Modifications of *Saccharomyces pastorianus* cell wall polysaccharides with brewing process

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ABSTRACT

The cell wall polysaccharides of brewers spent yeast *Saccharomyces pastorianus* (BSY) and the inoculum yeast (IY) were studied in order to understand the changes induced by the brewing process. The hot water and alkali extractions performed solubilized mainly mannoproteins, more branched for BSY than those of IY. Also, ^{31}P solid state NMR showed that the BSY mannoproteins were 3 times more phosphorylated. By electron microscopy it was observed that the final residues of alkali sequential extraction until 4 M KOH preserved the yeast three-dimensional structure. The final residues, composed mainly by glucans (92%), showed that the BSY, when compared with IY, contained higher amount of (1 $\rightarrow$ 4)-linked Glc (43% for BSY and 16% for IY) and lower (1 $\rightarrow$ 3)-linked Glc (17% for BSY and 42% for IY). The enzymatic treatment of final residue showed that both BSY and IY had (α 1 $\rightarrow$ 4)-linked Glc and (β 1 $\rightarrow$ 4)-linked Glc, in a 2:1 ratio, showing that *S. pastorianus* increases their cellulose-like linkages with the brewing process.

Keywords: brewers spent yeast; sequential extraction; mannoprotein; glucan; alcoholic fermentation stress.

1. Introduction

Mannoproteins and β -glucans represent the majority of the compounds of *Saccharomyces* brewing cell walls used as brewing yeasts. However, as they are highly entangled in the cell wall matrix, they are difficult to extract (Liu, Wang, Cui & Liu, 2008; Quirós, Morales, Pérez-Través, Barcenilla & Gonzalez, 2011), mainly the β -glucans, preventing their structural characterization (Pinto, Coelho, Nunes, Brandão & Coimbra, 2015).

Cell wall mannoproteins could be *N*-linked if carbohydrate moieties are linked to the nitrogen of asparagine or arginine, or *O*-linked if mannans have a glycosidic linkage to the hydroxyl oxygen of serine or threonine. *N*-linked mannoproteins can be extended with an outer chain of 50-200 ($\alpha 1 \rightarrow 6$) linked Man residues extensively branched with short ($\alpha 1 \rightarrow 2$)-Man side chains with terminal ($\alpha 1 \rightarrow 3$)-Man residues. *N*-linked type mannoproteins are the majority of yeast mannoproteins and are essentially composed by 90% carbohydrate and 10% protein, having a relevant structural function (Lesage & Bussey, 2006). These *N*-linked mannans can contain phosphate groups as mannosylphosphate residues that contribute for the ionic properties of yeast cell surface (Jigami & Odani, 1999). The *O*-linked mannoproteins are composed by 50% of protein and 50% of carbohydrates, bearing oligosaccharide chains containing 1 to 5 ($\alpha 1 \rightarrow 2$)-Man and ($\alpha 1 \rightarrow 3$)-Man units usually with one mannosylphosphate residue (Corbacho, Olivero & Hernández, 2005). These mannoproteins are responsible for several enzymatic activities (Lesage & Bussey, 2006).

The yeast cell wall inner layer is composed of glucans mainly constituted by ($\beta 1 \rightarrow 3$)-Glc residues (85%) and ($\beta 1 \rightarrow 6$)-Glc residues (15%). The outer layer of mannoproteins are covalently attached to these polymers (Klis, Boorsma & De Groot, 2006). Also, ($\alpha 1 \rightarrow 4$)-Glc residues with ($\alpha 1 \rightarrow 4,6$)-Glc linkages has been identified in *Saccharomyces* cell walls, covalently linked to ($\beta 1 \rightarrow 6$)-glucans. These polymers were

identified as insoluble glycogen-like polysaccharides and could represent 1 to 23% of yeast cell wall dry weight (Arvindekar & Patil, 2002; Kwiatkowski, Thielen, Glenney & Moran, 2009).

The existence of (β 1 $\rightarrow$ 4)-linked glucans in the BSY *Saccharomyces pastorianus* cell walls has been recently reported, accounting for 30% of the (1 $\rightarrow$ 4)-linked glucans (Pinto et al., 2015). *S. pastorianus* is an allotetraploid hybrid yeast derived from *S. cerevisiae* and an *S. bayanus* (Rainieri et al., 2006; Tamai, Momma, Yoshimoto & Kaneko, 1998). *S. pastorianus* is a *lager* brewing strain that, contrary to *S. cerevisiae*, sinks on fermentation tank because of its more hydrophilic surface (Dengis, Nelissen & Rouxhet, 1995).

In order to evaluate the modifications of *S. pastorianus* cell wall polysaccharides with brewing process, the yeast of inoculum (IY) and the BSY were studied. For that, a sequential extraction of yeast polysaccharides was performed. Yeast intracellular material was released by sonication and yeast cell walls were prepared as alcohol insoluble residues (AIRs), and then subjected to sequential extraction with alkali solutions. Sugars and methylation analysis were performed. Also, the differences in mannoprotein phosphorylation were evaluated by ^{31}P solid state NMR and the glucans that remained in the insoluble residues were enzymatically treated with α -amylase and cellulase to determine the anomeric configuration of the (1 $\rightarrow$ 4)-Glc linkages.

2. Materials and methods

2.1. Samples

Inoculum yeast (IY) and brewers spent yeast (BSY) were provide by Unicer Bebidas SA., Portugal. IY was collected from the 4th generation of yeasts adapted to the fermentation broth, and BSY was collected from the bottom of fermentation reactor after 3-7 fermentative cycles.

2.2. Sequential extraction of yeast polysaccharides

An aqueous suspension of yeast cells (IY and BSY) were submitted to 15 min of sonication, and the solubilized material was separated from the insoluble residue by centrifugation at 4°C and 24,652g during 20 min. The soluble fractions were precipitated with 80% (v/v) ethanol at 4°C during 2 h. The yeast cells residues were boiled with 80% (v/v) ethanol during 10 min in order to prepare the alcohol insoluble residues (AIR). Each AIR was submitted to a hot water extraction during 15 min at 100°C with an AIR/water ratio of 1:16 (w/v). The residue left was submitted to an alkali extraction with 0.1 M KOH + 20 mM NaBH₄ using oxygen-free solutions to prevent peeling reactions. The alkali extraction was performed at room temperature during 2 h with continuous stirring, and the insoluble material was separated from the soluble one by centrifugation. The extracted material was acidified to pH 5 and then dialyzed against water with 12-14 kDa molecular weight cut off membranes. After dialysis, the precipitated extracted material with 0.1 M KOH was separated by centrifugation at 24,652g, 4°C during 20 min. The sequential extraction proceeded with 1 M and 4 M KOH solutions using the same procedure described above. The residue left after 4 M KOH extraction was suspended in 200 mL of water, neutralized to pH 5 with glacial acetic acid, dialyzed, and centrifuged, allowing to obtain the final residue (FR) and a material that remained soluble inside the dialysis membrane (snFR) (Fig.1). Each supernatant, precipitate, and the FR collected were suspended in water, concentrated by rotary evaporation under reduced pressure, frozen, and freeze-dried.

Fig. 1: Schematic representation of sequential extraction of inoculum yeast (IY) and brewers spent yeast (BSY).

2.3. Sugar analysis

The sugars composition of the polysaccharides were determined by gas chromatography-flame ionization detection (GC-FID) as alditol acetates and quantified using 2-deoxyglucose as internal standard (Blakeney, Harris, Henry & Stone, 1983). Monosaccharides were released from polysaccharides (1-2 mg of sample) by pre-hydrolysis in 0.2 mL of 72 % H₂SO₄ (w/w) for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H₂SO₄ at 100°C (Selvendran, March & Ring, 1979). After 1h hydrolysis, it was collected 0.5 mL of solution for uronic acids determination. Monosaccharides were reduced with NaBH₄ (15% in 3 M NH₃) during 1 h at 30 °C and then acetylated, with acetic anhydride (3 mL) and 1-methylimidazole (450 µL), during 30 min at 30 °C. The alditol acetates were separated by liquid-liquid extraction with water and dichloromethane and after evaporation of organic solvent, were solubilized in anhydrous acetone and analysed by a GC-FID Perkin Elmer-Clarus 400 with a capillary column DB-225 (30 m length, 0.25 mm inner diameter and 0.15 µm film thickness). The oven temperature program was as follows: 200 °C to 220 °C at a rate of 40 °C/min (7 min), increasing to 230 °C at a rate of 20 °C/min (1 min). The temperature of injector was 220 °C and the detector was 230 °C. Hydrogen was used as carrier gas at a flow rate of 1.7 mL/min.

Uronic acid content was determined by the *m*-phenylphenol colorimetric method (Blumenkrantz & Asboe-Hansen, 1973) using galacturonic acid as standard. To the 0.5 mL of diluted hydrolysed sample (1:4) was added 3 mL of boric acid 50 mM H₂SO₄ 98% (w/w), after shaken the test tubes were heated at 100 °C during 10 min. After cooling, 100 µL of *m*-phenylphenol was added, reacting 30 min in dark, and the absorbance was measured at 520 nm.

2.4. Glycosidic-linkage analysis

The glycosidic-linkage composition was determined by gas chromatography-quadrupole mass spectrometry (GC-qMS) of partially methylated alditol acetates (Ciucanu & Kerek, 1984; Nunes & Coimbra, 2002). The samples (1-2 mg) were dissolved in 1 mL of anhydrous dimethylsulfoxide (DMSO), and then powdered NaOH (40 mg) were added under an argon atmosphere. The samples were methylated with CH₃I (80 µL) during 20 min with stirring, following by a second addition of 80 µL CH₃I and stirring for another 20 min. CHCl₃/CH₃OH (1:1, v/v, 3 mL) was added, and the solution was dialyzed (membrane with a pore diameter of 12-14 kDa) against 3 lots of 50% ethanol. The dialysate was evaporated to dryness and the material was remethylated using the same procedure. The remethylated material was hydrolysed with 2 M TFA (500 µL) at 120°C for 1 h, and then reduced and acetylated as previously described for sugar analysis, using NaBD₄ instead of NaBH₄. The partially methylated alditol acetates were separated and analysed by GC-qMS using an Agilent Technologies 6890N Network. The GC was equipped with a DB-1 (J&W Scientific, Folsom, CA, USA) capillary column (30 m length, 0.25 mm of internal diameter, and 0.15 µm of film thickness). The samples were injected in “split” mode during 5 min using an injector temperature of 220°C, during 5 min. The temperature program used was as follows: initial temperature was 50°C, with a linear increase of 8°C/min until 140°C (5 min), followed by a linear increase of 0.5°C/min until 150°C followed by a linear increase of 40°C/min until 250°C (1 min). The helium carrier gas had a flow rate of 1.7 mL/min and a column head pressure of 14.4 psi. The GC was connected to an Agilent 5973 mass quadrupole selective detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500, 1 s cycle, in a full scan mode acquisition.

2.5. Enzymatic hydrolyses

The FR samples were treated with α -amylase and cellulase as described for [Pinto et al. \(2015\)](#). The hydrolysis of (α 1 $\rightarrow$ 4)-Glc linkages were performed with a 62 U/mg α -amylase from *Bacillus subtilis* (Sigma-Aldrich, EC 3.2.1.1) on 10 mg of sample, suspended in 5 mL of 50 mM phosphate buffer pH 7.0, during 2 h at 25 °C with continuous stirring. The hydrolysis of (β 1 $\rightarrow$ 4)-Glc linkages was held with a 1.5 U/mg cellulase from *Aspergillus niger* (Sigma-Aldrich, EC 3.2.1.4) and 10 mg of sample, suspended in 5 mL of 20 mM acetate buffer pH 5.0, during 4 h at 37 °C, with continuous stirring. The enzymatic reactions were stopped by boiling for 10 min, and samples were dialyzed, frozen and then freeze-dried.

2.6. ^{31}P solid-state NMR

The ^{31}P solid-state magic angle spinning nuclear magnetic resonance (MAS-NMR) with high power decoupling (HPDEC) experiments were acquired using a Bruker Avance III 400 MHz spectrometer. It was performed 1,424 scans analysis with a relaxation delay (D1) of 60 s, pulse length (P1) of 3 μ s, power level (PL1) of 10 dB, decoupler pulse length (PCPD2) of 6.5 μ s, power level for decoupler (PL12) of 4.8 dB and flip angle of 90°.

2.7. Scanning electron microscopy

Scanning electron microscopy (SEM) images were acquired using a field emission gun-SEM Hitachi SU70 microscope operated at 15 kV. Samples were deposited on a sample holder and coated with carbon, as described by [Sousa, Matos, Pinto, Freire and Silvestre \(2014\)](#).

3. Results and discussion

3.1. Carbohydrate composition of IY and BSY

The IY and BSY had a moisture content of 85 and 84 %, respectively. IY dry weight was composed by 56 % of carbohydrates, namely 60 mol% of glucose and 33 mol% of mannose, and BSY proportion of carbohydrates was 47 % dry weight, mainly composed by 70 mol% of glucose and 19 mol% of mannose (Table 1).

3.2. Sequential extraction and structural analysis of IY and BSY polysaccharides

The sequential extraction of yeast polysaccharides was performed with an initial treatment of IY and BSY with ultrasounds in order to increment the release of the intracellular material and preparation of an AIR of cell walls, with further hot water, 0.1, 1.0 and 4.0 M KOH alkali extractions. The mass yields and sugars composition of the extracts and final residues of IY and BSY extractions are shown in Table 1 and the glycosidic linkages composition are shown in Table 2.

Table 1: Extraction yields and carbohydrate composition of the fractions obtained by sequential extraction of IY and BSY.

Fraction	Yield (%) (w/w)	Yield (%) * in extracted carbohydrates	Carbohydrates (mol%)									Total carbohydrate (µg/mg)
			dRib	Rha	Rib	Ara	Xyl	Man	Gal	Glc	HexA	
IY	-	-	1	-	2	1	1	33	-	60	1	555
US Et80	10.2	11.7	1	-	1	3	4	15	1	74	2	635
US EtSn	9.9	4.8	1	-	0	3	4	2	-	88	3	267
sn AIR	5.6	1.1	5	1	2	1	3	2	-	81	4	110
AIR	59.3	40.7	1	-	6	-	-	45	-	45	2	381
sn H ₂ O _{100°C}	4.1 (6.9)	2.8 (6.8)	2	-	13	1	1	50	-	30	2	374
sn 0.1 M KOH	2.3 (3.9)	2.0 (5.0)	2	-	20	1	1	67	-	5	3	487
pp 0.1 M KOH	3.1 (5.2)	0.6 (1.5)	5	-	70	-	-	18	-	3	3	111
sn 1M KOH	1.3 (2.1)	1.1 (2.8)	1	-	1	2	2	81	-	10	2	500
pp 1M KOH	1.9 (3.2)	0.3 (0.6)	12	-	1	4	2	26	-	51	3	74
sn 4M KOH	2.9 (4.9)	3.7 (9.2)	1	-	1	1	2	83	-	9	4	712
pp 4M KOH	2.4 (4.0)	0.8 (2.1)	4	-	-	1	1	8	-	82	3	197
sn FR	1.8 (3.0)	2.3 (5.7)	-	-	-	-	-	66	-	30	3	726
FR	17.2 (29.1)	15.9 (39.0)	-	-	-	-	-	6	-	91	2	511
BSY	-	-	2	-	2	1	1	19	-	70	5	469

US Et80	8.7	11.3	2	-	-	2	3	3	-	82	6	607
US EtSn	11.8	6.6	4	1	-	2	3	1	-	86	7	262
sn AIR	5.9	8.0	8	2	-	2	3	2	-	72	9	638
AIR	57.0	62.7	1	-	3	1	-	26	-	64	5	511
sn H ₂ O _{100°C}	4.1 (7.7)	3.4 (5.9)	1	-	8	1	1	45	-	38	6	392
sn 0.1 M KOH	2.6 (4.8)	1.5 (2.6)	3	1	35	1	-	50	-	8	3	276
pp 0.1 M KOH	2.1 (4.0)	0.3 (0.5)	12	4	39	-	-	22	-	10	14	63
sn 1M KOH	1.9 (3.5)	1.9 (3.3)	3	1	1	1	-	81	-	9	4	474
pp 1M KOH	1.5 (2.8)	0.2 (0.4)	16	5			-	30	-	42	7	69
sn 4M KOH	4.4 (8.2)	5.8 (10.0)	2	1	1	1	-	82	-	9	4	624
pp 4M KOH	2.0 (3.7)	0.3 (0.5)	23	5	-	-	-	46	-	26	1	70
sn FR	1.3 (2.4)	2.1 (3.6)	2	-	1	1	-	52	-	41	3	756
FR	23.1 (43.0)	33.6 (57.4)	-	-	-	-	-	4	-	92	4	681

Values between brackets correspond to yields calculated in AIR mass basis. * The yield of extracted carbohydrates was calculated considering the concentration of carbohydrates (total of carbohydrates in mg/g) and the mass (g) of respective fraction. With these carbohydrates mass values it was possible to calculate the yield of carbohydrates extracted in initial yeast or AIR basis.

3.2.1. Yeasts pre-treatment

The pre-treatment of yeasts with sonication solubilised 17 % of IY carbohydrates and 18 % of BSY (sum of yields of US EtSn and US Et80). The extracted material that precipitated with ethanol was mainly composed of glucose, namely 74 mol% for IY and 82 mol% for BSY. Glycosidic-linkage analysis of US Et80 showed that glucose was mainly (1→4)-Glc (44.4 mol% for IY and 56.3 mol% for BSY) and (1→4,6)-Glc (10.5 mol% for IY and 16.7 mol% for BSY), which are probably the linkages of intracellular glycogen. The ultrasounds treatment allowed the extraction of intracellular glycogen probably through the increasing permeability and disruption of the cell wall (Liu, Zeng, Sun & Han, 2013; Zhang, Jin, Xie, Wu & Wu, 2014).

From the residues of yeasts ultrasonic treatment, it was prepared the AIR of both yeasts cell walls. The AIR of IY represented 59% of initial yeast dry weight and 57% for BSY, with a yield of carbohydrates higher for BSY than IY AIR, 63% and 41%, respectively. Also, the BSY AIR was richer in carbohydrates (51%) than IY AIR (38%) and with a higher proportion of glucose (64 mol%). The glycosidic linkage analysis shows that the BSY AIR glucose was mainly (1→4)-Glc (38 mol%), 4 times higher than that present in IY AIR, indicating a possible higher proportion of (1→4)-Glc in the

BSY cell walls. In AIR yeast cell walls was also present (1→3)-Glc (10.8 mol% for IY and 6.7 mol% for BSY) and (1→6)-Glc (3.1 mol% for IY and 5.8 mol% for BSY), possibly constituents of β -glucans (Klis, Mol, Hellingwerf & Brul, 2002). Both yeasts had similar proportions of (1→2)-Man (10.1 mol% for IY and 5.8 mol% for BSY) and (1→2,6)-Man linkages (11.6 mol% for IY and 9.4 mol% for BSY), showing that the mannoproteins of IY and BSY AIR are highly branched.

Table 2: Glycosidic-linkage composition (mol%) of IY and BSY fractions of sequential extraction.

Glycosidic linkages	IY						BSY					
	US Et80	AIR	sn H ₂ O 100°C	sn 4M KOH	sn FR	FR	US Et80	AIR	sn H ₂ O 100°C	sn 4M KOH	sn FR	FR
t-Ara	5.2	0.4	t	-	-	-	2.1	0.6	-	0.1	-	-
Total	5.2 (4)	0.4 (0)	t (1)	-	-	-	2.1 (2)	0.6 (1)	-	0.1 (1)	-	-
4-Xyl	3.3	0.5	t	-	-	-	2.1	1.5	6.1	0.3	-	-
Total	3.3 (3)	0.5 (0)	t (1)	-	-	-	2.1 (3)	1.5 (0)	6.1 (1)	0.3 (0.3)	-	-
t-Man	6.5	13.5	13.0	39.0	23.3	3.6	0.4	8.7	15.1	29.6	21.1	2.4
2-Man	3.3	10.1	13.5	19.0	14.6	3.9	0.2	5.8	10.8	20.0	13.1	1.2
3-Man	-	4.0	t	10.1	8.2	-	-	-	t	7.4	4.3	-
4-Man	-	0.4	-	t	0.3	-	-	-	0.9	0.4	0.5	-
6-Man	0.4	1.3	1.2	1.8	1.3	1.0	0.4	1.2	1.1	2.2	1.2	t
2,6-Man	3.9	11.6	10.4	13.1	22.1	3.5	2.1	9.4	12.6	29.5	13.4	2.5
3,6-Man	-	1.3	t	1.2	0.5	-	-	0.8	1.0	0.7	-	-
2,3,6/2,4,6-Man	-	-	-	0.5	0.7	-	-	-	-	0.6	0.6	-
2,3,4,6-Man	3.0	14.3	13.8	0.4	0.5	0.3	0.1	1.5	2.2	0.4	1.3	0.9
Total	17.1 (15)	56.5 (45)	54.1 (50)	85.1 (83)	71.5 (65)	12.3 (6)	3.1 (3)	28.0 (26)	43.6 (45)	90.7 (82)	55.6 (52)	7.0 (4)
t-Glc	15.5	2.8	5.3	1.3	2.6	8.6	16.9	6.9	4.9	0.5	5.4	10.0
3-Glc	0.2	10.8	1.4	1.4	0.5	41.8	1.0	6.7	1.7	0.2	0.8	17.3
4-Glc	44.4	9.3	27.3	8.0	21.1	15.9	56.3	38.0	30.3	6.6	27.1	42.7
6-Glc	0.7	3.1	2.3	0.7	0.4	11.3	1.5	5.8	1.9	0.6	1.2	9.3
2,3-Glc	-	0.9	-	-	-	0.7	-	0.8	t	-	-	1
3,4-Glc	-	0.4	-	-	-	0.2	-	0.5	t	-	-	0.4
3,6-Glc	-	1.2	-	-	-	3.3	-	1.0	t	-	-	2.2
4,6-Glc	10.5	1.4	5.0	0.8	2.3	1.2	16.7	3.8	3.5	-	1.7	5.4
2,3,4,6-Glc	3.19	11.3	6.9	1.5	1.2	3.4	0.2	4.6	5.5	0.8	6.6	3.7
Total	74.4 (74)	41.2 (45)	46.0 (30)	13.7 (9)	28.2 (30)	86.4 (89)	92.6 (82)	68.2 (64)	47.7 (38)	8.7 (9)	42.9 (41)	92.0 (90)
4-GlcNAc	-	0.8	-	0.9	0.1	1.1	-	1.5	1.3	0.1	0.6	0.5
Total	-	0.8 (n.d.)	-	0.9 (n.d.)	0.1 (n.d.)	1.1 (n.d.)	-	1.5 (n.d.)	1.3 (n.d.)	0.1 (n.d.)	0.6 (n.d.)	0.5 (n.d.)

Values between brackets correspond to data from sugar analysis. t = trace; n.d. = not determined in sugar analysis.

3.2.2. Hot water and alkali polysaccharides extraction

Hot water and alkali extractions led to the extraction mainly of mannoproteins (38 % for IY and 59 % for BSY) and a low content of glucans (12 % for IY and 7 % for BSY) from yeast cell wall AIRs. The extraction with hot water solubilized 6.8% and 5.9% of AIR carbohydrates of IY and BSY, respectively. The snH₂O of IY had 37% of carbohydrates and was mainly composed by 50 mol% of mannose, 30 mol% of glucose and 13 mol% of ribose. The snH₂O of BSY had 39% of carbohydrates, namely 45 mol% of mannose, 38 mol% of glucose and 8 mol% of ribose. In the previously work of Pinto et al. (2015), with hot water extraction it was solubilized a fraction with 70% carbohydrates that have a high contribution of intracellular material, namely glucose. In the present study, due to yeasts' pre-treatment, the hot water extraction performed after

releasing of the intracellular material led to recover a fraction with higher recovery of mannose, consistent with extraction of cell wall mannoproteins. The mannose residues were mainly (1→2)-Man and (1→2,6)-Man residues, present in similar proportions for both yeasts. The glucose solubilised in hot water had the characteristic glycosidic linkages of glycogen, namely (1→4)-Glc (27 mol% for IY and 30 mol% for BSY) and (1→4,6)-Glc (5 mol% for IY and 3.5 mol% for BSY).

The sequential alkali extraction with 0.1M KOH allowed the solubilization of 6.5% and 3.1% of AIR carbohydrates from IY and BSY, respectively (sum of yields of sn and pp fractions). These fractions were poor in carbohydrates and were mainly constituted by ribose, with 70 mol% for IY. As the presence of ribose in yeast cell wall is only described to the yeast *Ustilago maydis* (5%), (Ruiz-Herrera, Leon, Carabez-Trejo & Reyes-Salinas, 1996), the extracted ribose probably comes from yeasts' RNA.

The extraction with 1 M KOH solubilised 2.8 and 3.3% polysaccharides from IY and BSY AIR, respectively, with about 50% carbohydrates and 81 mol% of mannose residues, attributed to yeast mannoproteins. The alkali extraction with 4 M KOH led to obtain a yield of 11% in relation to the AIR, which was 3 times higher than that extracted with 1 M KOH. The material solubilised with 4 M KOH was rich in sugars (62-71 %) and was mainly composed by mannose (82-83 mol%), with 17% and 32% yield of extraction of mannoproteins, from IY and BSY AIR, respectively. The glycosidic-linkage analysis (Table 2) reveals that the mannoproteins extracted have the higher proportion of terminally-linked mannose (39 mol% for IY and 30 mol% for BSY), followed by (1→2,6)-Man (13 mol% for IY and 30 mol% for BSY), (1→2)-Man (19 mol% for IY and 20 mol% for BSY) and (1→3)-Man linkages (10 mol% for IY and 7.4 mol% for BSY). The higher mol% of terminally-linked mannose and lower mol% of branched mannose for IY could indicate that the 4 M KOH solution extracted lower molecular weight mannoproteins from IY than from BSY. The proportion of

(1→2,6)-Man to the total of mannose residues shows that BSY has 1 branching for each 6 residues and BSY has 1 branching for each 3 residues. The extraction of higher branched structure in BSY suggests that after brewing process the yeasts exhibit more branched mannoproteins. These higher branched mannoproteins structures could be important to yeast flocculation and protection against fermentative stress (Soares, 2011). During environmental brewing stress, it was shown that *Saccharomyces pastorianus* increase the amount of cell wall mannoproteins, the resistance to lytic enzymes, and the lectin-receptor interactions (Gibson, Lawrence, Leclaire, Powell & Smart, 2007; Smart, 2009). In turn, flocculation provides protection of yeasts against ethanol stress and prevents the losing of cell viability (Soares, 2011).

Another important feature for yeasts survival under stress conditions is the presence of phosphorylated mannoproteins. As the sn4M KOH was the fraction with higher content of mannoproteins extracted, it was performed a ³¹P solid NMR analysis to investigate the presence of phosphate in these samples. As shown in Fig. 2, the ³¹P solid NMR analysis reveals one peak between -10 and 10 ppm, a range that includes phosphates in phosphodiester linkages (Sang, Prakash & Seib, 2007). As NMR was performed in same HPDEC conditions for both samples, we can conclude that the sn4M of BSY had 3 times more phosphate than sn4M of IY. This result suggests that mannosylphosphorylation in yeast cell wall have been induced under stress conditions. Mannosylphosphates give a net negative charge to cell wall surface, providing a more hydrophilic and rigid structure to the yeasts and increasing their tolerance against osmotic stress and lytic enzymes (Jigami et al., 1999).

Fig. 2: ^{31}P solid NMR spectra of sn4M from a) IY and b) BSY. Both spectra are at same scale.

3.2.3. Polysaccharides recovered upon dialysis of alkali final residue

The material that remained insoluble after the alkali sequential extraction was neutralised, dialyzed and centrifuged, allowing to obtain the material that became soluble in water and retained inside the dialysis membrane (snFR) separated from the insoluble final residue (FR). The snRF fraction had a high carbohydrate recovery (5.7 % for IY and 3.6 % for BSY) and content of sugars (73 % for IY and 76 % for BSY). The snRF was mainly composed by mannose (65 mol% for IY and 52 mol% for BSY) and glucose residues (30 mol% for IY and 41 mol% for BSY), which indicates that it was possible, by this separation and recovery of snRF, to obtain a large proportion of remaining mannose and a high content of glucans. After the 4 M KOH extraction, the recovery of yeast glucans increased, and the higher amount of (1→4)-Glc (21 mol% for IY and 27 mol% for BSY) solubilized with (1→4,6)-Glc linkages (2.3 mol% for IY and 1.6 mol% for BSY) is in accordance with the presence of glycogen in yeast cell wall, as reported for BSY (Pinto et al., 2015) and *S. cerevisiae* (Arvindkar et al., 2002).

The FR represents 17% and 23% of IY and BSY initial dry weight, respectively, mainly composed of glucans (91 mol% for IY and 92 mol% for BSY). The presence of mannose residues in FR (6 mol% for IY and 4 mol% for BSY), and the carbohydrate

content of 51% for IY and 68 % for BSY, indicate the presence of insoluble mannoproteins even after extensive alkali extractions. The oxidation of BSY FR with chlorite and extraction with 1 M KOH led to the solubilisation of mannoproteins with a low carbohydrate moiety and allowing the recovery of a residue very rich in carbohydrates (92%), with 99 mol% of glucose (unpublished results). Thus, combining the data from these two works, it is possible to infer that the mannoproteins remaining insoluble after alkali extraction should have a higher protein than carbohydrate moiety, which is in accordance with the existence of *O*-mannoproteins in the yeast inner cell wall. The presence of mannoproteins in the yeast inner cell wall of *Saccharomyces cerevisiae* was reported by Moukadiri, Armero, Abad, Sentandreu and Zueco (1997) and Valentin, Herrero, Rico, Miragall & Sentandreu (1987) [ENREF 23](#).

The alkali extractions led to the solubilization of 46% of IY and 66% of BSY, respectively, of mannoproteins present in the AIR. However, the final residues only contained 5% of IY and 8% BSY of the AIR mannoproteins. These figures allow to hypothesise that 48% of the IY AIR mannoproteins and 27% of BSY ones should have been lost during the dialysis process. Since the IY mannoproteins are less branched and have lower molecular weight than those of BSY, this could explain the higher percentage of loss during dialyses. Regarding the AIR glucans, 76% of IY and 84 % of BSY remained insoluble in the FR, which is in accordance with the low yield of glucans extracted by the sequential extraction (16 mol% for IY and 9 mol% for BSY). These results are in accordance with Pinto et al. (2015), where only 9% more of BSY glucans were solubilized with alkali extractions until 8 M KOH.

The glucans present in FR have higher proportion of (1→3)- than (1→6)-Glc either for IY (41.8 mol% and 11.3 mol%, respectively) and for BSY (17.3 mol% and 9.3 mol%), which should correspond to (β1→3) and (β1→6) glucans (Klis et al., 2006; Lesage et al., 2006). The percentage of (1→4)-Glc linkages was 3 times higher in BSY

FR (42.7 mol%) than in IY FR (15.9 mol%), which seems to reveal a change in yeast' polysaccharides composition and structure along the brewery fermentative process.

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

In order to identify the anomeric configuration of the (1→4)-linked Glc yeasts FRs, the samples were enzymatically treated with α -amylase and cellulase. With the treatment with α -amylase, the relative amount of (1→4)-Glc in IY FR decreased from 15.9 mol% to 5.3 mol%. Also, the treatment of IY FR with cellulase allowed to show a decrease in (1→4)-Glc, from 15.9 mol% to 11.0 mol%. Taking into account to extent of these two reactions, it can be inferred that 67 mol% of IY FR (1→4)-Glc linkages were (α 1→4)-linked and 31 mol% were (β 1→4)-linked. Also, the hydrolysis of BSY FR with α -amylase and cellulase led to a decrease from 42.7 mol% (1→4)-Glc to 17 mol% and 29.3 mol%, respectively. These results showed that 60 mol% of (1→4)-Glc linkages were (α 1→4)-linked and 13 mol% were (β 1→4)-linked. As 27% of (1→4)-linkages, even after repetition of the hydrolysis, were not cleaved it was not possible to identify their anomeric configuration. The results herein obtained for enzymatic hydrolysis of BSY FR were in agreement with [Pinto et al. \(2015\)](#) when reporting the occurrence of 55 mol% of (α 1→4)-linked and 30 mol % of (β 1→4)-linked glucose residues for BSY FR. These results show that *S. pastorianus* seems to modify the cell wall composition during the brewing process increasing in same proportion both (α 1→4)- and (β 1→4)-Glc residues (Fig. 3), as decreasing the (1→3)-Glc residues (Table 2).

As the glycogen content of yeast cell wall depends on the nutritional state of the cells, the isolation method, and the growth phase, it is possible that the BSY have higher glycogen content due to the stress conferred by the brewing process, which is essential to the osmotic tolerance of yeast during the recycle of yeasts between the fermentative process ([Lillie & Pringle, 1980](#)). The yeast cell wall glycogen exponentially increases 3

times more than intracellular glycogen, until 8 % (v/v) of ethanol (Dake, Khetmalas & Amarapurkar, 2011). Also, the increase of cellulose-like (β 1 $\rightarrow$ 4)-Glc linkages could be related with an increase in the cell wall rigidity and strength necessary to prevent dissolution of the cell wall.

Fig. 3: Molar percentage of (α 1 $\rightarrow$ 4)-Glc and (β 1 $\rightarrow$ 4)-Glc of IY and BSY in relation to total carbohydrates in the FR. The ?-(1 $\rightarrow$ 4)-Glc represents the relative amount of not identified anomeric configuration.

3.4. Scanning electron microscopy of IY and BSY final residue

Figure 4 shows the SEM images of IY FR (Fig. 4a and 4c) and BSY FR (Fig. 4b and 4d) after the sequential extraction, revealing that the brewer's yeast cells original structure was preserved in FR. The preservation of yeast structure and shape by glucans after removal of outer layer of mannoproteins has been already reported for the spent yeast *S. cerevisiae* (Liu, Wang, Cui & Liu, 2008). However, the cell wall structure was completely destroyed after enzymatic hydrolysis with α -amylase and cellulase for IY FR (Fig 4e) and BSY FR (Fig 4f), showing that α and β (1 $\rightarrow$ 4)-Glc linkages are important for preservation of the cell wall integrity, maintaining the network of glucans and possibly the inner layer mannoproteins. This is supported by the observations of

Arvindekar et al. (2002) and Jadhav et al. (2008), which confirmed that the glycogen is covalently linked to the cell wall ($\beta 1 \rightarrow 3$)-glucans through a ($\beta 1 \rightarrow 6$)-linkage.

Fig. 4: Scanning electron microscopy (SEM) images of **a)** IY FR with 400 $\times$ of magnification and **c)** with 10,000 $\times$ of magnification; **b)** BSY FR with 400 $\times$ of magnification and **d)** with 10,000 $\times$ of magnification; enzymatic hydrolysed residues **e)** IY FR with 10,000 $\times$ of magnification and **f)** BSY FR with 10,000 $\times$ of magnification.

4. Conclusions

The results of this work show that BSY polysaccharides have different features from those of IY. This was shown in the mannoproteins extracted with hot water and alkali solutions, where higher branched and phosphorylated polymers were observed for

BSY. The modifications of *S. pastorianus* mannoproteins with the brewing process could be important since they could led to an increase of yeast flocculation and an increase of negative charge of yeast surface, important to yeast protection against osmotic stress, lytic enzymes and preservation of yeast viability.

Contrarily to mannoproteins, the glucans of *S. pastorianus* were poorly extracted from both IY and BSY cell walls. IY glucans were mainly composed by (1→3)-Glc residues while the BSY glucans were mainly composed by (1→4)-Glc linkages. Although with different (1→4)-Glc content between IY and BSY, both had 1/3 of (β1→4) and 2/3 of (α1→4)-linked glucose. These results showed that *S. pastorianus* seems changing their glucans during the brewing process, incrementing in the same proportion the content of (α1→4) and (β 1→4)-Glc over the content of (1→3)-Glc linkages. The higher proportion of both (1→4)-Glc anomeric configurations in BSY could result from adaptation of *S. pastorianus* to fermentative stress, since glycogen is important to yeast osmotolerance and cellulose-like structures could be important to increases cell wall strength.

Other important feature observed for *S. pastorianus* was the preservation of the three-dimensional structure of the yeast after the sequential polysaccharides extraction. The yeast final residue seems to form a hollow structure that was only destroyed after enzymatic hydrolysis of its (1→4)-Glc linkages. This allows to infer that these linkages are important for maintenance of the yeast structure. The observation of the existence of these capsules and how they can be formed could open new perspectives for yeast valuation.

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490

- Graphical Abstract

Saccharomyces pastorianus

IY – Inoculum Yeast
BSY – Brewers Spent Yeast

Mannoproteins