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Valuation of brewers spent yeast polysaccharides: A structural characterization approach

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ABSTRACT

Brewers spent yeast (BSY) is a by-product from beer industry that can be exploited as source of glucans and mannoproteins, with potential biological activities. In order to solubilize these carbohydrate-rich polymeric materials, a sequential extraction with hot water and alkaline solutions (0.1–8 M KOH) was performed. Mannoproteins were mainly (85%) extracted with 4 M KOH whereas glucans were extracted with 8 M KOH and in an amount that accounted only for 34% of total glucose. Final residue still accounted for 34% of the initial glucans and contained 98% of glucose. Cellulase and α-amylase treatments showed the presence of both α- and β-(1→4)-Glc linkages. To promote total solubilization of these insoluble glucans, the final residue was submitted to a partial acid hydrolysis. This work is the first report showing that the most abundant polysaccharides in BSY are polymers that contain structural features similar to cellulose, thus justifying their resistance to alkaline extractions, acid hydrolysis, and insolubility in water.

1. Introduction

Brewers Spent Yeast (BSY) represents 1.7–2.3 g/L of beer, used mainly in animal feed formulations. This by-product has, however, potential to be valued, particularly those which may result from carbohydrate polymers. BSY polysaccharides are mainly glucans and mannoproteins (Klis, Boorsma, & De Groot, 2006; Lipke & Ovalle, 1998), described as having immunostimulatory (Bohn & BeMiller, 1995; Chen & Seviour, 2007; Herre, Gordon, & Brown, 2004; Liu, Wang, & He, 2011), antioxidant (Jaehrig, Rohn, Kroh, Fleischer, & Kurz, 2007; Kogan et al., 2005; Križková, Ďuračková, Šandula, Sasinková, & Jánošík, 2001) and anti-tumoral activities (Križková et al., 2001; Lesage & Bussey, 2006; Liu, Wang, Cui, & Liu, 2008; Mantovani et al., 2008), and with emulsifying (Barriga, Cooper, Idziak, & Cameron, 1999; Cameron, Cooper, & Neufeld, 1988; Dikit, Maneerat, Musikasang, & H-kittikun, 2010) and prebiotic effects (Chen & Seviour, 2007; Laroche & Michaud, 2007). Consequently, they can be used as the basis of value-added products.

Brewing yeasts can be divided into two classes: top fermenting *ale* beer that uses *Saccharomyces cerevisiae* strains and bottom fermenting *lager* beer that uses *Saccharomyces pastorianus* or *S. carlsbergensis* strains, a natural allotetraploid hybrid from *S. cerevisiae* and *S. bayanus* (Ferreira, Pinho, Vieira, & Tavarela, 2010; Tamai, Momma, Yoshimoto, & Kaneko, 1998). Top- and bottom-fermenting brewing strains have similar surface ultrastructure, but different cell wall elasticity, degree of hydrophobicity, and polysaccharide properties. The surface of *S. pastorianus* is poor in proteins and much more hydrophilic than top fermenting species, explaining their tendency to sink, contrary to the floating properties of the *S. cerevisiae* due to their hydrophobic association with CO_2 bubbles (Alsteens et al., 2008; Dengis, Nélissen, & Rouxhet, 1995).

The information available relative to Saccharomyces cell wall polysaccharides concerns to *S. cerevisiae*. The β-glucans represent 50–60% of the cell wall composition, mannoproteins and chitin account for 35–40% and 1–3%, respectively (Klis et al., 2006; Lipke & Ovalle, 1998) and glycogen is between 1–23% (Aklujkar, Sankh, & Arvindekar, 2008; Arvindekar & Patil, 2002; Deshpande, Sankh, & Arvindekar, 2011; Northcote, 1953).

The β-glucans from *S. cerevisiae* are composed of β-(1→3)- and β-(1→6)-linked glucose residues (Bohn & BeMiller, 1995; Klis, Mol, Hellingwerf, & Brul, 2002; Lipke & Ovalle, 1998; Liu et al.,

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2008; Mantovani et al., 2008), in the proportion of 5:1 (Lipke & Ovalle, 1998; Liu et al., 2008). Glycogen, composed by α-(1→4)- and α-(1→4,6)-Glc linkages can be associated to β-glucans through covalent linkages, forming α,β-glucan complexes (Arvindekar & Patil, 2002; Kwiatkowski, Thielen, Glenney, & Moran, 2009). The *S. cerevisiae* mannoproteins are constituted mainly by mannose residues (89–96%) forming a highly branched and short chain structure, where most of the mannose residues are terminally linked and α-(1→2,6)-linked, together with (1→2)- and (1→3)-linked linear residues. Small amount of other sugar residues such as glucose, galactose, and xylose, linked to the protein moiety, are also present (Barriga et al., 1999; Jigami & Odani, 1999; Lipke & Ovalle, 1998).

The yield of extraction of *S. cerevisiae* carbohydrate polymers that can be calculated based on the data available in the bibliography is 14% for mannoproteins (Freimund, Sauter, Kappeli, & Dutler, 2003) and 22–26% for glucans (Freimund et al., 2003; Wang, Yao, & Wu, 2003). This allows to infer that the major part of these polymers remains insoluble in the cell wall matrix. To define the appropriate application for the valuation of this by-product, these structural features of this insoluble material should be solubilized and characterized.

In order to study the structural features of *S. pastorianus* glucans and carbohydrate moiety of its mannoproteins, a sequential extraction with water and alkali solutions with increasing concentrations (0.1 M, 0.5 M, 4 M, and 8 M KOH) was performed. In addition, the polysaccharides that remained unextracted upon this procedure were submitted to a partial acid hydrolysis and to α- and β-glucanase enzymatic hydrolysis. All fractions were analyzed concerning sugar and glycosidic linkage composition.

2. Materials and methods

2.1. Materials

BSY (*S. pastorianus*) was provided by the brewery Unicer Bebidas, SA, Portugal. The lot had 10% solids content, composed mainly by carbohydrates (49%) and proteins (27%) determined, respectively, as alditol acetates (section 2.6) and by the bicinchoninic acid method (Wrolstad et al., 2001). The carbohydrates were mainly glucose (71%) and mannose (19%), with lesser amounts of uronic acids (7%), arabinose (2%), xylose (1%), and galactose (<1%).

2.2. Sequential extraction of the BSY polysaccharides

The BSY polysaccharides were sequentially extracted with hot water and alkali solutions (Fleet & Manners, 1976; Liu et al., 2008). The suspension of BSY (64.5 g dry matter) polysaccharides were sequentially extracted with [1] hot water (1 L, 100 °C, 5 min); [2] 0.1 M KOH + 20 mM $NaBH_4$ (500 mL, 20 °C, 2 h); [3] 0.5 M KOH 20 mM + $NaBH_4$ (500 mL, 20 °C, 2 h); [4] 1 M KOH + 20 mM $NaBH_4$ (500 mL, 20 °C, 2 h); [5] 4 M KOH + 20 mM $NaBH_4$ (500 mL, 20 °C, 2 h); [6] 8 M KOH + 20 mM $NaBH_4$ (500 mL, 20 °C, 2 h). The alkali extractions were carried out with O_2-free solutions under nitrogen atmosphere. After each extraction, solubilized polymers were separated from the insoluble residue by centrifugation at 15,000 rpm, at 4 °C during 20 min. All extracts were acidified to pH 5 with acetic acid prior to dialysis (12–14 kDa cut off). The precipitates formed during dialysis of alkaline extracts were collected separately.

The final residue (FR1) that remains after the last alkali extraction (8 M KOH + 20 mM $NaBH_4$) was suspended in 400 mL of distilled water and the solution was acidified to pH 5 and dialyzed. The suspension was centrifuged and the supernatant solution (Sn-FR) was collected separately by centrifugation from the final residue (FR2). All extracts collected after dialyses were freeze-dried (Fig. 1).

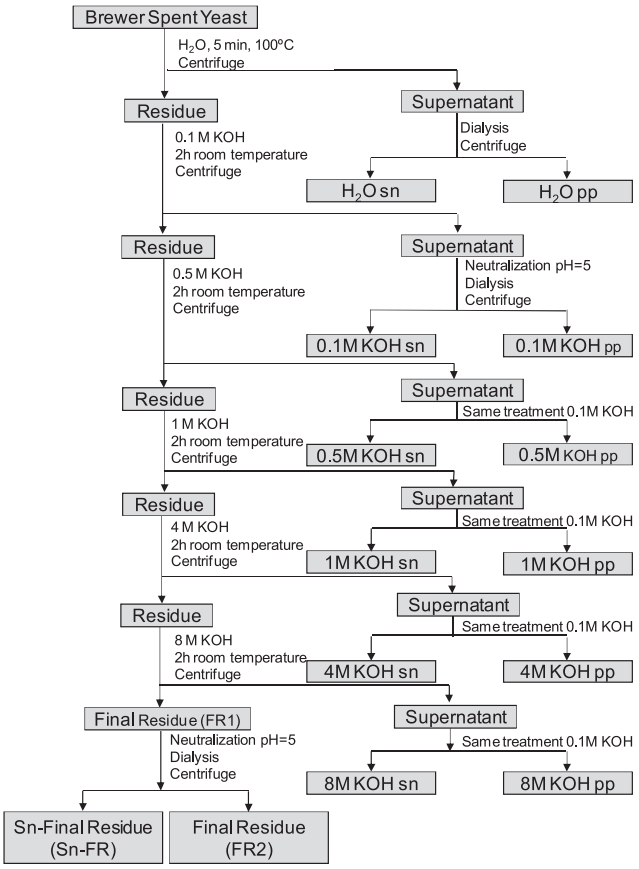

Fig. 1. Schematic representation of sequential extraction from brewer spent yeast polysaccharides.

2.3. Partial acid hydrolysis

The final residue was submitted to a partial acid hydrolysis according to the procedure described by (Nunes, Reis, Silva, Domingues, & Coimbra, 2008). The sample (10 mg) was suspended in 2 mL of TFA 250 mM, at 70 °C, during 30 min. The residue was then separated from the supernatant solution by centrifugation at 4000 rpm, during 20 min. The residue obtained was resuspended in TFA 250 mM and a new TFA treatment under the same conditions was performed. These steps were repeated until the complete solubilization of the material. For this propose seven cycles of partial acid hydrolysis were necessary. In each cycle the supernatant obtained was collected and evaporated under reduced pressure at 30 °C. The compounds obtained in each supernatant were fractionated by size exclusion chromatography on Biogel P-30 and submitted to methylation analysis.

2.4. Size exclusion chromatography

Preparative size exclusion chromatography was performed on a Pharmacia Biotech XK 26 chromatography column (42.5 cm × 1.6 cm column) containing Biogel P-30, using a flow rate of 0.08 mL/min. Exclusion and total volume were calibrated with blue dextran and glucose, respectively.

The freeze-dried material was dissolved in 1 mL of 50 mM sodium–phosphate buffer, pH 6.5, and loaded on the column previously equilibrated with the loading buffer. Fractions (2 mL) were collected and assayed for sugars with the phenol–H_2SO_4 method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956).

The fractions of each chromatographic peak were pooled and rotary evaporated, dialyzed and then freeze-dried.

2.5. Enzymatic hydrolyses

The final residue was treated with α- and β-glucanase in order to achieve the anomeric configuration of the (1→4)-Glc linkages. The enzymatic hydrolysis was performed according to Dikit, Methacanon, Visessanguan, H-kittikun, and Maneerat (2010).

For the treatment with α-glucanase, the final residue (10 mg) was suspended in 5 mL of 50 mM phosphate buffer pH 7.0. Then, the sample was hydrolyzed with α-amylase (EC 3.2.1.1) from *Bacillus subtilis* 62 U/mg (Sigma–Aldrich, Steinheim, Germany) by incubating at 25 °C for 2 h at 0.58 μg/mL. The reaction was stopped by boiling for 10 min and then the material was dialyzed and freeze-dried.

For the treatment with β-glucanase, the final residue (10 mg) was suspended in 5 mL of 20 mM acetate buffer pH 5.0. Then, the sample was hydrolyzed with a cellulase (EC 3.2.1.4) from *Aspergillus niger* 1.5 U/mg (Sigma–Aldrich, Steinheim, Germany) by incubating at 37 °C for 4 h at 1.2 μg/mL. The reaction was stopped by boiling for 10 min and then the material was dialyzed and freeze-dried.

2.6. Sugar and glycosidic-linkage analysis

Monosaccharides were released by a pre-hydrolysis in 0.2 mL of 72% H_2SO_4 (w/w) for 3 h at room temperature followed by 2.5 h hydrolysis in 1 M H_2SO_4 at 100 °C (Selvendran, March, & Ring, 1979). Neutral sugars were analyzed as their alditol acetates by gas-chromatography-flame ionization detection (Blakeney, Harris, Henry, & Stone, 1983). The hydrolysis of all fractions was performed in duplicate. Results used have less than 5% variability in the major component sugars. Uronic acids (UA) were quantified by a modification of the method of (Blumenkrantz & Asboe-Hansen, 1973) of the 3-phenylphenol colorimetric method.

Glycosidic-linkage composition was determined by gas chromatography-quadrupole mass spectrometry (GC-qMS) of the partially methylated alditol acetates based on Ciucanu and Kerek (1984), Nunes and Coimbra (2001) and Coelho, Rocha, and Coimbra (2011). 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 CH_3I (80 μL) during 20 min with stirring, following by a second addition of 80 μL CH_3I and stirring for another 20 min. $CHCl_3$/MeOH (1:1, v/v, 3 mL) was added, and the solution was dialyzed (membrane with a pore diameter of 12–14 kDa) against three lots of 50% EtOH. The dialysate was evaporated to dryness and the material was remethylated using the same procedure. The remethylated material was hydrolyzed with 2 M TFA (1 mL) at 120 °C for 1 h, and then reduced and acetylated as previously described for neutral sugar analysis (using $NaBD_4$ instead of $NaBH_4$). The partially methylated alditol acetates (PMAA) were separated and analyzed by gas chromatography–mass spectrometry (GC–MS) (Agilent Technologies 6890 N 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 with the injector temperature of 220 °C, during 5 min. The temperature program used was as follows: initial temperature was 45 °C, with a linear increase of 10 °C/min until 140 °C, followed by a linear increase of 0.5 °C/min until 170 °C, followed by a linear increase of 15 °C/min until 280 °C. 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 in a 1 s cycle in a full scan mode acquisition.

2.7. Mid infrared spectroscopy

Mid infrared spectra of the samples were obtained in an ATR Golden Gate FT-IR Bruker IFS-55 with a resolution of 8 cm^{-1} and 128 scans. Spectra were recorded in the absorbance mode from 4000 to 600 cm^{-1}.

The spectra were transferred via a *JCAMP.DX* format into the data analysis software package developed by Barros and Rutledge (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998; Coimbra, Barros, Rutledge, & Delgadillo, 1999) and a Principal Component Analysis (PCA) was applied to the FT-IR region of interest (1200–600 cm^{-1}).

3. Results and discussion

3.1. Sequential extraction of the BSY polysaccharides and structural characterization

The yields of the sequential extraction of BSY polysaccharides and the sugar composition of the extracts obtained are shown in Table 1. The extraction with hot water allowed to obtain 17% of the BSY carbohydrates. From these, 10% remained soluble in cold water and 7% precipitated after dialysis. The fraction that remained soluble in cold water was composed by 70% of carbohydrates, rich in glucose (52 mol%) and mannose (26 mol%). Table 2 shows that the glucose was present mainly (1→4)-linked (23.2 mol%) and (1→4,6)-linked (4.5 mol%), which are linkages typically associated to the presence of glycogen (Arvindekar & Patil, 2002; Deshpande et al., 2011; Northcote, 1953), easily extracted with hot water. The glucose characteristic of β-glucans, namely the (1→6)- and the (1→3,6)-linked, occurred in small amounts (1.2 and 1.9 mol%), showing that no significant contents of β-glucans can be extracted with hot water. The mannose present in this fraction was composed mainly by terminally linked (18.2 mol%), (1→2)-linked (11.1 mol%), and (1→2,6)-linked mannose residues (15.7 mol%), which are characteristic of the presence of mannoproteins (Coelho et al., 2011).

The extraction with 0.1 M, 0.5 M and 1 M KOH solutions solubilized low amounts of carbohydrates, while extractions with strong alkali solutions allowed to solubilize a highest amount, namely 7% with 4 M KOH and 19% with 8 M KOH. The majority of carbohydrates solubilized (16.5%) was obtained upon dialysis of the neutralized residue resultant from the 8 M KOH solution.

The polymeric material recovered with 0.1 M KOH was poor in carbohydrates (26%), which is in accordance with the higher abundance of ribose (46%), a sugar constituent of RNA. According to Table 1, the 0.1 M KOH and the hot water extracts recovered the majority of the nucleic acid derived material present in BSY. The material soluble in cold water and extracted with 0.5, 1, and 4 M KOH solutions accounted for 43, 37 and 41% of carbohydrates, respectively, mainly mannose (90, 81, and 85 mol%) and glucose (8, 11, and 13 mol%). The higher amount of mannose and the lower carbohydrate content are indicative of the presence of mannoproteins. This can be confirmed in Table 2 by the presence of terminally linked-Man (38.9 mol%), (1→2)-Man (18.8 mol%), (1→3)-Man (10.9 mol%), and (1→2,6)-Man (10.9 mol%). The proportion of (1→2,6)-Man to the total mannose residues showed one branching for each six residues. That glycosidic linkages obtained were consisting to what it is described for yeast mannoproteins. (Coelho et al., 2011).

Concerning the glucose present in the 4 M KOH extract, the (1→4) was the main linkage (16.8 mol%), followed by the terminally linked glucose (1.7 mol%), and in lesser amount the (1→3)-, and (1→6)-Glc, being respectively 0.6 mol% and 0.2 mol%. There

Table 1
Extraction yields and carbohydrate composition of the fractions obtained by the sequential extraction of brewer spent yeast.

Fraction	Yield (%) (m/m)	Yield of extracted carbohydrates (%)	Carbohydrates (mol %)						Total carbohydrate[a] (μg/mg)
			Rib	Ara	Xyl	Man	Gal	Glc	
H_2O, 100 °C									
sn	0.4	10.1	4	9	8	26	1	52	703
pp	0.5	7.0	9	4	4	16	1	65	364
sn residue			3	2	1	21	–	74	269
Residue			3	3	t	20	–	73	331
0.1 M KOH									
sn	5.4	2.9	46	1	1	32	–	19	258
pp	1.2	0.4	47	2	1	31	–	20	146
sn residue			2	4	t	22	–	72	215
Residue			1	1	t	17	–	81	430
0.5 M KOH									
sn	4.4	4.0	–	1	1	90	–	8	434
pp	4.2	0.6	–	2	2	59	–	39	69
sn residue			–	2	t	28	–	70	401
Residue			–	1	t	9	–	89	428
1 M KOH									
sn	3.6	2.7	–	5	3	81	–	11	371
pp	3.6	1.0	–	1	1	41	–	57	135
sn residue			–	2	–	43	–	55	404
Residue			–	2	–	4	–	94	505
4 M KOH									
sn	7.1	6.0	–	1	1	85	–	13	410
pp	4.5	1.1	–	4	–	29	–	67	121
sn residue			–	2	–	27	–	72	387
Residue			–	1	–	2	–	97	608
8 M KOH									
sn	1.2	1.9	–	1	–	65	–	34	760
pp	0.9	0.7	–	1	–	10	–	89	346
sn final residue	11.6	16.5	–	t	–	12	–	88	693
Final residue	20.9	24.2	–	1	–	1	–	98	566

t – traces.
[a] Values expressed in μg of anhydrous sugar by mg.

is also evident the presence of (1→4,6)-Glc (0.5 mol%) that has a lower branching degree comparatively to molar percentage of (1→4,6)-Glc of water extraction (4.5 mol%). The material soluble in cold water upon extraction with 8 M KOH was richer in sugars (76%), showing also higher content in mannose (65 mol%) and glucose (34 mol%). However, this extract was less rich in mannose and was richest in glucose than the other alkali extracts, resultant of a higher solubilization of glucans at this alkali strength. The material solubilized during the dialysis of the neutralized residue left after the 8 M KOH extraction was composed by 69% of sugars, mainly glucose (88 mol%), presenting (1→4) and (1→4,6) linkages (68.7 and 10.7 mol%, respectively) (Table 2), which is consistent with the presence of glycogen (Arvindekar & Patil, 2002; Deshpande et al., 2011; Northcote, 1953). Also, this fraction contained a small amount of mannose (12 mol%), presenting (1→2,6)-, (1→2)-, and terminally linkages in the proportion of 4.2, 3.8, and 3.1 mol%, respectively.

The final residue was composed only by glucose (98 mol%), showing that all mannoproteins were exhaustively extracted with the alkali solutions. Taking into account the amount of sugars in the final residue (57%), it was possible to calculate that 34% of the glucans present in the initial BSY sample remained insolubilized even after the strong alkali solutions used. According to the methylation analysis (Table 2), these glucans occur almost as (1→4)-linked (56.6 mol%), (1→3)-linked (16.7 mol%), (1→4,6)-linked (10.2 mol%), terminally linked (8.9 mol%), (1→6)-linked (4.2 mol%), and (1→3,6)-linked glucose (1.6 mol%), which are characteristic of the presence of glycogen (Arvindekar & Patil, 2002; Deshpande et al., 2011; Northcote, 1953) and β-glucans (Klis et al., 2006; Lesage & Bussey, 2006; Lipke & Ovalle, 1998).

3.2. Structural analysis of BSY insoluble polysaccharides

When the final residue was hydrolysed with cellulase, the (1→4)-linked glucose decreased from 56.6% to 39.8% (Table 2). This result suggests that approximately 30% of (1→4)-Glc present in the final residue was β-(1→4)-linked. In order to confirm the presence of α-(1→4) glucose in BSY, the final residue was also treated with α-amylase, allowing to observe a decrease from 56.6% to 25.2% of (1→4)-Glc (Table 2). Based on this result, it can be inferred that 55% of the (1→4)-glucose of the final residue was α-(1→4)-linked. It was not possible to identify the anomeric configuration of 15% of the (1→4)-linked glucose residues as they were not enzimatically hydrolysed.

According to the literature, BSY are mostly constituted by β-glucans, being the majority of the glycosidic linkages β-(1→3)-Glc, with a small amount of β-(1→6)-Glc. There is no reference to the presence of β-(1→4) glucans in BSY (Klis et al., 2006; Lesage & Bussey, 2006; Lipke & Ovalle, 1998). The presence of α-(1→4)- and α-(1→4,6)-linked glucose residues on yeasts, corresponding to α-glucans (glycogen), has been reported by Northcote (1953) and by Arvindekar and Patil (2002). The yeast glycogen occurs in two pools: (1) in the cytosol, on soluble form and (2) in the cell wall, on insoluble form, covalently linked to β-(1→3) glucans through β-(1→3,6) linkages. These linkages are essential to maintain the components in their right positions, as well as preventing dissolution/lixiviation of soluble polysaccharides (Deshpande et al., 2011). The occurrence of β-(1→4) glucans in BSY together with glycogen may contribute in a large extend to the high water insolubility of the BSY.

To validate the results obtained by the enzymatic assays, namely the presence of both α- and β-anomeric configurations of (1→4)-linked glucose in BSY, the final residue and the hydrolysates

Table 2
Glycosidic-linkage composition of fractions obtained by brewer spent yeast sequential extraction and final residue treated with cellulase and α-amylase.

Glycosidic linkages	Fractions (% mol)[a]					
	sn H_2O, 100 °C	sn 4 M KOH	Sn-FR	FR	FR-Cellulase	FR-α-Amylase
t-Ara	3.6	–	–	–	–	–
2-Ara	0.2	–	–	–	–	–
3-Ara	0.1	–	–	–	–	–
5-Ara	0.5	–	–	–	0.6	1.5
Total	4.4 (9)				0.6	1.5
t-Xyl	0.2	–	–	–	–	–
4-Xyl	4.2	–	–	–	–	–
Xylitol	2.1	–	–	–	–	–
Total	6.5 (8)					
t-Man	18.2	38.9	3.1	0.9	1.3	1.7
2-Man	11.1	18.8	3.8	–	0.5	–
3-Man	1.5	10.9	–	–	–	–
6-Man	2.6	0.8	–	0.3	–	–
2,6-Man	15.7	10.9	4.2		0.7	0.9
3,6-Man	0.6	–	–	–	–	–
Mannitol	0.1	–	–	–	–	–
Total	49.8 (27)	80.3 (85)	11.1(12)	1.2 (1)	2.5	2.6
t-Glc	5.5	1.7	8.2	8.9	13.0	16.4
3-Glc	2.1	0.6	0.2	16.7	21.5	31.2
4-Glc	23.2	16.8	68.7	56.6	39.8	25.2
6-Glc	1.2	0.2	1.0	4.2	7.6	8.7
2,3-Glc	–	–	–	0.3	0.3	0.7
2,4-Glc	–	–	–	0.2	0.2	0.1
3,4-Glc	–	–	0.2	0.2	1.0	0.4
3,6-Glc	1.9	–	–	1.6	2.2	3.1
4,6-Glc	4.5	0.5	10.7	10.2	11.9	10.0
Glucitol	0.5	–	–	–	–	–
Total	38.9 (53)	19.7 (13)	89.8 (88)	98.8 (98)	97.5	95.8

[a] Sugar analysis between brackets.

resultant from the enzymatic treatments were analyzed by mid infrared spectroscopy (FT-IR).

The FT-IR spectrum of the residue treated with α-amylase (Fig. 2a) shows the presence of a band at 890 cm^{-1}, characteristic of the presence of β-(1→4) glucose (Galichet, Sockalingum, Belarbi, & Manfait, 2001; Kacuráková & Wilson, 2001; Oliveira, Barros, Delgadillo, Coimbra, & Santos, 2009), confirming the existence of β-(1→4) glucans on BSY. The residue treated with cellulase shows a band between 870–840 cm^{-1}, which is within the interval characteristic of α-glucose (Galichet et al., 2001; Kacuráková & Wilson, 2001; Oliveira et al., 2009). The principal component analysis (PCA) was performed in the 1200–600 cm^{-1} spectral region

Table 3
Glycosidic-linkage composition of fractions by the different cycles of partial acid hydrolysis.

Glycosidic linkage	Fraction									
	1st cycle		2nd cycle		3rd–6th cycles		7th cycle			
	A_1	B_1	A_2	B_2	A_{3-6}	B_{3-6}	A_7	B_7	C_7	D_7
t-Ara	19.6	35.3	7.0	20.4	0.2	1.1	3.3	t	t	0.4
3-Ara	t	–	–	–	–	–	–	–	–	–
5-Ara	2.5	3.6	1.0	6.4	0.4	7.8	3.9	4.8	1.7	4.1
Total	22.1	38.9	8.0	26.8	0.6	8.9	7.2	4.8	1.7	4.5
t-Man	0.7	–	–	–	1.1	0.6	–	–	–	1.6
2-Man	–	–	–	–	0.4	0.7	–	–	–	1.0
2,6-Man	–	–	–	–	0.8	–	–	–	–	–
Total	0.7	–	–	–	2.3	1.3	–	–	–	2.6
t-Glc	11.7	9.9	11.4	17.6	11.5	8.9	6.4	17.3	7.9	6.7
3-Glc	–	–	0.7	–	–	2.5	–	–	–	0.8
4-Glc	52.5	44.0	59.8	37.6	64.9	68.1	85.6	75.2	90.4	68.8
6-Glc	4.3	4.2	11.1	18.0	6.3	3.0	0.8	2.7	–	1.2
3,4-Glc	–	–	0.5	–	0.7	1.0	–	–	–	1.2
3,6-Glc	–	–	–	–	1.2	0.2	–	–	–	–
4,6-Glc	7.0	2.9	8.5	–	12.6	6.1	–	–	–	2.5
Total	75.5	61.0	92.0	73.2	97.2	89.8	92.9	95.2	98.3	81.2

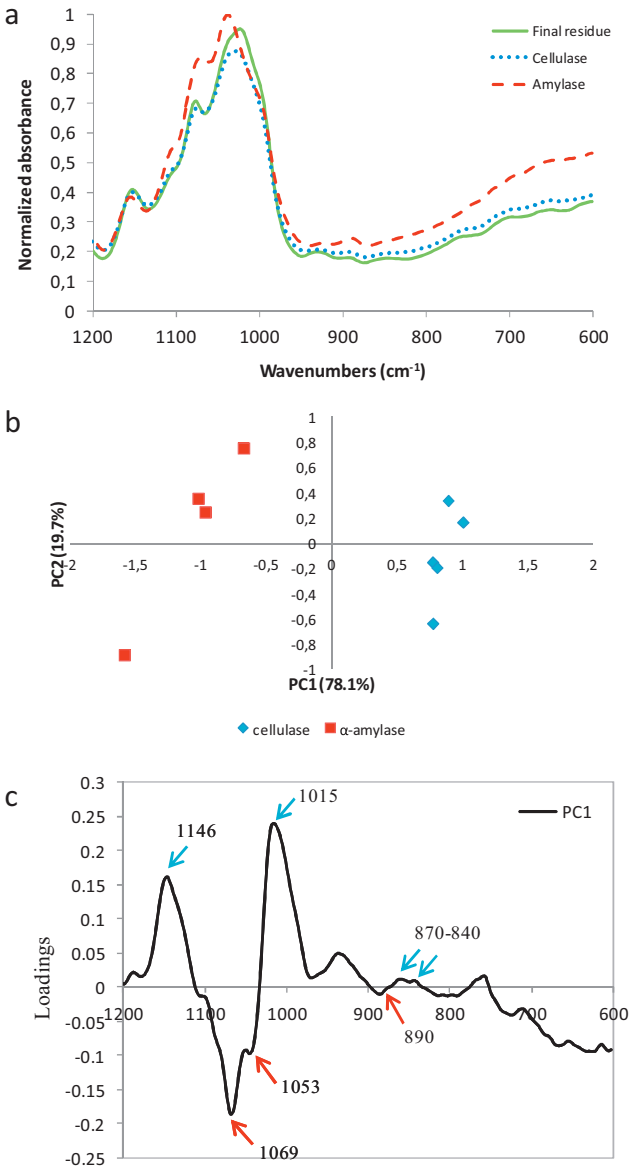

Fig. 2. (a) FTIR spectral in the 1200–600 cm^{-1} wave number region of final residue and final residue obtained after treatment with cellulase and α-amylase; (b) PC1 vs PC2 scores scatter plot of final residue hydrolysed with cellulase and with α-amylase; (c) PC1 Loading profile of FTIR region 1200–600 cm^{-1}. The blue arrows indicate peaks characteristics of α-(1→4) glucans and red arrows represent peaks characteristics of β-(1→4) glucans.

(Fig. 2b), PC1 represents 78.1% of total variability and shows a clear discrimination between the samples treated with cellulase and α-amylase. According to loadings profile (Fig. 2c), bands at 1146 and 1015 cm^{-1} on positive PC1 side are related to the presence of α-(1→4) glucans (Oliveira et al., 2009). Moreover, loadings plot shows two bands on PC1 negative side, at 1069 and 1053 cm^{-1}, characteristic of β-(1→4) glucans (Oliveira et al., 2009). It was also observed bands at 870–840 cm^{-1} (positive PC1) and 890 cm^{-1} (negative PC1), assigned to anomeric carbon (Tipson, 1968).

Pichia pastoris, from family Saccharomycetaceae, contains 5% of β-(1→4)-Glc linkages as insoluble material (Cocuron et al., 2007). It was also described the existence of β-(1→4) glucans on dimorphic pathogen *Sporothrix schenckii* cell wall (Previato, Gorin, Haskins, & Travassos, 1979).

Since *Pichia pastoris* and *S. pastorianus* belong to the same family, they might be sharing genes that are involved on the synthesis of β-(1→4) glucans, being the expression of these genes more evident on BSY. A hypothesis that eventually might justify these results is the osmotic and ethanol stress during brewing that could be enhanced by the several fermentative cycles that yeasts are submitted, conducing to the expression of these genes. Under fermentative stress conditions the expression of some genes was observed (James, Campbell, Donnelly, & Bond, 2003). In order to confirm the hypothesis that these stress conditions might activate some genes, and in this case, a gene involved on the synthesis of these cellulose-like polymers, it would be necessary study the polysaccharides from inoculum and from other yeasts strains submitted to fermentative stress.

Enzymatic hydrolysis and FTIR analyses showed and confirmed that final residue was constituted by α-(1→4)-Glc (55%) and β-(1→4)-Glc (30%), presented highly insoluble properties similar to cellulose. Thus, a partial acid hydrolysis was promoted in order to solubilize the glucans present in the final residue.

3.3. Partial acid hydrolysis and size exclusion chromatography

Following the treatment with 250 mM TFA, at 70 °C during 30 min, the soluble material was eluted through a size exclusion column with exclusion and inclusion limits of 40 and 2.4 kDa, respectively, allowing to obtain a fraction in the exclusion limit (A_1) (Fig. 3a). Also, the material included in the gel permeation medium was pooled and dialysed through 12–14 kDa membrane pore size to recover the polymeric material (A_2). Table 3 shows that fraction A_1 was composed mainly by (1→4)-Glc (52.5 mol %) and terminally linked arabinose (19.6%). Arabinose is usually associated to proteins and is very acid labile (Kolarová, Grešík, & Šmondrková, 1995). It is possible that it results from proteic material still present in the residue, which was composed by 56.6% of sugars (Table 1), which was confirmed by a chlorite treatment for oxidation of proteic material, allowing to obtaining a residue almost pure in carbohydrates. Although the arabinose only accounted for 1 mol% of the final residue (Table 1), the high amount of arabinose found in fraction A_1 may be justified by the small amount of material in this fraction, recovering all the arabinose. Together with the arabinose, this fraction contained also glycogen, as observed by the presence of (1→4)-Glc, (1→4,6)-Glc and terminally linked glucose. The fraction B_1 had the same composition of A_1, allowing to infer the occurrence of polymers with the same composition but with different molecular weights.

Because the majority of the material remained insoluble after the partial acid hydrolysis performed, a second treatment was performed under the same conditions and the material was recovered after elution through the BioGel P30 in two fractions (A_2 and B_2) (Fig. 3b). The glycosidic composition of this material was comparable to the previous fractions, except that the content of terminally linked arabinose decreased, with a concomitant increase of the (1→4)-Glc. To completely solubilize the material component of the final residue, it was necessary seven partial acid hydrolysis cycles. The material recovered after cycles 3–6 and that remained excluded from the BioGel column was pooled in fraction A_{3-6} and the material included in the gel permeation media was pooled in fraction B_{3-6} (Fig. 3c). In all these fractions (1→4)-Glc, (1→4,6)-Glc, and terminally linked glucose were the major linkages, allowing to infer the presence of glycogen. In the last cycle, a different chromatographic profile was obtained, with a negligible amount of size excluded material (Fig. 3d).

The major fraction (C_7) was composed exclusively by glucose, whereas (1→4)-Glc represented 90.4 mol% (Table 3).

The partial acid hydrolysis performed did not allow to obtain polysaccharides with (1→3)-Glc, although it accounted for

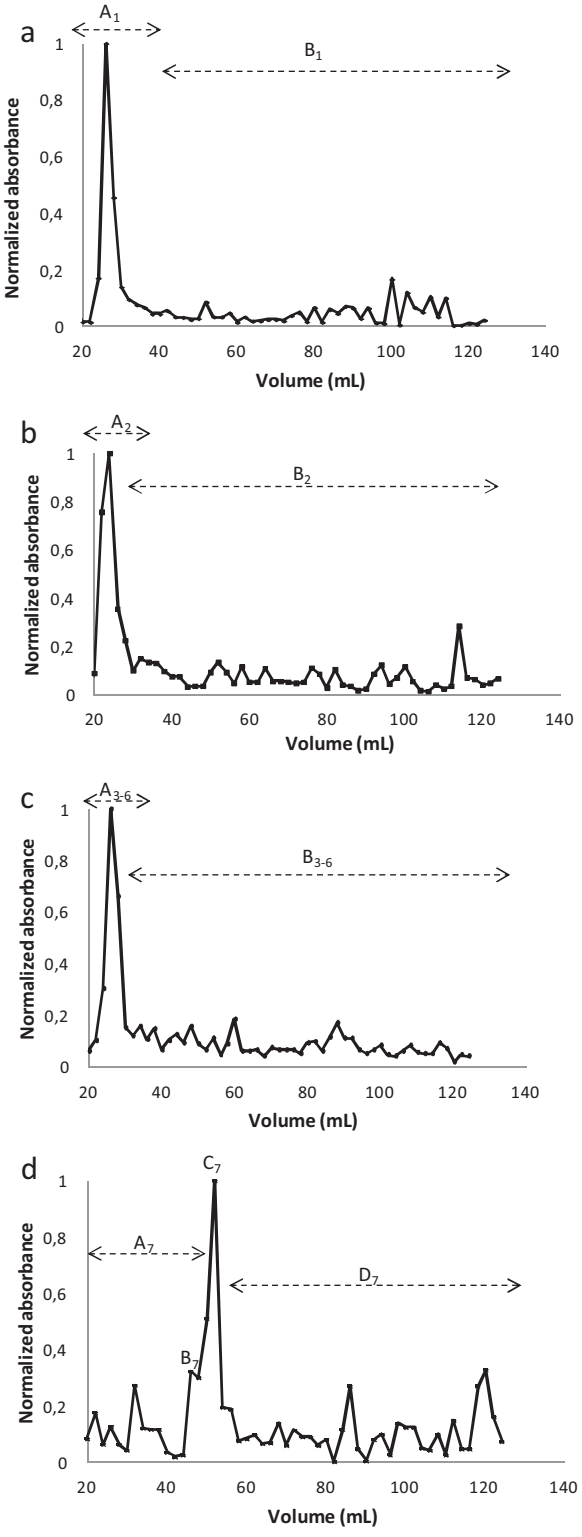

Fig. 3. Size-exclusion chromatography of the polysaccharides solubilized by partial acid hydrolysis. (a) 1st cycle, (b) 2nd cycle, (c) poll of 3rd to 6th cycles, and (d) 7th cycle. Letters A–D and the respective associated subscript numbers refer to subfractions of cycles 1–7.

16.7 mol% of the final residue. This shows that this material should have been lost during the dialysis step.

According to the literature, β-glucans with molecular weight ≥16 kDa are insoluble polymers (Mantovani et al., 2008). However, in this work, the glucans obtained had higher molecular weight (≥40 kDa), were soluble in water, and contained (1→4,6)-linked glucose residues, suggesting the presence of glycogen and not β-glucans.

4. Conclusions

The results presented allow to conclude that BSY polysaccharides are mainly constituted by insoluble material (83%). To solubilize them it was necessary to use strong alkali solutions, allowing to promoting a partial solubilization of only 34% of the mass. The residue left, which was resistant to strong alkali conditions, still accounted with 24% of total polysaccharides. The main polysaccharides present in BSY were mannoproteins, glycogen and β-glucans and the main glycosidic linkage found in this yeast was (1→4)-linked glucose that persists, and even increased, after exhaustive and strong alkali extractions. Beyond the (1→4)-Glc, BSY also shows, on minor amounts, (1→3)- and (1→6)-linked glucose branched with (1→4,6)- and (1→3,6)-Glc. The final residue has 56.6 mol% of (1→4)-Glc, presenting insoluble properties in water and alkali solutions, similar to those of cellulose. Cellulase and α-amylase treatments allowed to conclude that 30% corresponds to β-(1→4)-glucose and 55% to α-(1→4) glucose, these results were confirmed by FT-IR and chemometric analysis.

Partial acid hydrolysis of final residue was successfully applied in order to solubilize the water insoluble glucans. These results showed that it was possible to obtain soluble glucans with molecular weight ≥40 kDa. However, due to the presence of higher amounts of (1→4)- and (1→4,6)-linked glucose, contrarily to the residual amount of (1→3)-Glc, suggested that these soluble glucans are α-glucans (glycogen).

As far as we know, this is the first report on the occurrence of β-(1→4) glucose linkages in Saccharomyces. More studies in this subject should be done to detail these new structural features and explain its industrial relevance and valuation as soluble dietary fiber among other nutraceutical applications.

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