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Cell wall polysaccharides released during the alcoholic fermentation by *Schizosaccharomyces pombe* and *S. japonicus*: quantification and characterization

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Abstract

The present work demonstrates that yeasts belonging to the *Schizosaccharomyces* genus release a high quantity of polysaccharides of cell wall origin starting from the onset of the alcoholic fermentation. By the end of the alcoholic fermentation, all of the *Schizosaccharomyces* yeast strains released a quantity of polysaccharides approximately 3-7 times higher than that released by a commercial *Saccharomyces cerevisiae* yeast strain under the same fermentative conditions of synthetic juice. A higher content of polysaccharide was found in media fermented by *Schizosaccharomyces japonicus* with respect to that of *Schizosaccharomyces pombe*. Some of the strains evaluated were also able to produce high levels of pyruvic acid, which has been shown to be an important compound for color stability of wine. The presence of strains with different malic acid consumption patterns along with high polysaccharide release would enable production of naturally modified wines with enhanced mouth feel and reduced acidity. The chemical analysis of the released polysaccharides demonstrated divergence between the two yeast species *S. pombe* and *S. japonicus*. A different mannose/galactose ratio and a different percentage of proteins was observed on the polysaccharides released by *S. pombe* as compared to *S. japonicus*. Analysis of the proteins released in the media revealed the presence of a glycoprotein with a molecular size around 32-33 kDa only for the species *S. japonicus*. Mass spectrometry analysis of carbohydrate moieties showed similar proportions among the *N*-glycan chains released in the media by both yeast species but differences between the two species were also observed. These observations suggest a possible role of rapid MALDI-TOF screening of *N*-glycans compositional fingerprint as a taxonomic tool for this genus. Polysaccharides release in the media, in particular

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galactomannoproteins in significant amounts, could make these yeasts particularly interesting also for the industrial production of exogenous polysaccharide preparations.

Keywords

wine; polysaccharide; mannoprotein; galactomannoprotein; *Schizosaccharomyces pombe*; *Schizosaccharomyces japonicus*; MALDI-TOF

1. Introduction

The addition of commercial products containing polysaccharides derived from yeast cells wall to wine (in particular mannoproteins), is becoming a common practice during the winemaking process (Pozo-Bayón *et al.*, 2009). Positive enological properties associated with the polysaccharides and mannoprotein content of wine have been reported: reduction in protein and tartrate instability (Brown *et al.*, 2007; Dupin *et al.*, 2000; Gerbaud *et al.* 1997; Gonzalez-Ramos *et al.*, 2008; Moine-Ledoux and Dubourdieu, 1999; Lubbers *et al.*, 1993; Waters *et al.*, 1994), improvement of mouth-feel (Vidal *et al.*, 2004), increase of sweetness and roundness (Guadalupe and Ayestarán, 2007; Rosi *et al.*, 1998), decrease in astringency (Escot *et al.*, 2001; Quijada-Morín *et al.*, 2014), prevention of tannin aggregation and precipitation (Poncet-Legrand *et al.*, 2007), addition of complexity and aromatic persistence (Chalier *et al.*, 2007; Lubbers *et al.*, 1994), stabilization of the colour of red wines (Fuster and Escot, 2002; Riou *et al.*, 2002), and stability of the foam of sparkling wine (Vanrell *et al.* 2007). In contrast, Guadalupe and Ayestarán (2008), using commercial mannoprotein-rich preparations in Tempranillo must, observed a reduction of the content of wine proanthocyanidins and wine stable pigments. Thus the nature of the polysaccharide is an important factor in defining the potential impact on the wine.

Guadalupe *et al.* (2010) suggested that using yeasts during the alcoholic fermentation production that are able to release mannoproteins would be less costly than use of exogenous mannoproteins. However yeast release variable portions of mannoproteins and the typical yeast present during fermentation, *S. cerevisiae*, releases low amounts of polysaccharides, normally ranging from 50 to 150 mg/L (Rosi *et al.*, 2000). Therefore, an interesting alternative to the addition of these commercial exogenous polysaccharides-based products would be the use of yeasts able to release high quantity of polysaccharides during the alcoholic fermentation. Several studies have shown that non-*Saccharomyces* yeasts are generally characterized by the capacity to release a high quantity of polysaccharides (Comitini *et al.*, 2011; Domizio *et al.*, 2011a, 2011b, 2014; Giovani *et al.*, 2012; Gobbi *et al.*, 2013). In most of these analyses, the concentration of polysaccharides released by the non-*Saccharomyces* yeasts was much higher when compared with *S. cerevisiae* yeasts used as controls under the same conditions.

Schizosaccharomyces yeast strains have found application in winemaking because of their ability to reduce malic acid in grape juice and/or wine (Ciani, 1995; Dharmadhikari and Wilker, 1998; Gao and Fleet, 1995; Magyar and Panyik, 1989; Munyon and Nagel, 1977; Rankine, 1966; Silva *et al.* 2003; Snow and Gallender, 1979; Thornton and Rodríguez, 1996; Yokotsuka *et al.*, 1993). Recently, Benito *et al.* (2012, 2014) reported benefits in addition to

the demalication activity deriving from using *S. pombe* yeast in wine fermentation, such as the production of pyruvic acid and the possibility to reduce ethyl carbamate in wine, through the removal of its urea precursor, as a consequence of urease activity. Pyruvic acid seems to be of particular interest for the color stability of the wine. Indeed, a strong correlation between the amount of pyruvic acid released and the formation of vitisin A (a pyranoanthocyanin, a natural polyphenol found in grapes) has been observed (Morata *et al.*, 2003). Moreover, malic acid consumption by *Schizosaccharomyces* yeasts permit non-bacterial biological deacidification and averting production of amines. In this context, in order to avoid the risk of biogenic amines formation by lactic bacteria during malolactic fermentation, Benito *et al.* (2015) proposed mixed fermentation by using two different yeasts: *S. pombe*, to consume malic acid, and *Lachancea thermotolerans* to produce lactic acid to balance the acidity of wines produced from low acidity musts. *S. pombe* is also able to utilize D-gluconate as an alternative carbon and energy source for growth during glucose starvation (Tsai *et al.*, 1995). Therefore, the possibility to reduce gluconic acid in wines produced from rotten grapes by using *S. pombe* strains could represent an interesting approach. However, glucose addition rapidly inhibits gluconate degradation. For this reason, *S. pombe* use has been proposed as a way to remove gluconic acid from wine to be subsequently subjected to biological aging (Peinado *et al.* 2004). However, in following studies, Peinado *et al.* (2007, 2009), successfully used glucose-transport-deficient mutants of *S. pombe* mutant strains to reduce the content in gluconic acid also of grape juice obtained from rotten grapes. Thus this yeast has already been proposed for use in wine production. The ability to release polysaccharides with beneficial effects would also be of interest.

A study comparing the quantity of polysaccharides released at the end of alcoholic fermentation by eighty-nine non-*Saccharomyces* yeasts strains, found that the only *Schizosaccharomyces* strain tested released the highest level of polysaccharides (712 mg/L), about 5 times higher than the average of those released by three *S. cerevisiae* strains, used as controls (Romani *et al.*, 2010). In contrast, Giovanni *et al.* (2012) found that the only *S. pombe* strain tested released a quantity of polysaccharides (203 mg/L) lower than that released by three *S. cerevisiae* strains (ranging from 225 to 264 mg/L) when tested under the same conditions. Both of these studies used a single strain of *Schizosaccharomyces* and the differences could be due to strain effects or to the growth conditions of the studies.

The presence of a high quantity of polysaccharides from the beginning of the alcoholic fermentation process could promote the formation of polysaccharide-tannin complexes that stabilize the reactive tannins and in turn enhance the mouthfeel of red wine as well as enable other types of interactions of benefit to wine stability. Therefore, considering the elevated quantity of polysaccharides released by the strain of *Schizosaccharomyces* (712 mg/L), as previously reported by Romani *et al.*, (2010) and the variability in release reported in the literature (Giovanni *et al.*, 2012), in the present work we evaluated the ability of different strains belonging to the genus *Schizosaccharomyces* to release polysaccharides during the alcoholic fermentation.

2. Materials and methods

2.1. Yeast strains

Nine yeast strains belonging to the genus *Schizosaccharomyces* from the yeast culture collection of the Department of Agricultural, Food and Forestry Systems (GESAAF, University of Florence, Italy) and the Department of Viticulture & Enology University of California-Davis, (Davis) were used (Table 1). Six strains were ascribed to the species *Schizosaccharomyces pombe* and three to the species *Schizosaccharomyces japonicus* by D1-D2 domain analysis.

A commercial strain, Lalvin EC1118 (Lallemand Inc., Montreal, Canada), was used as reference strain for *S. cerevisiae* and for comparison determinations.

2.2. Fermentation trials

The fermentations were carried out in duplicate at 27°C in 200 mL Erlenmeyer flasks containing 150 mL of a synthetic grape juice medium “Minimal Must Medium” (MMM) (Spiropoulos *et al.*, 2000). The medium was sterilized by filtration. The flasks were inoculated at optical density of 0.1 (OD_{600 nm}), with 48-h pre-cultures grown in 10 mL of YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) (Oxoid Unipath Ltd, Hampshire, UK), at 25 °C in a roller drum. The levels of sugar and total assimilable nitrogen were 220 g/L and 208 mg/L, respectively. The assimilable nitrogen concentration was obtained by using 0.2 g/L of L-arginine and 0.5 g/L of ammonium phosphate. The flasks, continuously agitated at 150 rpm, were stoppered with silicone vent bungs (Ferm-Rite), allowing the CO₂ to escape. The flasks were weighed daily until the end of fermentation (defined as a constant weight for two consecutive days) to monitor the fermentation kinetics.

2.3. Biomass determination

Samples were taken from each flask during the alcoholic fermentation to monitor the growth kinetics by OD_{600 nm}. At the end of the fermentation, the dry weight biomass was determined gravimetrically by filtering 5 ml of sample from each culture on pre-weighed 0.45 µm nitrocellulose membranes. Membranes were washed twice with distilled water and placed in oven at 100 °C for 24 h before being weighed again, and the difference in starting and final weight was used to calculate biomass.

2.4. Analytical determinations of the fermentation products

Ethanol and residual sugars were determined by high performance liquid chromatography (HPLC) using an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA, USA) coupled with a refractive index (Hewlett Packard HP-1047A) detector. After filtration through 0.45 µm nitrocellulose membranes and after appropriate dilution in water, 20 µL of each sample was injected into the HPLC apparatus. Isocratic separation was performed at 75 °C on a (300 × 7.7 mm) Hi-Plex H column (Agilent, Palo Alto, CA, USA). The mobile phase was 4 mM H₂SO₄ at a flow rate of 0.4 mL/min. The compounds were identified and quantified by comparisons with external calibration curves for each compound.

The areas of the peaks of interest were integrated using the ChemStation Data Analysis System, version A.10.2 (1757) (Agilent, Palo Alto, CA, USA). All the analyses were carried out in duplicate.

2.5. Polysaccharide purification

After thirteen days of alcoholic fermentation, the fermented juice was centrifuged (8000 g, 4°C, 10 min) to separate the yeast cells. The purification of the polysaccharidic fraction was performed by ethanol precipitation, as previously described (Domizio *et al.*, 2014). The dried pellets obtained during this step were then rehydrated with nanopure water for the successive analytical determinations.

2.6. Polysaccharide characterization

2.6.1. Monosaccharide composition—After acid hydrolysis of the rehydrated pellets, as previously reported (Domizio *et al.*, 2014), the hydrolyzed samples were neutralized with Ba(OH)₂, as proposed by François (2006), with some modifications. Briefly, sulfate ions were precipitated by the addition of saturated Ba (OH)₂ until neutral pH was reached (checked with pH paper). The total volume was adjusted with nanopure water to 1 ml and BaSO₄ precipitates were pelleted by centrifugation at 3700 g for 5 min. The supernatant was removed and left at 4°C overnight to allow precipitation of the remaining sulfate ions which was removed by a second centrifugation at 3700 g for 5 min. The monosaccharide composition of the polysaccharides was then determined by high-performance anion exchange chromatography with pulsed amperometric detection (Thermo Scientific HPAEC-PAD ICS-5000, Sunnyvale, CA) equipped with a detector/chromatography module including a pulsed amperometry electrochemical detector, an electrochemical cell with a disposable gold working electrode, a pH-Ag/AgCl reference electrode, an autosampler and a single pump. After filtration through a 0.22- μ m membrane (Pall, Port Washington, NY) and after appropriate dilution in nano pure water, 25 μ L of each sample was injected into the CarboPacPA200 analytical column (3 \times 250 mm, Dionex, Sunnyvale, CA) and a CarboPacPA200 Guard Column (3 \times 50 mm, Dionex). The separation was performed with isocratic elution (18 mM NaOH; 1.2 mL/min; 25 °C). Quantification of the monosaccharides was performed in comparison with an external calibration curve with concentrations of each compound ranging from 1 to 60 mg/L.

2.6.2. Protein quantification—The protein concentration was determined by dye-binding Bradford assay (Bradford, 1976) using Bio-safe Coomassie blue G-250 dye reagent (Bio-Rad). Bovine serum albumin (BSA) (Sigma-Aldrich) was used to constitute the external calibration curve.

2.6.3. Protein profiling by gel electrophoresis—Electrophoresis was performed by using 10% Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) to characterize the released proteins and mannoproteins. Based on the protein concentration, the rehydrated pellets obtained from the precipitated polysaccharides were further diluted with ultrapure water to have a protein concentration ranging from 0.2 to 0.3 mg/mL. Then 20 μ L of the diluted sample was treated with 6.65 μ L of 4X Laemmli buffer (Bio-Rad) and 2.75 μ L of 1M dithiothreitol (DTT) (Acros Organics) and heated at

95 °C for 5 min. After this treatment, 15 µL of the mixture was loaded onto the gel. The CandyCane molecular weight standard (a mixture of glycosylated and un-glycosylated protein standards) (Invitrogen, Carlsbad, CA, USA) at a concentration of 750 ng/lane, was used. In addition, 10 µL of Blue precision plus protein standard (Bio-Rad) was loaded.

The SDS-PAGE was developed in a Mini Protean II apparatus (Bio-Rad) at 45 V within the stacking gel and at 104 V in the developing gel (until the tracking dye bromophenol blue ran off the gel). Gels were stained with a Pro-Q Emerald 488 glycoprotein stain (Molecular Probes, Inc., Eugene, OR, USA) to detect the glycoproteins according to the manufacturer's protocol. The resulting green-fluorescent signal produced by the Pro-Q Emerald 488 dye was visualized by a TyphoonTrio Plus variable mode imager (GE Healthcare) with a 488 nm excitation laser and a 520 nm long-pass emission filter. Following detection of glycoproteins, total protein profiles were subsequently displayed using the Bio-Safe Coomassie G-250 stain (Bio-Rad).

2.6.4. N-glycan isolation and MALDI-TOF analysis—Analysis of the *N*-glycans released by PNGase F from the glycoprotein was performed as previously described (Domizio *et al.*, 2014) in order to characterize the mannoproteins released in the media at the end of alcoholic fermentation by the different *Schizosaccharomyces* yeast strains. The *N*-glycans released from the pool of proteins were detected according to the method reported in Bordiga *et al.* (2012), with some modifications (Domizio *et al.*, 2014). MALDI-TOF analysis was performed on sodiated species in the *m/z* scan range from 1600 Da to 4000 Da on a Bruker Ultraflex II MALDI-TOF (BrukerDaltonics, Bremen, Germany) in positive ion mode. Each sample was spotted in triplicate from biological duplicate extractions and the mass spectra were collected by using 100 laser shots. Resulting mass spectra were analyzed using the Flex-Analysis software from Bruker Daltonics (Bremen, Germany).

3. Results and Discussion

3.1. Fermentation performance

Nine different *Schizosaccharomyces* yeast strains, belonging to two different species, *S. pombe* and *S. japonicus* (Table 1), were selected to be evaluated for polysaccharide production over the course of fermentation using a synthetic grape juice in comparison with a commercial strain of *Saccharomyces*, Lalvin EC1118. The *Schizosaccharomyces* yeasts selected were isolated from wines of different origins. Based on the quantity of CO₂ released during the alcoholic fermentation (Fig. 1), it was possible to distinguish different fermentative behaviors across the strains. The three strains belonging to *S. japonicus* were characterized by the low fermentative activity, reaching the maximum quantity of CO₂ produced (~ 4.5g/100 mL) just after two days (strain #1) or after five days (strains #8 and #9) of alcoholic fermentation. After thirteen days of fermentation, the residual sugar concentration ranged between 11-12% (w/v) and the ethanol level was roughly 6% (v/v) (Fig. 2). In contrast, among the six strains of *S. pombe* two different fermentation patterns were observed (Fig. 1). The first group of *S. pombe* strains (# 3, #4 and #7) showed an exponential increase in fermentation between days 1 and 3 of the fermentation after which they were characterized by a slower constant production of CO₂ until day 9 when

fermentation rates increased again for strains #3 and #4 but stayed constant for strain #7. *S. pombe* strain #7 overall presented the lowest rate of CO₂ loss but it still exceeded that of any of the *S. japonicus* strains. The second group of *S. pombe* strains (#2, #5 and #6), presented fermentation profiles more similar to that of the strain of *S. cerevisiae* used as a reference with fermentation rates decreasing sooner between the third and fourth day of fermentation. After 10 days of fermentation, strains #5 and #6 produced the same quantity of CO₂ as that released by the *S. cerevisiae* strain. Different fermentative behaviors among *S. pombe* strains have been previously reported (Benito *et al.*, 2012, 2014) that also describe strains with slow and strong fermentative ability. The analysis of glucose and fructose consumption demonstrated that all the *Schizosaccharomyces* strains are glucophilic, similar to *Saccharomyces* yeasts (Figure 2).

An inter and intra specific variability of the demalication activity was also observed among the *Schizosaccharomyces* strains. In particular, within the species *S. japonicus*, strains #8 and #9 showed a similar trend in malic acid consumption, with a 73-80% reduction in malate levels after five days of alcoholic fermentation. However, 92% of the malic content was reduced by strain #1 within this same time frame. Among the yeast strains belonging to the species *S. pombe*, strain #2 showed no malic consumption. In contrast, almost a complete reduction of malic acid was observed after five days of fermentation for strains #5 and #6, and after eight days for strain #7. Strains #3 and #4 reduced the malic content by approximately 55% after 5 days of fermentation and no further decrease was then observed. Similar variability among *Schizosaccharomyces* strains in the malic acid degradation has been observed previously by other authors (Gao and Fleet, 1995; Magyar and Panik, 1989; Silva *et al.*, 2003; Snow and Gallander, 1979; Taillandier *et al.*, 1995; Thornton and Rodriguez, 1996). Some strains appeared to consume malate simultaneously with glucose and fructose (strains #5 and #6) consuming all substrates by day ten of the fermentation while others showed a preference for one substrate over the other: malate for strain #1 and glucose and fructose for strain #2, and malate and glucose over fructose for strains #8 and #9 (Figure 2). Such variability could be advantageous during wine production if malate depletion was desired for example or retention of sugar substrates for *Saccharomyces*.

With respect to acetic acid, strains #4, #5 and #6 of *S. pombe* produced the highest levels ranging from 0.4 to 0.5 g/L. All the other strains of *Schizosaccharomyces* produced a lower concentration of acetic acid, ranging from 0.17 g/L to 0.34 g/L, which was consistent also with their lower fermentation capacity. High levels of acetic acid (ranging from 0.86 g/L to 1 g/L) have been often reported for *S. pombe* yeasts during the alcoholic fermentation of grape juice and represents one of the major problems observed among these types of yeast (Benito *et al.*, 2012). In order to find strains with positive enological potential, Benito *et al.* (2014) evaluated one hundred strains of *Schizosaccharomyces* and found only five strains producing less than 0.4 g/l of acetic acid, about 20% of strains producing a concentration of acetic acid ranging from 0.3 g/L to 0.6 g/L and most of the strains producing a quantity around 0.8 g/L. In a recent study, Benito *et al.* (2016) evaluated 75 strains of *S. pombe* and found that only 8% of the strains produced less than 0.4 g/L of acetic acid, while the majority of the strain (65%) produced acetic acid ranging from 0.4 g/L to 0.7 g/L and 15 % between 0.8 and 1.4 g/L of acetic acid. In general, we observed lower levels of acetic acid production in the

present study; however this may be impacted by medium composition and further studies using actual winemaking conditions and juices are warranted.

All *S. pombe* strains except strain #8 produced more pyruvic acid than the *Saccharomyces* yeast during the course of fermentation. In particular, strain # 6 produced 0.43 g/L of pyruvate after five days of fermentation, six times higher than that produced by the *S. cerevisiae* strain (0.072 g/L) and this level remained high at the end of fermentation (Figure 2). A lower quantity of pyruvic was found in the media fermented by strain #9 of *S. japonicus* (0.066 g/L) and pyruvate was undetectable at day 14. A high spike in pyruvate formation was observed at eight days of fermentation, for strain #1 of *S. japonicus*. The results here obtained are consistent with those previously published (Benito *et al.*, 2012, 2014), where strains of *S. pombe* were reported to be able to produce levels of pyruvic acid ranging from 0.2 to 0.42 g/L during the alcoholic fermentation.

High variability among the *Schizosaccharomyces* strains was also observed for the final glycerol content, ranging from 8.3 g/L to 10.5 g/L for the *S. japonicus* strains and from 9 g/L to 11.4 g/L for the *S. pombe* strains (Fig. 2) and was not significantly different with respect to glycerol production by the *S. cerevisiae* strain (9.9 g/L). Similar levels of glycerol have been reported by Benito et al (2016) with values ranging from 8.14 to 8.9 g/L. Also in this case, the values were similar to those obtained for the two *S. cerevisiae* strains used as reference.

3.2. Polysaccharide release and cell growth

The release of polysaccharides for each strain was compared to the optical density observed at the time of assay (Figure 3). Four strains were flocculent, strain #2 and all three *S. japonicus* strains (#1, #8, and #9). However, *S. japonicus* strains started to de-flocculate after about five days of fermentation. Hence, when the strains were flocculent, it was not possible to obtain accurate assessments of biomass using optical density. All *Schizosaccharomyces* strains started growth immediately upon transfer and released polysaccharides throughout fermentation with the greatest release ranging from 70% and 86% with respect to the final content occurring before day 3 suggesting that release of polysaccharides in these strains accompanies active growth and cell division. A slightly lower percentage of polysaccharide (63% -67%) was detected for strains #2, #6 and #7. The flocculent strains also released polysaccharides immediately upon transfer to the medium with the bulk of the release occurring by day 2. At the end of the alcoholic fermentation, the level of polysaccharides detected in the media ranged from 542 mg/L to 786 mg/L for all strains of *S. pombe* with the exception of strain # 6 (900 mg/L). The level of polysaccharides for the three strains of *S. japonicus* ranged from 930 mg/L to 1386 mg/L. In any case, at the end of the alcoholic fermentation all the *Schizosaccharomyces* yeast strains released a higher quantity of polysaccharides as compared to the commercial *S. cerevisiae* yeast EC1118 strain and, specifically, 2.6 - 4.3 times higher for *S. pombe* yeast species and 4.4 - 6.6 times higher for *S. japonicus* yeast species.

At the end of the alcoholic fermentation, the amount of polysaccharides released in the media was normalized to cell biomass (polysaccharides / cell dry weight (mg/g)) (Fig. 4). This ratio was also calculated for the three strains of *S. japonicus*, which was possible

because after the fifth day until the end of the fermentation, cells were no longer flocculent and dry weight could be determined. Strain #2 of *S. pombe* remained flocculent throughout and as a consequence it was not possible to determine the polysaccharide/dry weight ratio. The yeast strain of *S. cerevisiae* EC1118 produced the lowest ratio (68 mg/g), confirming our previous results (Domizio *et al.*, 2014). In contrast, the three strains of *S. japonicus* produced the highest amounts of polysaccharides, ranging from 265 to 411 mg/g, while all the *S. pombe* strains produced an intermediate quantity, ranging from 98 to 208 mg/g (Fig. 4).

High levels of polysaccharides released within the first days of alcoholic fermentation have been observed for other non-*Saccharomyces* yeasts and have been related to the cell response to external stimuli, causing cell wall stress, or to the cell growth (Domizio *et al.*, 2014). In the budding yeast *Saccharomyces* polysaccharide release during the alcoholic fermentation has been related to the production of beta-glucanases during the growth phase which serves to weaken the cell wall and allow bud emergence (Fleet, 1991). Yeasts belonging to the genus *Schizosaccharomyces* divide instead by binary fission with the formation of a septum between the cells. This septum is a three-layered structure composed of a middle layer named the primary septum, bordered at both sides by the secondary septum (Johnson *et al.*, 1973; Sipiczki, 2007). Cell separation represents the last step of cytokinesis and requires degradation by glucanases of the primary septum and the contiguous cell wall, which presents an outer layer rich in galactomannoproteins (Cortés *et al.*, 2012; Horisberger and Rouvet-Vauthey, 1985; Pérez, and Ribas, 2004). Therefore, the polysaccharides found in the media likely derive from the cell wall degradation by the action of glucanase during the separation process between the daughter cells (Dekker *et al.*, 2004; Garcia *et al.*, 2005) thus explaining the association of growth and polysaccharide release. The higher level of polysaccharide released in the media by all the *Schizosaccharomyces* yeast strains, compared to that released by the *S. cerevisiae* strain, could be due to their osmophilic character and, as consequence, characterized by a cell wall richer of resistance elements, likely polysaccharides, useful to support high osmotic pressures (Kopeckà *et al.*, 1995). Moreover, a greater proportion of the cell wall surface is likely involved during the binary division of *Schizosaccharomyces* yeast with respect to that occurring during budding. Indeed, the erosion of outer surface of old cell wall covers the entire cell diameter around the edge of the primary septum, while in budding yeast it concerns only the thin mother-bud neck.

Based on this last observation, we can also speculate that the higher content of polysaccharide found in media fermented by *S. japonicus* with respect to that of *S. pombe* is due to the fact that *S. pombe* is characterized by a smaller cell size with respect to *S. japonicus* (Hironori, 2014). Moreover, the higher level of polysaccharide after the third day of fermentation released by *S. japonicus* versus *S. pombe*, could also be due to the faster generation time characterizing yeasts belonging to the species *S. japonicus* as compared to *S. pombe* (Klar, 2013).

The increased polysaccharide release of *S. japonicus* could alternately be a consequence of the sexual cycle that this species undergoes during normal growth. Microscopic observation of *S. japonicus* cells during the alcoholic fermentation revealed that after two days most of

the cell had already started a conjugation process, resulting in the ascospore formation (Fig. 5). Indeed, in fission yeasts meiosis proceeds after karyogamy and zygotes give rise to asci immediately. After a few days the ascus wall surrounding the ascospores dissolves, releasing the ascospores into the media. All the three strains of *S. japonicus* started to flocculate after one or two days of fermentation and de-flocculate after about five days of fermentation. Calleja *et al.* (1971, 1977) report that the flocculation phenomenon in *S. pombe* represents an initial and essential step in the process of sexual conjugation. After cell flocculation, covalent bonds (copulation) are formed between the two cells in order to stabilize the flocs. Following floc formation a series of events occur (conjugation tube initiation, cross-wall formation and 2-layer cross-wall erosion, expansion of contact area, conjugation-tube expansion, perforation of 2-layer cross wall, crimping at site of union) permitting nuclear migration and fusion, with the consequent zygote formation. At the end of this process, almost all of the cross-wall is gone and a discontinuity along the site of union between the walls of copulating cells remains until the walls are repaired during a de-differentiation process of site of union. This last step consists in the deposition of new wall material in the gap at the periphery of the contact area of the walls or in the removal of the protein-rich outer surface which fills the gap (Calleja *et al.*, 1977). In this latter case, the galactomannoproteins, participating in the cell wall structure, could be released, contributing to further increase the quantity of polysaccharides in the media. After cell fusion and zygote formation, cells normally start the sporulation process that ends in the formation of asci containing mature ascospores (Tanaka and Hirata, 1982). After that, as final step of the sexual cycle, the ascus wall surrounding the ascospores dissolves, releasing the ascospores and permitting their dispersion into the media. The spore release from the ascus is assured by the action of hydrolytic enzymes such as α -glucanase and β -glucanase (Dekker *et al.*, 2007; Dedo *et al.*, 2009). Since the ascus wall corresponds to the cell wall of conjugating haploids or to the cell wall of the diploid cell, it is predicted to have a composition similar to that of the vegetative cell wall. Therefore, the high content of polysaccharide released in the media by the three strains of *S. japonicus* could be a natural consequence of the cell conjugation process as well as of the spore release from the ascus.

Further, Calleja *et al.* (1977) observed that, during the conjugation process, a significant portion of cells usually lyse spontaneously, probably as a consequence of faulty fusion or uncontrolled lytic activity during cross-wall removal. This spontaneous lysis of the cells could be another possible reason to explain the high level of polysaccharide found in the media fermented by *S. japonicus*. In contrast strain #2 of *S. pombe*, which flocculated during the entire time course of fermentation did not display zygote formation after cell aggregation. Therefore, this was likely a non-sexual flocculation.

The conjugation process of the three strains of *S. japonicus* started within the first two days of fermentation (as observed via microscopic observation) of the synthetic grape juice. Since *S. japonicus* cells usually enter into meiosis following the deterioration of nutrient conditions (Hironori, 2014), the early onset of the conjugation process could indicate the lack of specific nutrient. The slower fermentation capacity observed in the three strains of *S. japonicus* could also be an indication of nutrient limitation. Further investigation is necessary to clarify the fermentation needs of this species.

3.3. Characterization of the released polysaccharides

The nature of the released polysaccharide component was analyzed for the nine yeast strains of *Schizosaccharomyces*. Proteins and glycoproteins content were characterized by gel electrophoresis and the carbohydrate composition was analyzed by HPAEC-PAD and by mass spectrometry.

The overall pattern of the proteins released in the media as analyzed by SDS PAGE, was similar for the two yeast species. The protein profiles spanned a wide variety of molecular weights, ranging from 25 kDa to greater than 250 kDa for all nine strains (Fig. 6 a-f).

The three strains of *S. japonicus* presented an evident band around 32-33 kDa that corresponded to a glycosylated protein as demonstrated after gel staining with the Pro-Q Emerald 488 gel stain kit (Fig. 6 a-b) that is specific for glycoproteins. To further validate our findings, we applied enzymatic digestion with PNGase F and we observed a molecular size decrease of the 32-33 kDa band that was consistent with the release of glycan moieties (Fig. 6 e-f). Other glycosylated proteins were also identified using this same procedure.

Treatment of the pool of proteins released by all the *S. pombe* strains at the end of fermentation with PNGase F enabled de-glycosylation prior to running the gel. Subsequent staining of the gel with Pro-Q Emerald 488 revealed that most of the released proteins were glycosylated. Their greater mobility in the gel compared to the native glycoproteins was evident (Fig. 6 c-f). None of the *S. pombe* strains had a 32-33 kDa band. These results are in agreement with those of Herrero *et al.* (1987) who found in comparing the cell wall proteins liberated by Zymolyase from several ascomycetous yeast, that only the *S. pombe* strain did not present any band in the region of 33 kDa in SDS-polyacrylamide gels. Moine-Ledoux and Dubourdiou (1999) identified a N-glycosylated 32 kDa mannoprotein, corresponding to a parietal invertase fragment of *S. cerevisiae*. This mannoprotein, released by *S. cerevisiae* after alcoholic fermentation, was considered responsible for improving protein stability in wine and showed a higher stabilizing effect than other mannoproteins with higher molecular weights. In the fission yeast invertase is a high-molecular-mass glycoprotein (205 kDa) located outside the plasma membrane and, differently from *S. cerevisiae*, it contains not only mannose but also galactose (Moreno *et al.*, 1990). Further studies are necessary in order to identify the 32-33 kDa protein from *S. japonicus* and to explore its possible role in wine stability.

The presence of glycoproteins in the stacking gel is likely due to their high molecular masses. In agreement, the existence of a high-molecular-mass glycoprotein component has been observed in the Zymolyase-released glycoprotein material from isolated walls of *S. pombe* (Herrero *et al.*, 1987).

Bands with high molecular weight were particularly evident in the SDS-polyacrylamide gels for most of the *S. pombe* strains. This last observation is consistent with the presence of an early elution peak, (retention time of about 6.7 minutes) in the relevant chromatograms of the polysaccharides, analyzed by HPLC, and extracted at the end of the alcoholic fermentation (Fig. 7 a-b). These earlier peaks were particularly evident for the strains # 3, #4, #5 and #6. Indeed, the elution of polysaccharides in HPLC/RI is based on size, with

longer polymers eluting before shorter ones. Commercial mannans extracted from *S. cerevisiae*, and used as reference standard for the quantification of polysaccharide in the present study, showed a peak at a retention time of about 7 minutes (Fig. 7 c), consistent with when most of the polysaccharide fractions extracted from the fermented media of all the *Schizosaccharomyces* strains have been detected. Palomero *et al.* (2009) observed a similar early peak in the HPLC chromatograms of the polysaccharides produced by *S. pombe*. This earlier peak was no evident in the chromatograms of the three strains of *S. japonicus* (Fig. 7 b). Further research is needed to identify these earlier peaks and to evaluate the influence of these larger size polymers on the chemical properties of wine.

The monosaccharide composition of the total polysaccharide fraction released into the media was also analyzed and revealed high percentages of mannose (ranging from 44% to 55%) and galactose (ranging from 33% to 45%) (Fig. 8). In particular, the *S. japonicus* strains showed a higher percentage of mannose (ranging from 50% to 55%) and lower percentage of galactose (ranging from 33% to 34%) with respect to *S. pombe* (ranging from 44% to 47% of mannose and ranging from 36% to 45%, of galactose). The mannose/galactose ratio was higher in the *S. japonicus* strains (1.45-1.66) versus *S. pombe* (0.98-1.24). Moreno *et al.* (1990), during the purification and characterization of the glycoprotein invertase from *S. pombe*, found equimolar amounts of mannose and galactose consistent with our data. Only yeasts belonging to *Schizosaccharomyces* genus have been reported to have galactomannans located in the outer layer of the cell wall, which is composed of galactomannan, 9–14%; alkali-soluble α -1,3- linked glucans, 18–28%; alkali-soluble β -1,3-linked glucans, 24%; β -1,6-linked glucans, 2%; alkali-insoluble β -1,3- linked glucans, 18% (Manners and Meyer, 1977).

The percentage of glucose on the polysaccharides of all of the *Schizosaccharomyces* strains ranged from 9.4% and 15%, with exception of strain #7, which showed the highest percentage of glucose (18.4%). The percentage of glucosamine ranged from 0.26 % to 0.34 % for all *Schizosaccharomyces* strains, again with the exception of *S. pombe* strain # 7 (0.60%). Glucosamine likely derived from acid hydrolysis of chitin (Hardy *et al.*, 1988), a long-chain polymer of N-Acetylglucosamine, which has been shown to be present, in small quantity (0.5%), in the *S. pombe* cell wall (Sietsma and Wessels, 1990).

The protein percentage of the polysaccharide was lower in all of the *S. pombe* strains (ranging from 0.7% to 1.21%), with the exception of the strain # 7 (2.28%) as compared to the three *S. japonicus* yeast strains (ranging from 1.57% to 1.82%). In any case the protein concentration of the glycoprotein component was lower than that of the strain EC1118 of *S. cerevisiae* (3.68%), as previously reported (Domizio *et al.*, 2014). These results, besides confirming the difference between the two yeast species, highlight the different features of *S. pombe* strain #7 with respect to the other *S. pombe* strains evaluated. The higher percentage of glucose of this strain could be due to a higher release of a small part of its inner layer of the cell wall composed of β (1-3), β (1-6), and α (1-3) glucans (Pérez and Ribas, 2004), or to a different cell wall architecture.

The analysis of the released *N*-glycans (now oligosaccharides) by MALDI-TOF (Fig. 9) provided a snapshot of the most likely composition of the complex polysaccharides present.

The *S. pombe* strains, showed a core of *N*-glycans with mass ranging from 9 up to 15 hexose residues. This is consistent with the reported structure of *S. pombe* galactomannoproteins, containing a heterogeneous small “core” oligosaccharide fraction linked to asparagine with sugar compositions that range from $\text{Man}_9(\text{GlcNAc})_2$ to $\text{Gal}_4\text{Man}_{10}(\text{GlcNAc})_2$. (Gal: Galactose, Man: Mannose, GlcNAc: N-Acetylglucosamine) (Ballou *et al.*, 1994). These compounds represent the smaller members of a series of a much larger N-linked glycans that have been shown to have an average composition of $(\text{Gal})_{52}(\text{Man})_{64}(\text{GlcNAc})_2$ (Gemmill and Trimble, 1996). The smallest glycan detected in the present study among the *S. pombe* strains, with the exception of the strain #7, was $(\text{Hex})_9(\text{GlcNAc})_2$. This is in agreement with previous work reporting that *S. pombe*, unlike *S. cerevisiae*, lacks an endoplasmic reticulum $\text{Man}_9\text{-}\alpha$ 1,2-mannosidase and therefore is not able to trim $\text{Man}_9\text{GlcNAc}_2$ to $\text{Man}_8\text{GlcNAc}_2$ prior to incorporation into hybrid and complex sugars (Gemmill and Trimble, 1996; Ziegler *et al.*, 1994). $\text{Man}_9\text{-}\alpha$ 1,2-mannosidase is an important enzyme involved in glycoprotein quality control and is important for the targeting of misfolded glycoprotein for degradation. However, the presence of a smaller glycan $(\text{Hex})_8(\text{GlcNAc})_2$ found in *S. pombe* #7, is particularly interesting and could confirm what Movsichoff *et al.* (2005) reported that *S. pombe* yeast exhibits little α -mannosidase activity against misfolded glycoproteins.

In contrast to *S. pombe*, but in common with the budding yeasts such as *S. cerevisiae*, a smaller glycan unit $(\text{Hex})_8(\text{GlcNAc})_2$ was present in the polysaccharides of the three strains of *S. japonicus*. This is a unique feature among the eukaryotes (Gemmill and Trimble, 1996) and is consistent with our previous results (Domizio *et al.*, 2014) where all the non-*Saccharomyces* yeasts displayed glycan $(\text{Hex})_8(\text{GlcNAc})_2$ as the smallest glycan unit.

The structure of the glycoproteins released by *Schizosaccharomyces* yeasts in the media are expected to be similar to that of the yeast cell wall, as has been observed for glycoproteins released into the wine for *S. cerevisiae* (Llaubères *et al.*, 1987). An intraspecific similarity of the glycans profile, with comparable intensity for some of the glycans within each yeast species, was seen (Fig. 9). This observation suggests that rapid glycan fingerprint by MALDI-TOF could potentially be used as a taxonomic tool for these yeasts. Different *N*-glycan chains profiles were obtained from eight non-*Saccharomyces* yeasts, each belonging to different genera (Domizio *et al.*, 2014). A different cell wall composition at the genera, species, and strain level has been reported (Ballou, 1976).

4. Conclusion

Given the ability of *Schizosaccharomyces* yeasts to release polysaccharides during the alcoholic fermentation, further research on strain diversity among *Schizosaccharomyces* yeasts is necessary. Indeed, Jeffares *et al.* (2015) showed that strains of *S. pombe* harbor genomes with an average nucleotide diversity on the same order of magnitude as that previously determined for *S. cerevisiae* (Schacherer *et al.* 2009). Particular attention should be given to the species *S. japonicus*. To our knowledge, use of *S. japonicus* in winemaking has not previously been proposed. *S. japonicus* was isolated and identified as a new species of fission yeast by Yukawa and Maki (1931). Since then, differences of some physiological features with respect to other fission yeasts have been reported (Hironori, 2014). In particular, *S. japonicus* yeasts present a larger cell size, different chromosomal behavior

during cell division and hyphal growth. Our results highlight differences between *S. pombe* and *S. japonicus*. The main differences found in the present study are the fermentation performances, the quantity of polysaccharide released and, particularly interesting, the different ability to trim Man₉GlcNAc₂ to Man₈GlcNAc₂ prior to incorporation into hybrid and complex sugars. However, further studies are necessary in order to confirm this ER-specific mannosidase activity, here observed for all the three strains of *S. japonicus*.

Importantly, the release of high quantity polysaccharides in the media, in particular galactomannoproteins, renders these yeasts particularly interesting for the industrial production of exogenous polysaccharide preparations that could then be purified and used not only for winemaking purposes but also as novel functional foods.

To date the galactomannans polysaccharides commercially available are obtained from the endosperm of plant seeds, and have found many applications as in the pharmaceutical, biomedical, cosmetics, and food industries (Silveira and Bresolin, 2011; Srivastava and Kapoor, 2005). Microbial galactomannans represent a small fraction, mainly because their higher production cost if compared to the inexpensive plant based-products. However, fission yeasts, besides being non-pathogenic, could represent an interesting alternative to plant based products because the protein glycosylation occurs through a mechanism more related to that found in animal cells, generating hybrid-type oligosaccharides in glycoproteins, compared to that found in *S. cerevisiae*, which generates glycoproteins with high-mannose-type oligosaccharides (Kornfeld and Kornfeld, 1985; Ziegler, *et al.*, 1994). In addition, the possibility to recover these compounds directly from the media could make these yeasts even more convenient from a production perspective for potential use as prebiotics as well. Many processes for the isolation and purification of polysaccharide have been developed. Some of them use enzymatic treatments in order to release the polysaccharide from the cell wall. Others use acids, hot alkali or a combination of both, which solubilize proteins and other polysaccharides. In this case, the acidic or alkaline conditions could lead to a more or less strong degradation of the sugar chains. Because the bioactivities of glycoprotein are significantly affected by structural features and molecular weight (Young *et al.*, 1998) and, in turn, the structural features are affected by the extraction method, the possibility to keep the native structure of the glucan, avoiding drastic, glucan-destroying conditions, eliminating the necessity for cell wall enzymatic treatment, and maintaining at the same time an efficient yield of glycoproteins, could represent distinct advantages.

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Highlights

- We evaluated the ability of nine *Schizosaccharomyces* yeast strains to release polysaccharides during the alcoholic fermentation
- *Schizosaccharomyces* yeasts showed higher capacity to release polysaccharides as compared to *S. cerevisiae*
- *S. japonicus* strains released higher quantity of polysaccharide with respect to *S. pombe* strains
- Polysaccharides analysis showed divergence between *S. pombe* and *S. japonicus*
- An intraspecific similarity of the glycans profile was observed
- Rapid glycan fingerprint by MALDI TOF could be used as a taxonomic tool for these yeasts

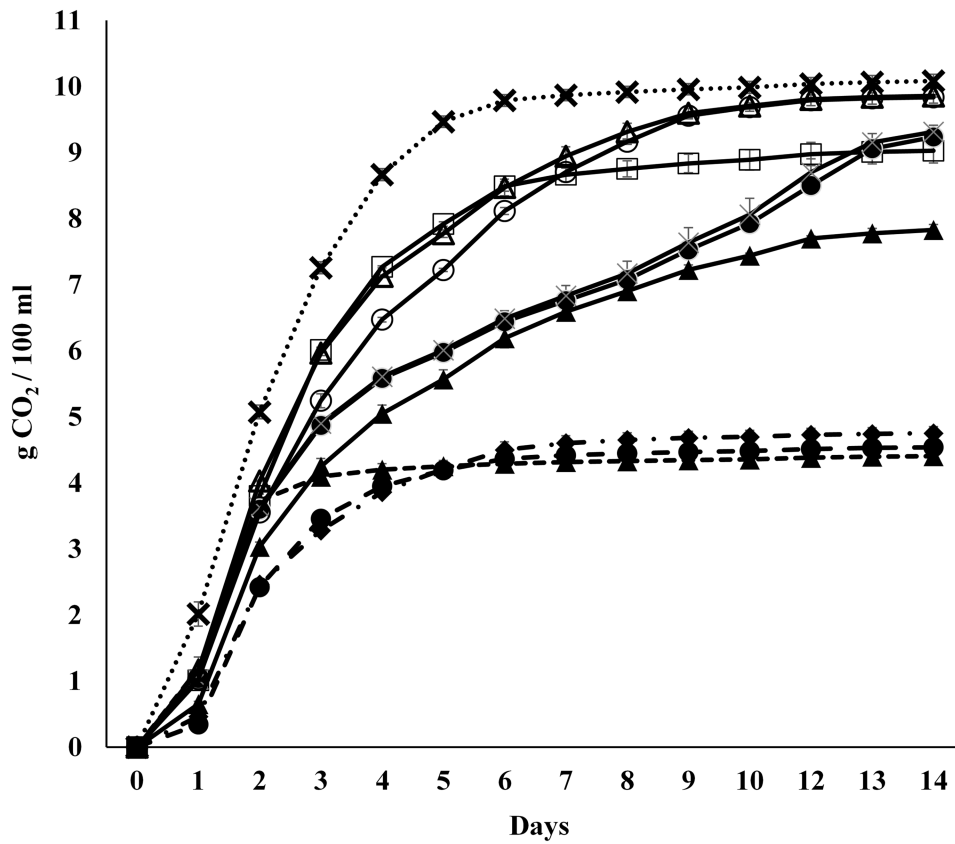


Figure 1. Fermentation kinetics of pure cultures of *S. japonicus* (dash lin -▲- #1, -◆- #8, -◄- #9), *S. pombe* (continuous line: ⊕ #2, ◆ #3, ✱ #4, ⊕ #5, ⊕ #6, ▲ #7) and *S. cerevisiae* (dot lines: ✱) inoculated in synthetic grape juice. Data are representative of two independent experiments. Error bars represent standard deviation of two independent experiments, each carried out in duplicate.

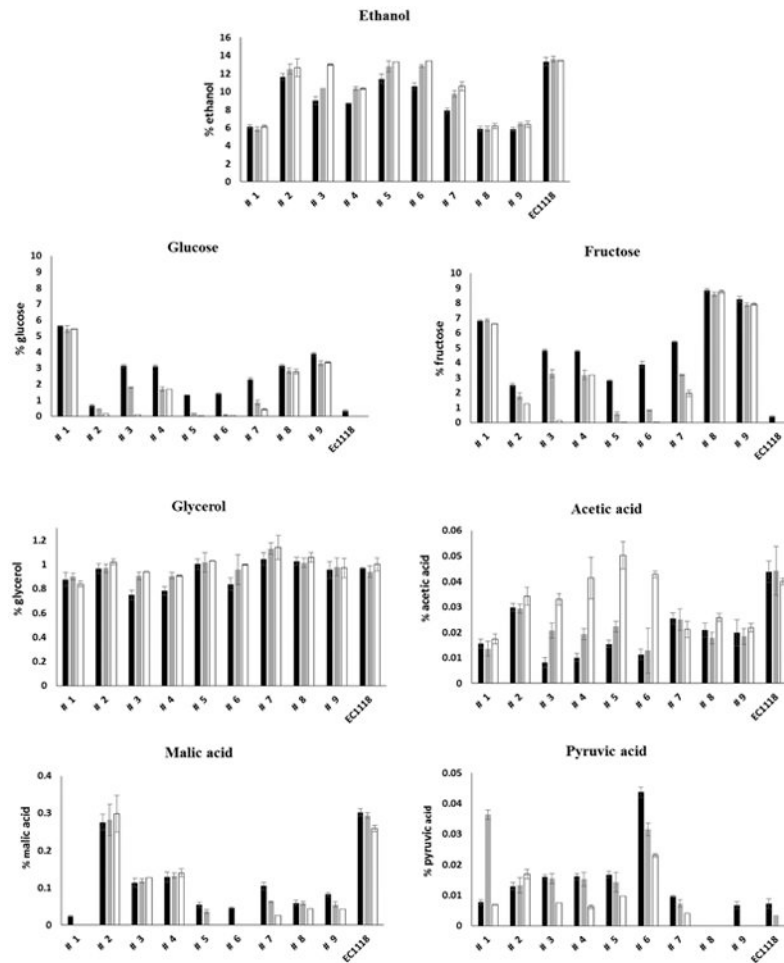


Figure 2. Fermentative performance of the nine *Schizosaccharomyces* yeast strains and the commercial strain of *S. cerevisiae* in terms of ethanol production and amounts of residual sugars, glycerol, acetic acid, malic acid and pyruvic acid at 5 ■, 8 ■ and 14 ■ days of alcoholic fermentation. Data are representative of two independent experiments. Error bars represent standard deviation of two independent experiments, each carried out in duplicate.

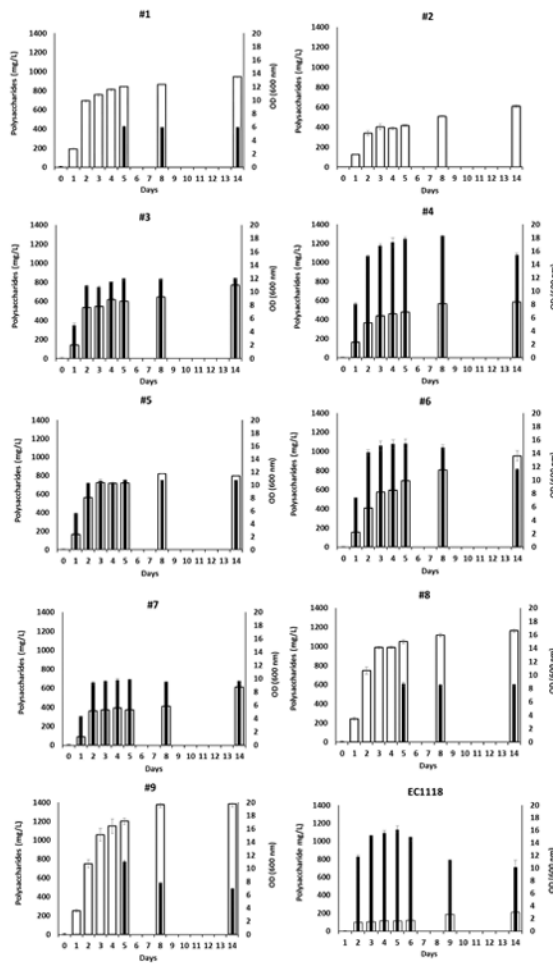


Figure 3. Growth (■) and total polysaccharide release (□) by *Schizosaccharomyces* and *S. cerevisiae* during the alcoholic fermentation. Data are representative of two independent experiments. Error bars represent standard deviation of two independent experiments, each carried out in duplicate.

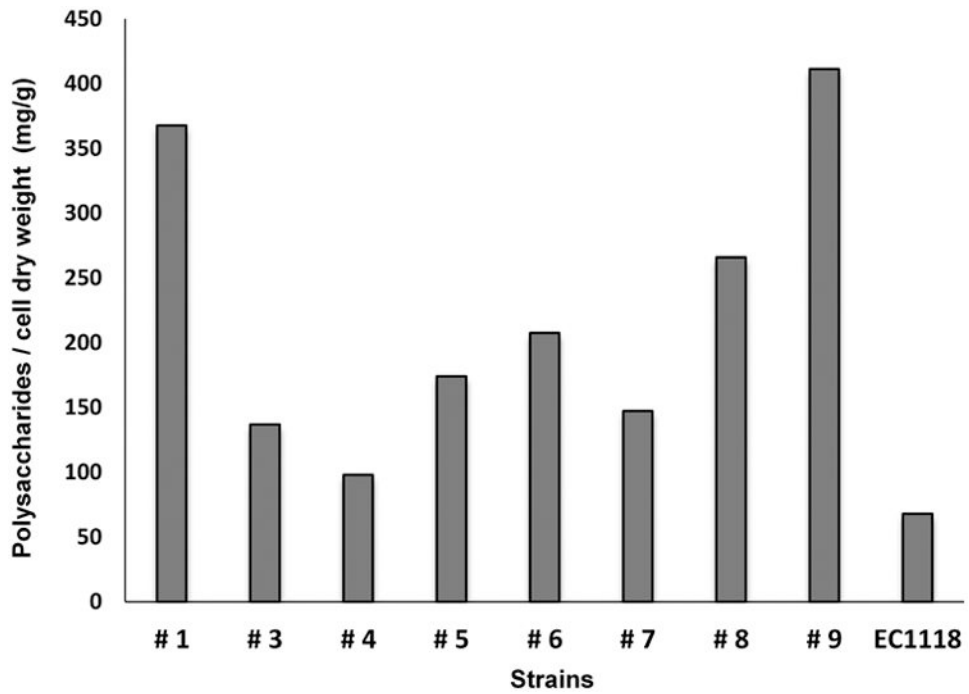


Figure 4. Ratio of polysaccharides (mg) to grams of cell dry weight (mg/g) of the nine *Schizosaccharomyces* strains and of the commercial strain of *S. cerevisiae*

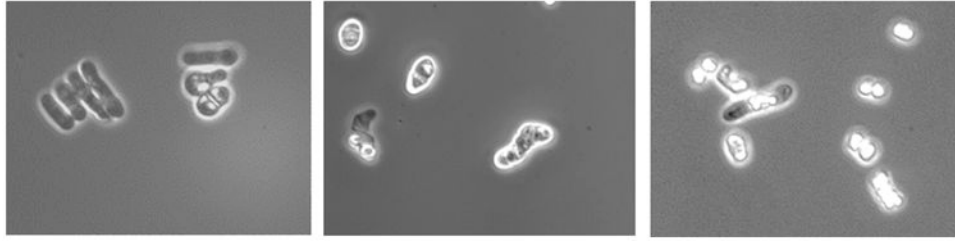


Figure 5. Microscopic observation of the strain #9 of *S. japonicus* during the alcoholic fermentation of a synthetic grape juice: conjugation process and ascospore formation.

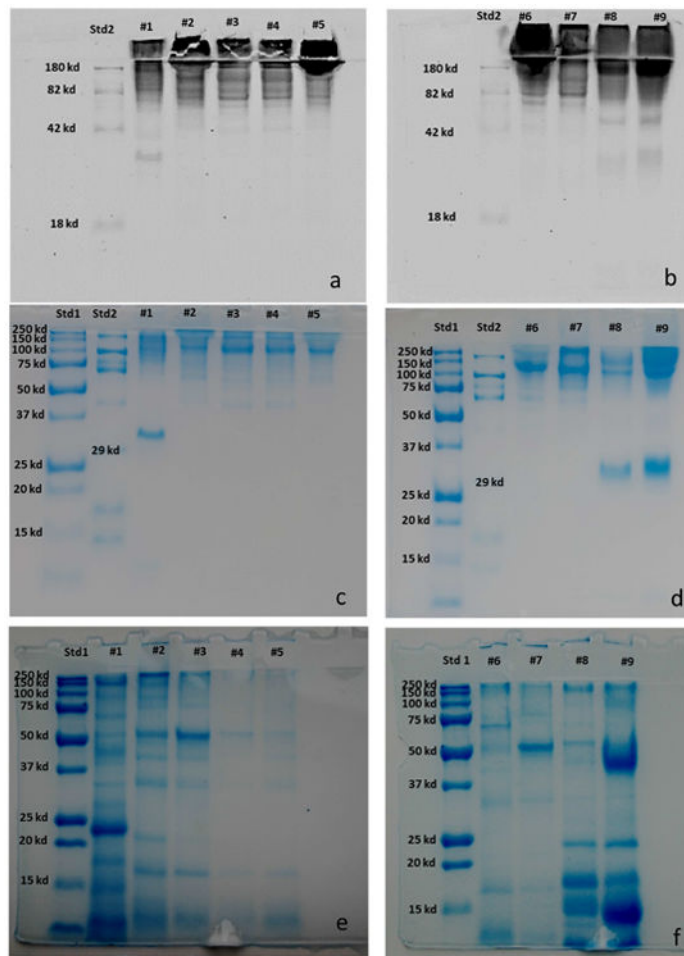


Figure. 6. SDS-PAGE electrophoresis of the proteins released into the media from *Schizosaccharomyces* yeasts. Glycoprotein visualization was performed using a two step procedure. The glycoproteins were first stained on electrophoretic gel with Pro-Q Emerald 488 glycoprotein gel stain kit (6 a-b). Then, the proteins were stained in the same gel with Bio safe Coomassie (6 c-d). The pool of proteins released in the media have been loaded in the gel after enzymatic de-glycosylation with PNGase F and then stained in the gel with Bio safe Coomassie (6 e-f). Std1: Blue precision plus molecular weight standard; Std2: CandyCane molecular weight standard.

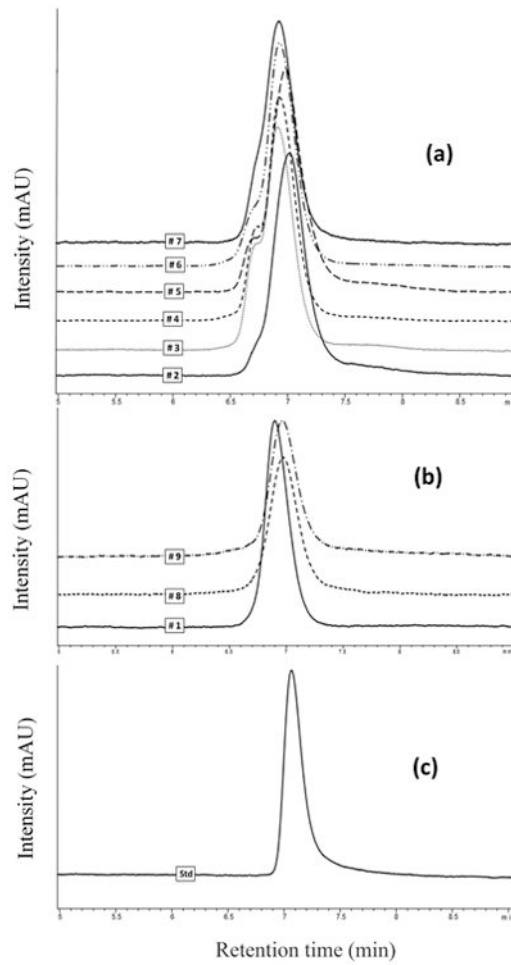


Figure 7. HPLC chromatograms of polysaccharides released in the media by *S. pombe* strains (a) and *S. japonicus* (b) at the end of the alcoholic fermentation, and of a standard solution of commercial mannan (c).

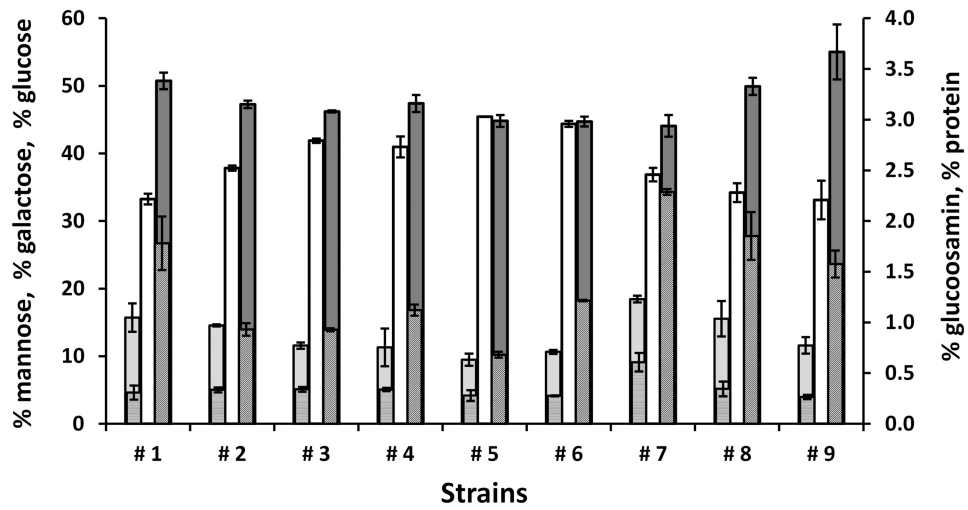


Figure 8. Sugar composition (■ glucose, ■ galactose, ■ mannose, □ glucosamin) and protein ▨ contents of polysaccharides released by *Schizosaccharomyces* strains after 14 days of alcoholic fermentation. Data are representative of two independent experiments. Error bars represent standard deviation of two independent experiments, each carried out in duplicate

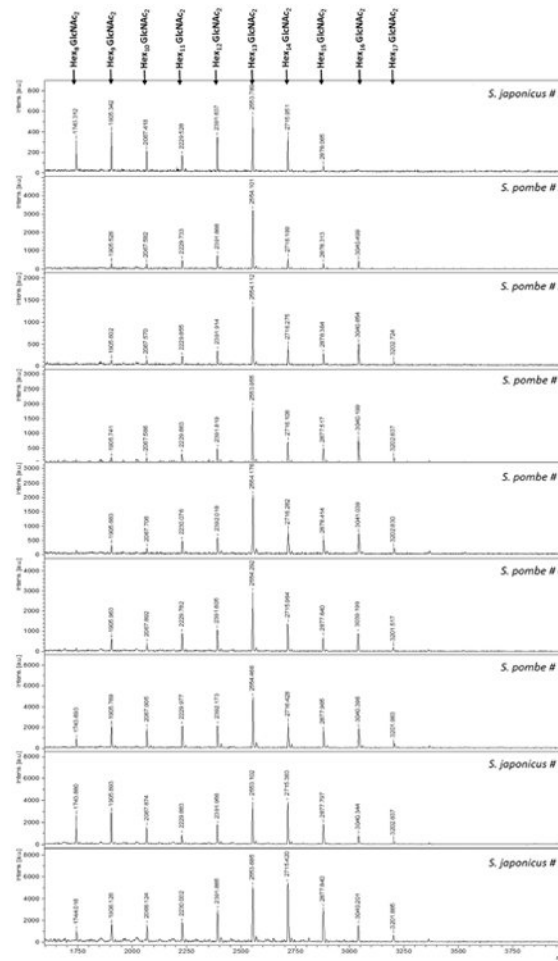


Figure 9. MALDI-TOF-MS profiles of *N*-Glycans prepared by PNGase F digestion. Digestion was conducted on the proteins released by each yeast strain at the end of the alcoholic fermentation. The chromatograms are representative of the analyses performed on each sample in triplicate from biological duplicate extractions. GlcNAc: N-Acetylglucosamine

Table 1
Origin and source of the *Schizosaccharomyces* strains used in the present study

CODE	Species	Strain	Origin	Source
# 1	<i>Schizosaccharomyces japonicus</i>	13	GESAAF ^a	Wine
# 2	<i>Schizosaccharomyces pombe</i>	227	UCD ^b	Unkown
# 3	<i>Schizosaccharomyces pombe</i>	582	UCD ^b	Sherry wine
# 4	<i>Schizosaccharomyces pombe</i>	583	UCD ^b	Sherry wine
# 5	<i>Schizosaccharomyces pombe</i>	584	UCD ^b	Wine
# 6	<i>Schizosaccharomyces pombe</i>	687	UCD ^b	Wine
# 7	<i>Schizosaccharomyces pombe</i>	807	UCD ^b	Unkown
# 8	<i>Schizosaccharomyces japonicus</i>	2096	UCD ^b	Wine
# 9	<i>Schizosaccharomyces japonicus</i>	2489	UCD ^b	Wine

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