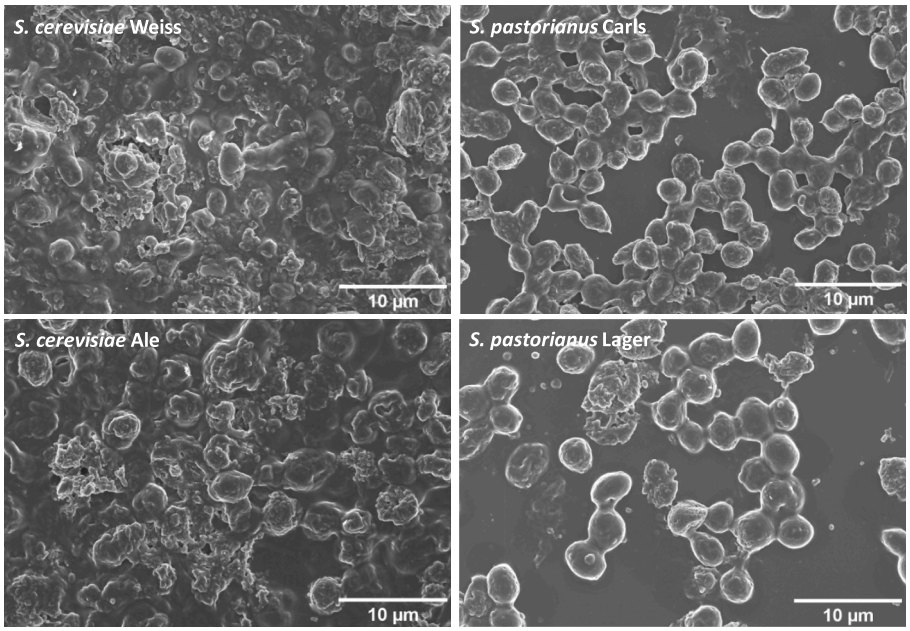

Fig. 5. Scanning electron microscopy (SEM) images of final residues (3000× magnification) obtained after extraction with hot water and subcritical water using MAE, preceded by a thermal autolysis of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

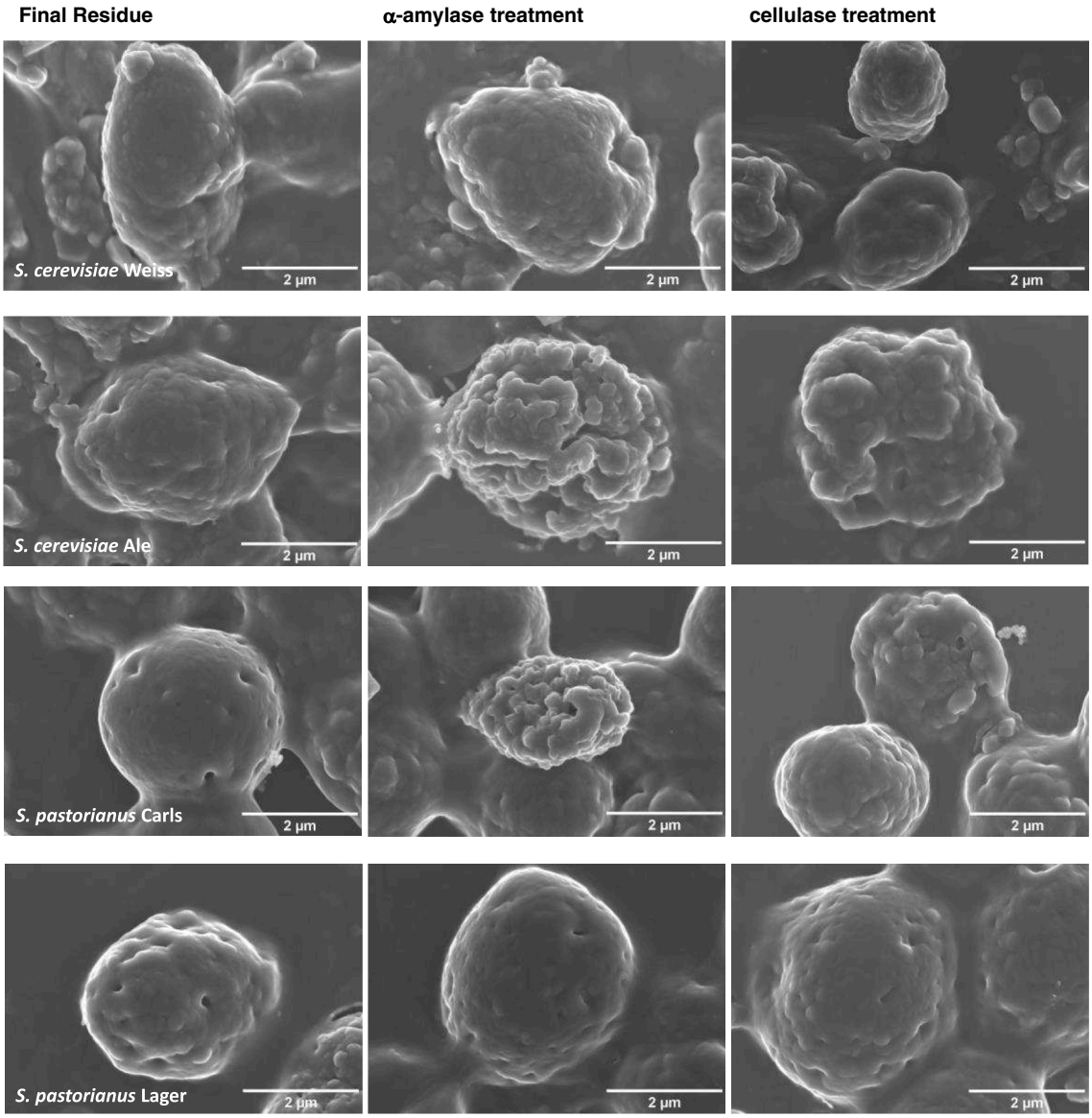

Fig. 6. Scanning electron microscopy (SEM) images of final residues (20,000 × magnification) before and after treatment of α-amylase and celullase, obtained after extraction with water and MAE, preceded by a thermal autolysis, of BSY of *S. cerevisiae* Weiss (1 fermentation), *S. cerevisiae* Ale (3 fermentations), *S. pastorianus* Carls (2 fermentations) and *S. pastorianus* Lager (6 fermentations).

which makes sense when looking to the residual mol% of 1,4-Glc linkages of BSY from this species being unaffected by both hydrolyses (Fig. 4).

The enzymatic treatments on *S. cerevisiae*_Ale and *S. pastorianus*_Carls residues had a different result from Weiss, some cells appear with rough and not smooth surfaces as before. This behaviour is stronger when using α-amylase than cellulase. This result suggests that, in some of the cells, the 1,4-Glc linkages are at the cell surface being available to the enzyme. If the solubilised material obtained by subcritical water extraction were representative of the BSY cell-wall surface, it may explain the weak binding of *S. cerevisiae*_Ale to (β1 → 3)-Glc specific binding proteins as seen in Fig. 2. In the case of *S. pastorianus*_Lager, the treatments have no influence on the three-dimensional structure of cells, but in this case 1,4-Glc linkages were higher than Weiss, and higher than Carls. This result leads again to the interspersed 1,4-Glc and 1,3-Glc linkages hypothesis. This occurrence could be triggered by successive fermentative stress induced by the repitching process or a particularity of this strain.

4. Conclusion

The main difference of solubilised material of BSY from *S. cerevisiae* and *S. pastorianus* was the first mainly composed of glucans and the second of mannoproteins. The yeast cell walls that remained after the extractions (final residues) still represented a considerable amount of carbohydrates in BSY from both *Saccharomyces* species, in which the ones with a smaller number of pitches have the least amount, where a maximum of yeast material and carbohydrates were solubilised. *S. cerevisiae*_Weiss residue stood out by the possible accumulation of lipids. *S. cerevisiae* cell-wall also modified with the fermentation process and repitching in a more extended way than *S. pastorianus*, probably due to the use of a different mechanism in the adaptation to the fermentative stress, seen by the low 1,3-Glc/1,4-Glc ratio. Furthermore, the β-configuration of 1,4-Glc linkages was again verified for *S. pastorianus* and for the first time reported for *S. cerevisiae*, and despite the uncertainty in the organization of the linkages of β1,4-Glc in the glucans network, its importance for maintaining the three-dimensional structure of yeast cells was clear. *S. cerevisiae* modifies the cell-wall composition

during the fermentative stress looking to the yeast osmotolerance over the cell-wall strength while for *S. pastorianus* both responses are balanced. The equilibrium between β1,4- and α1,4-Glc creates a very resistant cell-wall structure towards fermentative stress and could be the reason beyond the maintenance of *S. pastorianus* fermentation performance under serial repitching. The *S. cerevisiae* lacks this equilibrium between osmotolerance and cell-wall strength, disabling serial repitching due to performance loss. The different brewer's spent *Saccharomyces* yeasts used to produce the main types of beers, have different microarray binding profiles with immune receptors and may be used to develop ingredients with tailored immune responses, able to interact with Dectin-1, DC-SIGN, and Dectin-2.

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CRediT authorship contribution statement

Conceptualization, Elisabete Coelho (E.C.), Manuel A. Coimbra (M.A.C.); methodology, E.C., M.A.C., Angelina S. Palma (A.S.P.); investigation, Susana Messias (S.M.), Lisete M. Silva (L.M.S.), Sofia F. Reis (S.F.R.), Rita Bastos (R.B.), Vítor J. Martins (V.J.M.), Viviana G. Correia (V.G. C.), Benedita A. Pinheiro (B.A.P.); formal analysis, S.M., S.F.R., L.M.S.; Validation, A.S.P., E.C., M.A.C., S.F.R., L.M.S.; Resources, E.C., M.A.C., A.S.P.; writing—original draft preparation, S.M., S.F.R.; writing—review and editing, E.C., M.A.C., L.M.S., A.S.P., V.G.C., B.A.P., V.J.M., R.B.; Visualization, S.M., S.F.R., E.C.; Supervision, E.C., M.A.C., A.S.P.; Project administration, E.C.; Funding acquisition, E.C., M.A.C., A.S.P.; All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Professor Manuel A. Coimbra is Editor of Carbohydrate Polymers.

Data availability

Data will be made available on request.

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