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Full Paper

Evaluation of the Components Released by Wine Yeast Strains on Protein Haze Formation in White Wine

Ellen C. Giese[a,b], Maria Chacón Ocaña[a], Nuria Barrajón Simancas[a], Ana I. Briones Pérez[a], Robert F. H. Dekker[c,d], Aneli M. Barbosa[e*]

[a]*Universidad de Castilla - La Mancha, Departamento de Química Analítica y Tecnología de Alimentos, Facultad de Químicas, 13071, Ciudad Real, Spain.*
[b]*Centro de Tecnologia Mineral (CETEM), Coordenação de Processos Metalúrgicos e Ambientais, 21941-908, Rio de Janeiro, Brazil.*
[c]*Universidad de Castilla - La Mancha, Instituto de Regional Investigación Científica Aplicada (IRICA), 13071, Ciudad Real, Spain.*
[d]*Universidade Tecnológica Federal do Paraná, Programa de Pós-Graduação em Engenharia Ambiental, Campus Londrina, Londrina-PR, Brazil.*
[e]*Universidade Estadual de Londrina, Departamento de Química, 86051-990, Londrina-PR, Brazil.*

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Abstract: Cultures of 23 indigenous yeast strains (22 *Saccharomyces cerevisiae* and a non-*Saccharomyces*, *Torulaspora delbrueckii*), isolated from fermentation tanks at wineries in Castilla-La Mancha (Spain), and were performed under winemaking conditions using a synthetic must. Polysaccharide analysis and turbidity assays were conducted so as to observe the capacity of the released mannoproteins against protein haze formation in white wine, and 3 strains (2 *Saccharomyces cerevisiae* and *T. delbrueckii*) were chosen for further experiments. The action of a commercial β-glucanolytic enzyme preparation (Lallzyme BETA®), and a β-(1→3)-glucanase preparation from *Trichoderma harzianum* Rifai were evaluated to release polysaccharides from the different yeast strains' cell walls. Protection against protein haze formation was strain dependent, and only two strains (Sc2 and Sc4) presented >50% stabilization in comparison to controls. Addition of β-glucanases did not increase the concentrations of polysaccharides in the fermentation musts; however, a significant increase of polymeric mannose (mannoproteins) was detected using an enzymatic assay following total acid hydrolysis of the soluble polysaccharides. Enzymatic treatment presented positive effects and decreased protein haze formation in white wine.

Keywords: β-glucanases; β-glucosidases; indigenous yeast strains; protein haze formation; mannoproteins; wine

1. INTRODUCTION

The cell walls of yeasts such as *Saccharomyces cerevisiae* are constructed from (1→3)-β-D-glucans containing branches of (1→6)-β-linked D-glucan, chitin,α-mannoproteins, proteins and glycogen. The (1→6)-β-D-glucan acts as a linker between (1→3)-β-D-glucan, chitin and the mannoproteins, and stabilizes the whole structure making the yeast cell wall insoluble [1]. The α-glucan (glycogen) present is rendered water-insoluble owing to its covalent linkage to cell wall β-glucans [2]. Yeasts can produce more mannoproteins during and after alcoholic fermentation, and these compounds can be released either during fermentation, or through enzymatic action during autolysis when the wines are left on the lee (*sur lee*) [3, 4].

Mannoproteins (mannose-containing glycol-proteins) are composed of protein chains with one to four residues of mannose linked by α-(1→2) or α-(1→3) linkages as reviewed by Pérez-Serradilla and Luque de Castro [5]. These glycoproteins can be obtained from different treatments (physical, chemical and enzymatic) of yeasts cell walls resulting in different molar mass and composition [6]. Mannoproteins have been applied to different biotechnological areas: as bioemulsifiers [7], the encapsulation of flavors [8] and mainly in winemaking [9, 10].

*Corresponding author. E-mail: anelibarbosa@gmail.com

Mannoproteins constitute a principal group of macromolecules found in wines that contribute to their stability and quality. These polysaccharides are capable of retaining aroma compounds and are considered to be protective colloids since they can prevent aggregation, flocculation and thereby haze formation, and the crystallization of tartrate salts [11-13]. The oenological function of parietal mannoproteins includes also ochratoxin A adsorption and enhancement of malolatic fermentation [14]. However, the principal and expected effect of mannoproteins is the interaction of these compounds with aromatic compounds naturally present in wines, modulating aroma intensity and volatility [15, 16]. Yeast mannoproteins have been described to protect wine against protein haze formation [17, 18]. Protein haze could be caused by proteins present in white wines that precipitate and aggregate to form an undesirable visible haze. Mannoproteins derived from yeasts exert a competitive mechanism with the wine proteins that are able to form insoluble aggregates of denatured protein. As the concentration of these wine components decreases, due to the presence of mannoproteins, the particle size of the haze decreases and thus visible turbidity declines [19].

Mannoproteins can be released during cell wall autolysis but this process may be accelerated by the action of exogenous hydrolytic enzymes. Different enzyme preparations have been used in wine making to hydrolyze pectic substances [20], increase the aroma [21], release anthocyanins [22], and extract cell wall components that affect protein haze formation [23]. β-(1→3)-Glucanases from *Trichoderma harzianum* have been used to improve the clarification and filtration of wines, and have also been described to be involved in grape fermentation and juice clarification [24].

In this paper, we evaluated the capability of 23 indigenous yeast strains (22 *Saccharomyces cerevisiae* and a non-*Saccharomyces* strain, *Torulaspora delbrueckii*, isolated from fermentation tanks from wineries in the state of Castilla-La Mancha (Spain), and previously identified by PCR-RFLP) [25] to protect white wines against protein haze formation. We also examined the effects of addition of two different β-glucanolytic enzyme preparations (a commercial Lallzyme BETA®, and one obtained from *Trichoderma harzianum* Rifai) on the release of cell wall components during the yeast strains growth.

2. MATERIAL AND METHODS

Yeast strains and cultivation

The 23 indigenous yeast strains were isolated from fermentation tanks at wineries in the state of Castilla-La Mancha (Spain) previously inoculated with active dry yeast [25]. Inoculum was prepared by growing the yeast strains in 125-mL flasks containing synthetic medium (glucose (50 g L^{-1}), fructose (50 g L^{-1}) and YNB (yeast nitrogen base, 6.7 g L^{-1}), pH 6.0) for 48 h at 28 °C and 180 rpm. The yeasts were cultivated anaerobically in 250-mL flasks equipped with septa and Müller valves containing SO_2-saturated water, and filled with 50 mL of the synthetic medium. The flasks were statically incubated at 28 ºC for 6 days. Supernatants containing the fermentation fluids (FS) were recovered after centrifugation (1000 x *g*/15 min). The sedimented yeast pellets obtained were re-suspended in 4 mL of Milli-Q water and transferred to 50 mL Erlenmeyer flasks and then autoclaved at 105 ºC for1 h followed by agitation at 150 rpm during 5 min. After this treatment, the yeast cells were centrifuged (1000 x *g*/15 min) and the supernatants recovered (autoclaved yeast cells, AYC). Both the AYC and FS extracts were filtered (0.45 μm filter Millipore), dialyzed against distilled water, and desalted on an EconoPac 10DG desalting-column (Bio-Rad Laboratories, Inc., USA). In the experiments employing β-glucanolytic enzymes (a commercial preparation from Lallzyme BETA®, Lallemand Inc., Canada; 4 mL (30 Units assayed against laminarin) [26], and a β-(1→3)-glucanase preparation from *Trichoderma harzianum* Rifai) [27], the enzyme was added to the 6-day-old yeast strain culture media. The cultures were then homogenized and incubated at 28 ºC for a further 6 days. Thereafter, the FS and yeast cells received the same treatment as described above to obtain the AYC. Control samples without any enzyme added were also included.

Chromatography analysis

The molecular sizes of different mannoprotein families were analyzed by size-exclusion using high performance liquid chromatography with UV detection (HPLC/UV) using a Polysep GFC-P4000 column (300 x 7.8 mm, Phenomenex Inc., USA). Samples (20 μL) were injected using an auto sample injector device. The mobile phase used was ultrapure water (Milli-Q system, Merck Millipore, Germany) at a flow rate of 0.2 mL/min and detection at 220 nm. Chromatographic separation was carried out at 25 ºC. The molecular weight distribution was determined using a calibration kit (Sigma-Aldrich, Switzerland) employing gel

filtration molecular weight protein markers (β-amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa), bovine carbonic anhydrase (29 kDa) and cytochrome c from horse heart, 12.4 kDa)) as standards. Chromatographic separation of standards was performed under the same conditions described above.

Mannose and glucose determination

In the acid hydrolysis experiments to determine soluble polysaccharides in the FS and AYC fractions, 25 µL of 8 M HCl was added to 175 µL of each sample to give a final concentration of 1 M HCl. The samples were then heated at 100 ºC for150 min, followed by neutralization with 200 µL of 2 M NaOH. The concentrations of mannose and glucose liberated were determined using an enzymatic kit (Sigma-Aldrich, Switzerland) for the determination of glucose and mannose in yeast cell preparations.

The principle of this UV-method consists of an initial phosphorylation of glucose by the enzyme hexokinase (HK) and adenosine-5'-triphosphate (ATP) and subsequent oxidation of glucose-6-phosphate (G-6-P) by the enzyme glucose-6-phosphate dehydrogenase (G6P-DH) with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction is stoichiometric with the amount of glucose and is measured by the increase in absorbance at 340 nm. In a second step, mannose-6-phosphate (M-6-P) is converted to fructose-6-phosphate (F-6-P) by phosphomannose isomerase (PMI); which is subsequently converted to G-6-P by phosphoglucose isomerase (PGI). The G-6-P formed reacts in turn with $NADP^+$ forming gluconate-6-phosphate and NADPH, leading to a further rise in absorbance at 340 nm that is stoichiometric with the amount of mannose.

Protein stability

Efficacy of mannoprotein preparations on protein stability and haze formation was evaluated on 500 µL samples, which were transferred to a 2.0 mL micro centrifuge tubes, and to this was added 5 µL of bovine serum albumin (BSA, 10 g L^{-1}) and 495 µL of a commercial white wine. In the control, samples were replaced by water. Each tube was then heated for 60 min at 90 ºC, followed by immediately cooling down to 4 ºC and left for 18 h. After this time, the tubes were maintained at room temperature for 20 min and the turbidity determined by measuring the absorbance at 490 nm. The percentage stabilization (%) was calculated as:

$$\%\ \text{protein stability} = \left[1 - \frac{(A_1 - A_2)}{A_0}\right] \text{x } 100\ \%$$

where A_1 was the absorbance of the sample, A_0 was the absorbance of the control, and A_2 was the absorbance of the blank without sample. All data was analyzed by one-way variance analysis (ANOVA), and significant differences ($p < 0.05$) between means were determined by Tukey's test.

Analytical methods

Reducing sugars were determined by the cupro-arsenate method [28, 29]. Total sugars were measured by the phenol-sulfuric acid method of Dubois et al. [30]. Yeast biomass was determined gravimetrically after recovering the cells by centrifugation (1000 x *g*/15 min) and drying at 70 °C to constant weight.

3. RESULTS AND DISCUSSION

Soluble substances from the cultivation of 23 indigenous yeast strains (22 *Saccharomyces cerevisiae* and a non-*Saccharomyces* strain, *Torulaspora delbrueckii*) were isolated from fermented media and quantified as total sugars. Yeast cells were treated as described above and their soluble polysaccharides were also quantified (Table 1). Total sugars present in the soluble fraction from yeast cells after autoclave treatment (AYC) was higher in strains Sc4 (101 µg mL^{-1}) and Sc6 (88 µg mL^{-1}). In the case of total sugars in the fermentation supernatants (FS), the major content of sugars was detected in cultures from strains Sc7 (111 µg mL^{-1}), Sc8 (110 µg mL^{-1}) and Sc9 (121 µg mL^{-1}). Treatments by autoclaving have been used successfully to release mannoproteins from yeast cell walls [6]. In addition, Núñez et al. [31], compared foaming properties between mannoproteins from enzymatic digestion of yeast cell walls as well as from thermal treatment, and demonstrated that the proteins remained stable after thermal exposure.

To identify the different families of glycoproteins present in the fermented media and in yeast cell walls, size-exclusion chromatography was employed and samples fractionated on a Polysep GFC-

4000 column. Figure 1 shows chromatograms of the profile of the molecular exclusion for protein standards of molecular weights ranging from 12.4 to 200 kDa (Figure 1a), and that obtained of a representative yeast cell wall autolyzate, strain Sc4 (Figure 1b); the latter suggesting mannoproteins were present with molecular weights of less than 200 kDa (peaks at approximately 200, 150, 80, 60 and 10 KDa). Generally, mannoproteins released during the stationary phase of yeast growth comprised MW's within the range of 50-500 kDa [15], which is in agreement with our results. Alexandre et al. [32] identified a 49 kDa hydrophobic mannoprotein from the cell wall of velum yeast, and reported that this compound was implicated in velum formation. Chalier et al. [33] observed mannoproteins ranging from 5 to 100 kDa, and concluded that differences between yeast strains had consequences in aroma quality of wines.

Table 1. Total sugars present in the soluble fraction from yeast cells after autoclave treatment (AYC), and in the fermentation supernatants (FS).

Yeast Strains	Total sugars (μg mL^{-1})	
	AYC	FS
Sc1	42.72 ± 3.19	53.31 ± 2.17
Sc2	26.73 ± 1.67	32.72 ± 5.75
Sc3	31.27 ± 2.51	45.53 ± 3.83
Sc4	94.23 ± 4.98	82.27 ± 5.58
Sc5	65.70 ± 5.25	70.28 ± 1.18
Sc6	81.23 ± 6.25	38.35 ± 2.79
Sc7	62.05 ± 3.59	113.49 ± 5.08
Sc8	51.03 ± 4.10	109.96 ± 3.94
Sc9	53.22 ± 3.26	113.49 ± 8.71
Sc10	11.36 ± 2.07	84.79 ± 4.47
Sc11	21.09 ± 4.59	68.89 ± 0.42
Sc12	10.92 ± 0.30	55.98 ± 4.47
Sc13	6.96 ± 0.24	35.89 ± 2.90
Sc14	26.75 ± 1.63	49.83 ± 5.38
Sc15	15.35 ± 0.56	45.81 ± 3.51
Sc16	23.50 ± 0.38	36.07 ± 7.38
Sc17	20.45 ± 2.14	44.27 ± 3.21
Sc18	19.12 ± 4.54	55.98 ± 6.40
Sc19	20.04 ± 1.82	50.62 ± 5.40
Sc20	42.81 ± 5.11	85.54 ± 4.90
Sc21	48.70 ± 3.90	52.11 ± 7.86
Sc22	33.09 ± 2.33	74.76 ± 1.26
S23	44.78 ± 2.55	83.57 ± 0.79

The mannose and glucose content of the soluble polysaccharides isolated was determined after total hydrolysis (acid) of each sample. In FS samples (Figure 2a), 10 strains presented a percentage of polymeric mannose >80%, and the highest mannose contents were presented by strains Sc17 (94%) and Sc22 (93%). Strains Sc9, Sc10, Sc19 and Sc20 presented lower contents of glucose and mannose (≈ 30%). The monosaccharide composition of the hydrolyzed polymers extracted from yeast cells' autolyzates showed a totally different profile (Figure 2b). Only strains Sc18 and S23 presented higher mannose contents (70 and 77%, respectively). Other strains presented a combination of glucose and mannose over 50%. Some studies demonstrated that mannoproteins from *S. cerevisiae* contained approximately 60 mannose units. On the other hand, Chalier et al. [33] observed that the mannose composition of the mannoproteins secreted by *S. cerevisiae* strains ICV D21 and ICV D80 at the end of fermentation ranged between 73 and 79%, respectively.

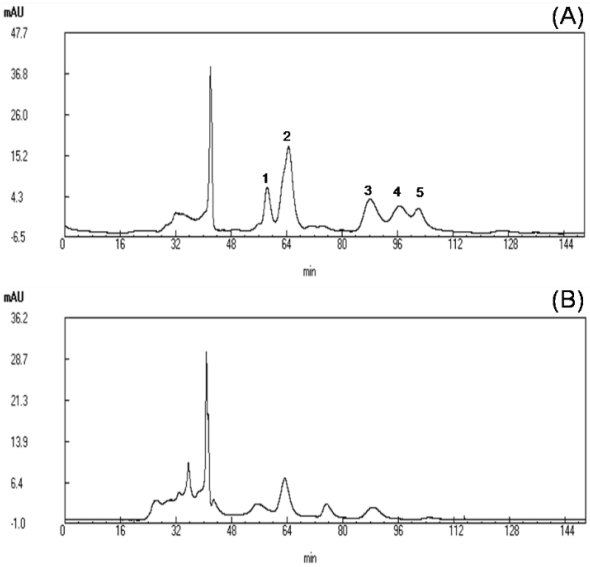

Figure 1. (A) Molecular gel exclusion chromatogram of the molecular weight markers [MW's: 200 (1), 150 (2), 66 (3), 29 (4) and 12.4 (5) kDa]. (B) Representative molecular gel exclusion chromatogram of an autolyzate from strain Sc4 after autoclave treatment. The peaks eluting before 48 min are due to the solvent.

Both extracts (FS and AYC) were added to a white wine in order to test the efficacy of the different glycoproteins obtained over the stabilization of the wine proteins. The turbidity assay on white wine was performed using bovine serum albumin as positive control since it is generally recognized that an increase

in mannoprotein content is related to the stabilization of protein hazes in wines [19]. The behavior of soluble polysaccharides in extracts FS and AYC for each of the yeast strains was different as can be observed in Figure 3.

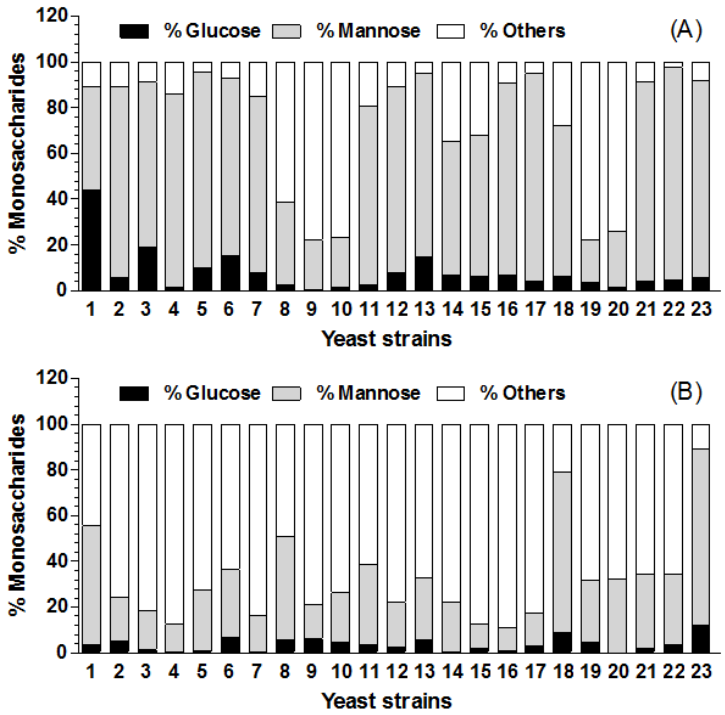

Figure 2. Composition of extracellular fluid (FS) (A), and yeast cells autoclaved (AYC) (B) after fermentation.

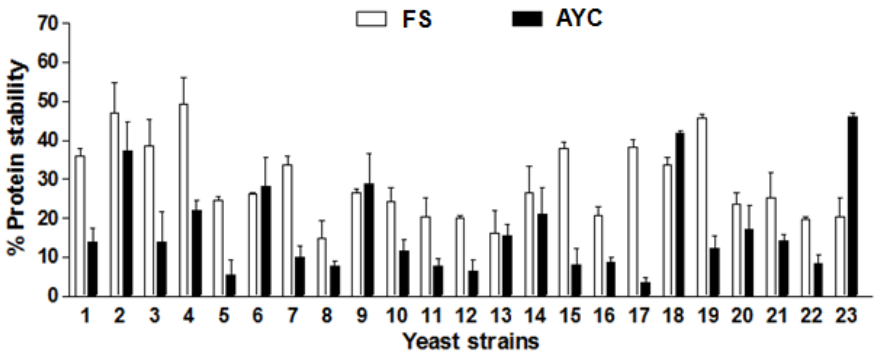

Figure 3. Test of effectiveness of compounds released from yeast cells on wine protein stabilization.

Maximum stability was achieved by the FS extracts of strains Sc2 and Sc4 (≈ 50%), which presented high amounts of mannose, and the lowest by strains Sc8 and Sc13 (≈ 18%). In the case of the yeast cell extracts, the maximum stability was observed in strains Sc18 and S23 (≈ 48%) that presented in its composition the higher mannose contents (70 and 77%, respectively) and minimum in strain Sc17 (≈ 5%).The difference between soluble polysaccharides found in FS and AYC from strains Sc6, Sc9, Sc13, Sc14 and Sc20 were not significant (p> 0.05). These data are in agreement with previously published results from recombinant *S. cerevisiae*, which was able to reduce turbidity between 18 to 35% [16]. However, it is important to highlight the importance of the appropriate selection of the yeast strain, in order to obtain successful protein stabilization. For the experiments under enzymatic treatment, yeast strains Sc4, Sc8 and S23 were selected because of the adverse profile observed in Figure 3. Strain Sc8 presented lower protein stability to both FS and AYC extracts, while Sc4 FS extract presented higher protein stability

(≈ 50%) while the S23 AYC-extract presented higher protein stability (≈ 48%).

β-Glucanases can act on cell walls and release mannoproteins and other carbohydrates during yeast autolysis, and storage of wines in contact with lees can enhance the content of mannoproteins as these oenological yeasts may produce extracellular hydrolytic enzymes [24, 34, 35]. An evaluation of two enzymatic preparations showing β-glucanase activity was conducted. A preparation containing 30 U of Lallzyme BETA®(commercial crude preparation of β-glucosidases from *Aspergillus niger*), and a fungal preparation of β-glucanases from *T. harzianum* Rifai were added separately to musts after 6 days of fermentation to verify their action on the release of polymeric materials from cell walls of the selected yeast strains, Sc4, Sc8 and S23. After this period, no further changes in yeast biomass and reducing sugars contents were observed, whilst reducing sugars increased almost 2-fold after enzyme addition in the culture medium from strain Sc4. Total sugars content did not increase after any treatments in comparison to the controls (data not shown). The addition of enzymatic preparations resulted in an increase of mannose contents in the hydrolyzed polysaccharides extracted from FS (Figure 4). No changes were observed in the glucose contents. For strain Sc4, mannose contents increased from 23% to approximately 30%, while for strain Sc8 an improvement of mannose from 18% to 34% was observed. The best results obtained were with strain S23, where an increase from 15% to 35% of mannose was detected in the treatment with Lallzyme BETA®, and 66% for the *T. harzianum* Rifai enzyme. In AYC treatments, the glucose and mannose content was higher than for the controls.

In the protein stability tests, the best effects were observed with strain Sc8, where addition of both enzyme preparations increased initial stability (20%) 2.4-fold for FS samples, and 2.8-fold (Lallzyme BETA®) and 3.2-fold (*T. harzianum* Rifai) for the yeast cell's soluble polysaccharides (Table 2). Generally, no correlation between the amounts of polysaccharides released and wine stability were found, as the latter depends upon the nature of the polysaccharides [36]. Charpentier et al. [37] observed that mannoproteins present in Sherry wine were partially hydrolyzed by β-(1→3)-glucanases liberated by yeasts from must. Palomero et al. [22] evaluated the action of β-glucanase activity on the cell wall from *S. cerevisiae* and *S. uvarum*, and the addition of enzymes resulted in more fragments with low molecular weights according to each of the three commercial β-glucanase preparations evaluated.

Table 2. Haze protective activity in white wine of enzymatic treated yeast cultures.

Strains	Treatment	% Stabilization	
		FS	AYC
Sc4	Control	47.84	27.34
	Lallzyme BETA®	46.83	37.74
	T. harzianum Rifai	49.96	41.64
Sc8	Control	19.60	13.37
	Lallzyme BETA®	47.12	36.10
	T. harzianum Rifai	47.87	41.14
S23	Control	31.84	52.99
	Lallzyme BETA®	43.43	58.26
	T. harzianum Rifai	48.17	59.07

FS, extracellular fluid; AYC, yeast cells autoclaved.

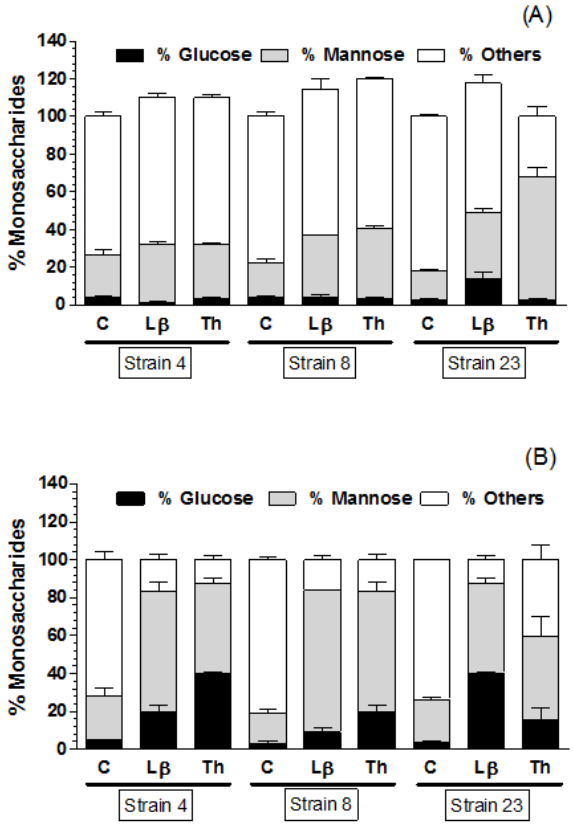

Figure 4. Composition of extracellular fluid (FS) (A) and yeast cells autoclaved (AYC) (B) after fermentation, and on addition of crude β-glucanase preparations. C, control; Lβ, Lallzyme BETA®; Th, fungal enzyme.

4. CONCLUSION

The components released during fermentation as well as those from yeast cell walls were found to be highly dependent upon each yeast strain examined, and showed different effects on protection of protein hazes in white wines. The addition of β-glucanase preparations could be important to improve beneficial effects of the released components from yeasts.

5. ACKNOWLEDMENTS

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