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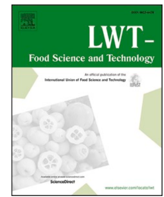

Use of *Hansensiaspora uvarum* and managing growth conditions increase polysaccharides and antioxidants content in yeast autolysates for winemaking

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ABSTRACT

Yeast derivatives are winemaking additives generally obtained from *Saccharomyces* spp., commonly used as fermentation and wine quality enhancers. Even though they are widely used, the production process - from initial biomass to their final composition, is not standardized and often not specific for winemaking purposes. In the present study, biomass production was performed by applying different temperatures and agitation conditions, to assess their effect on the chemical composition of the resulting derivatives, using *S. cerevisiae* and *H. uvarum* as starting microorganisms. The application of shaking mostly affected the chemical composition of yeast derivatives, resulting in the highest mean concentration of amino acids (146 mg/g in *S. cerevisiae*), polysaccharides (370 mg/g in *H. uvarum*), and glutathione (about 85 μmol/g in *S. cerevisiae*). Derivatives obtained from *H. uvarum* were characterized by a non-negligible amount of glutathione (35.6 μmol/g - 53.1 μmol/g) and cysteine (12.7 μmol/g - 26.2 μmol/g), regardless of the growth conditions previously applied; only for this strain, reducing proteins linked to cell wall residues were detected. The results obtained suggest that the chemical composition of yeast derivatives may be managed during biomass production, also using specific strains, thus possibly obtaining products naturally rich in compounds of enological interest.

1. Introduction

Yeast derivatives (YDs) are additives commonly used in winemaking as fermentation and wine quality enhancers. They are classified in four groups in relation to their manufacturing process: *i*) inactive yeasts obtained by thermal inactivation; *ii*) yeast autolysates obtained by thermal inactivation followed by a period of incubation, in order to allow the activity of endogenous enzymes and the release of cellular components; *iii*) yeast hulls (or walls), which represent the insoluble fraction of autolysates after drying; *iv*) yeast extracts, which represent the soluble fraction of autolysates after the separation of hulls (Pozo-Bayón, Andújar-Ortiz, & Moreno-Arribas, 2009).

Among YDs, mannoproteins (MP) are widely used in winemaking as wine quality enhancers, thanks to their positive effects towards tartaric and protein instability (Rodriguez-Nogales et al., 2012); furthermore, these compounds may be involved in reducing astringency (Escot et al., 2001) and in modulating the aroma profile and sensory perception of wine (Comuzzo et al., 2011). Together with β-glucans, MP are synthetized by yeasts as the main components of cell walls (Kollár et al., 1997); they are released into the wine during alcoholic fermentation, also depending on the strains (Domizio et al., 2014) and during wine aging on lees, when natural autolysis occurs (Comuzzo et al., 2021).

The International Organization of Vine and Wine (OIV) recommends that yeast derivatives should be produced using biomass from *Saccharomyces* spp.; only in the case of inactivated yeasts with guaranteed level of reduced glutathione (GSH), non-*Saccharomyces* yeasts (NSY) may be used as starting microorganisms. Nonetheless, the OIV has not specified the growth conditions required to obtain this kind of products or to enhance the natural production of GSH (International Organization of Vine and Wine, 2022). This tripeptide is well known for its antioxidant property, and it represents about 0.5%–1% of the yeast cell dry weight (Penninckx, 2002). To date, most of the studies till now carried out about the protective effect of lees or yeast derivatives towards wine oxidation are mainly focused on *Saccharomyces* spp. (Comuzzo et al., 2015); only recently, some authors have investigated the metabolisms of GSH in NSY during the production of active dry yeasts (Torrellas et al., 2020) or the ability of NSY strains to release GSH into the wine during alcoholic fermentation (Binati et al., 2021). Besides GSH, other compounds seem to be involved in the antioxidant properties of yeast lees or

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Abbreviations

OIV	International Organization of Vine and Wine
GSH	Reduced glutathione
NSY	Non-*Saccharomyces* yeasts
RPC	Reducing proteins containing cysteine residues
CYS	Cysteine
YDs	Yeast derivatives
MP	Mannoproteins

derivatives, such as some reducing proteins linked to the cell wall and containing cysteine residues (RPC) (Tirelli et al., 2010). However, to the authors' knowledge, no scientific data are currently reported in literature about the presence of these fractions in lees or derivatives from NSY.

Another issue related to YDs production concerns the propagation of yeast biomass at industrial level. YDs manufacturing is currently based on the use of baker's yeast as starting microorganism, whereas beet or cane molasses, supplemented with various macro and micronutrients, are the most common substrates used (Vieira et al., 2013). Generally, the biomass propagation is carried out in bioreactors in multiple batch fermentation steps; during this process, the right management of sugars concentration, pH, temperature and oxygen level is crucial to achieve an optimal growth rate and, consequently, maximum biomass production (Pérez-Torrado et al., 2015). Strains, type of sugars and nitrogen sources (Taccari et al., 2012), as well as the different preferences in terms of nutrients or the different behavior related to the Crabtree effect, may affect the specific growth rate of the strains. Generally, a temperature of 30°C and a pH value of 5.0 are considered suitable for the growth of different yeast strains (Schnierda et al., 2014).

During the growth phase, yeasts synthesize several compounds of enological interest, including polysaccharides and GSH; the production of these compounds may be potentially controlled by managing growth conditions.

During biomass propagation, the production of mannans by *S. cerevisiae* seems to be highly affected by the type and the concentration of sugars (with sucrose being the most preferred carbon source at a concentration of 5%), by nitrogen sources (*i.e.*, peptone and yeast extracts) and by the presence of glycerol (Liu et al., 2009). Additionally, the volume of culture medium, pH and temperature, starting inoculum (Liu et al., 2009) and dissolved oxygen level (Farinha et al., 2022) are other growth parameters that influence the production of polysaccharides during yeast biomass propagation. Lastly, shaking may also affect the production of polysaccharides, depending on the strain and on its carbon source preferences (Bzducha-Wróbel et al., 2018).

Regarding GSH, glucose and peptone (at 4%), and magnesium sulfate (at 0.5%) seem to be the best combination of nutrients promoting both biomass and GSH production by *S. cerevisiae* in batch fermentation conditions (Liu et al., 1999). The combination of batch and fed-batch cultivation, together with the addition of cysteine (CYS) and controlled oxygen level seem to be the most suitable conditions to enhance biomass yield (102 g/L) and total GSH production (981 mg/L) in *Candida utilis* (Liang et al., 2008). Nevertheless, to the authors' knowledge, little information is reported in literature about the effect of the growth conditions applied during biomass propagation on the chemical composition of YDs obtained from NSY for specific winemaking use.

Given the above considerations, the aim of the present work was to evaluate the potential of *Hanseniaspora uvarum* to produce yeast autolysates for winemaking purposes, also considering the influence of some growth parameters – mainly temperature and shaking - on biomass production and on the concentration of compounds of enological interest during the manufacturing process. Yeast cultivation under constant shaking and static conditions was performed at two temperatures (22°C and 30°C), using both *S. cerevisiae* and *H. uvarum* strains. The analytical evaluations mainly concerned cell viability and biomass production after the growth phase, whereas the concentrations of amino acids, polysaccharides, riboflavin and antioxidant molecules (GSH, CYS, RPC) were evaluated in yeast suspensions both after growth and after enzyme-assisted lysis.

2. Material and methods

2.1. Reagents

Sodium chloride for microbiology, bacteriological peptone, Malt Extract Broth, and technical agar were purchased from Oxoid (Milan, Italy). Glucose, fructose, sucrose, yeast extract, magnesium sulfate, tetracycline, sodium hydroxide, N-acetyl-L-cysteine, *o*-phthaldialdehyde (OPA), isoleucine, ethanol (96 % v/v), mannan from *S. cerevisiae*, sodium citrate, trifluoroacetic acid (TFA), riboflavin (RF), L-glutathione reduced, L-cysteine, *p*-benzoquinone, 3-mercaptopropionic acid, trichloroacetic acid (TCA) and acetaldehyde (ACS reagent, ≥99.5%) were purchased from Sigma Aldrich Italy (Milan, Italy). Methanol (HPLC grade) were purchased from VWR Chemicals (Milan, Italy). MilliQ water was produced by MilliQ Advantage A10 apparatus (Merck Millipore, Billerica, MA, USA) and microfiltered at 0.22 μm before use. Commercial β-glucanase preparation and active dry yeast preparation (*S. cerevisiae*) were supplied by Enologica Vason S.p.A. (San Pietro in Cariano, VR, Italy).

2.2. Yeast strains and optimization of growth medium

A commercial active dry yeast preparation of *Saccharomyces cerevisiae* and a strain of *Hanseniaspora uvarum* isolated from red grapes cv. Merlot (harvest 2021, Friuli Venezia Giulia, Italy) were used as starting microorganisms. This *H. uvarum* strain was selected for its ability to produce polysaccharides and antioxidant compounds and it was identified by molecular methods, as reported in Voce et al. (Voce et al., 2022). Each strain was purified on Malt Agar plates and single pure colonies were grown overnight in sterile tubes containing 3 mL of Malt Extract broth; then, 100 μL of yeast suspensions were transferred into sterile tubes containing 10 mL of the same culture medium. A preliminary test was carried out to evaluate the growth kinetics and to assess the most suitable growth factors for enhancing biomass production - in particular, the type of sugars (glucose, fructose, sucrose; 20 g/L) and the concentration of yeast extract (5 g/L and 10 g/L); magnesium sulfate (2.5 g/L) and peptone (10 g/L) were also added. The pH of the different culture media was adjusted to 5.0. The growth kinetics were evaluated using an absorbance microplate reader (Sunrise, Tecan Italia S.r.l., Cernusco sul Naviglio, MI, Italy), equipped with a shaker and temperature control unit, interfaced with a computer for processing the absorbance data (OD 600 nm). The inoculum was 6.3×10^4 cells/mL for *S. cerevisiae* and 2.7×10^5 cells/mL for *H. uvarum* and the kinetics were followed up to 72 h of incubation at 30°C. All the experimental trials were performed in triplicate.

2.3. Biomass production and enzyme-assisted lysis

After evaluating the effect of carbon sources and yeast extract concentration on the growth kinetics, the culture medium containing glucose (20 g/L), yeast extract (10 g/L), magnesium sulfate (2.5 g/L) and bacteriological peptone (10 g/L) was selected for the following step of biomass production. For each experimental trial, the inoculum was prepared starting from a single pure colony and incubating yeast suspensions overnight in the selected culture medium; yeast suspensions were then inoculated at 10 % (v/v) - corresponding to 6.4×10^6 cells/mL for *S. cerevisiae* and 4.7×10^6 cells/mL for *H. uvarum* respectively - in 200 mL-sterile flasks containing 90 mL of the same culture medium. For

biomass production, the conditions tested were temperature (30°C and 22°C) and shaking. Regarding the latter, yeast suspensions were incubated in an orbital shaker (SKI 4 shaking incubator, Argolab, MO, Italy) set at 150 rpm and also equipped with a temperature controller, in order to favor oxygen dissolution for biomass production (hereafter referred to as +), whereas static cultivation without shaking (*i.e.*, unshaken flasks) was performed to mimic fermentation condition (hereafter referred to as -) (Lemos Junior et al., 2021); all the tests were performed in triplicates. The biomass was recovered by centrifugation (13000 g for 10 min at 4°C) after 24 h of incubation, that approximately corresponded to the end of the exponential phase. The recovered yeast biomasses were washed twice with saline-peptone water (8 g/L of sodium chloride, 1 g/L of bacteriological peptone) and resuspended in 20 mL of citrate buffer at pH 5 for the following enzyme-assisted lysis. For inducing autolysis, a commercial β-glucanase preparation was added at a concentration of 3% (w/w); after incubation at 45 °C for 24 h, the autolyzed suspensions were centrifuged (3000 rpm for 15 min) and both the supernatants and pellets were subjected to the chemical evaluations as described below. The supernatant was used for determining the content of soluble molecules that are released as result of autolysis (cell wall polysaccharides and intracellular components, *i.e.*, amino acids and peptides, also including GSH); the pellet was analyzed due to the possible presence of antioxidant molecules, such as reducing proteins containing cysteine (RPC), linked to the insoluble cell wall residues. Concerning the analysis related to the growth phase, the culture medium was also subjected to the same chemical evaluation because of the presence of compounds (glutathione, polysaccharides, peptides, amino acids) deriving from yeast extract; the results were then used to correct the data related to the growth phase.

2.4. Analytical determinations

2.4.1. Cell viability and biomass production

Cell viability was determined after the growth phase, plating 100 μL of proper serial dilutions on Malt Extract Agar plates and incubating at 30°C for 48 h; the results were expressed in log CFU/mL. The production of biomass was evaluated by filtration under vacuum on pre-weighted 0.45 μm cellulose acetate membranes; membranes were washed with sterile water and placed in oven at 60°C up to constant weight. The difference between the initial and final weight was used to calculate the biomass, expressed in g/L.

2.4.2. Evaluation of free amino acids

The amino acids released after growth and enzyme-assisted lysis were quantified by *o*-phthaldialdehyde (OPA) derivatization as reported by Dukes & Butzke (Dukes & Butzke, 1998). Before analysis, yeast suspensions were centrifuged (3000 rpm for 10 min) and 50 μL of the supernatant were subjected to derivatization. The concentrations were calculated in relation to a calibration curve prepared with a standard solution of isoleucine (0–10 mM) and the results were expressed in mg/g of cell dry weight.

2.4.3. Evaluation of polysaccharides by SE-HPLC/RID

Yeast suspensions after growth and enzyme-assisted lysis were firstly centrifuged (3000 rpm for 10 min); the supernatants were then used for determining the content of water-soluble polysaccharides after ethanol precipitation, by SE-HPLC/RID. Five milliliters of supernatants were added with 5 vol of ethanol (96 % v/v) and stored at 0–4°C for 24 h. The precipitated pellet was separated by centrifugation, washed twice with ethanol (96% v/v), resuspended in 2 mL of Milli Q water and filtered on 0.22 μm cellulose acetate membrane before injection. SE-HPLC separation was achieved by using a binary pump Model LC 250 (PerkinElmer, Waltham, MA, US3A), equipped with a Rheodyne 7125 NS manual injection valve (Rheodyne, Rohnert Park, CA, USA) and a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). Separation columns were a PL Aquagel-OH MIXED-H (8 μm, 300 × 7.5 mm, Agilent Technologies, Santa Clara, CA, USA) and an Ultrahydrogel 250 (6 μm, 300 × 7.8 mm, Waters, Milford, MA, USA). Mobile phase was MilliQ water, and the separation was carried out in isocratic conditions, with a flow rate of 0.7 mL/min and an injection volume of 20 μL. The concentrations of polysaccharides were calculated in relation to a calibration curve prepared with standard solutions of mannan from *S. cereviasiae* (0–1000 mg/L).

2.4.4. Evaluation of riboflavin by RP-HPLC

Riboflavin content was determined on yeast suspensions after growth and enzyme-assisted lysis by RP-HPLC. Two (2) mL of yeast suspensions were filtered on 0.22 μm cellulose acetate membranes. The analysis was performed using a LC-2010 AHT liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an integrated autosampler and a UV–visible detector set at 440 nm. The separation column was a Zorbax Eclipse Plus C18 (5 μm, 150 × 4.6 mm, Agilent Technologies, Santa Clara, CA, USA), conditioned at 25°C. The mobile phase consisted of 0.05% v/v trifluoroacetic acid in Milli Q water (eluent A) and methanol (eluent B). The elution gradient used was set as follows: 30% B in the first 2 min, 60% B at 10 min, then up to 100% B at 11 min, held for 3 min; the equilibration time before the following injection was 3 min at the initial conditions. The flow rate was 0.6 mL/min, and the injection volume was 20 μL. The concentrations were calculated in relation to a calibration curve prepared with standard solutions of riboflavin (0–200 μg/L).

2.4.5. Evaluation of antioxidant compounds by RP-HPLC

The content of antioxidant compounds, *i.e.*, reduced glutathione (GSH), cysteine (CYS) and reducing proteins containing cysteine residues (RPC) linked to cell wall fraction, was determined on yeast suspensions after growth and enzyme-assisted lysis, following the method described by Tirelli et al. (Tirelli et al., 2010). The analysis was performed using a LC-2010 AHT liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an integrated autosampler and a UV–visible detector set at 303 nm. The separation column was a Zorbax Eclipse Plus C18 (5 μm, 150 × 4.6 mm Agilent Technologies, Santa Clara, CA, USA), conditioned at 25°C. The eluting solvents were 0.05% v/v trifluoroacetic acid in MilliQ water (eluent A) and methanol (eluent B), the latter increased from 10% to 35% in 18 min, then up to 100% at 20 min held for 3 min; equilibration time before the following injection was 3 min at the initial conditions. The flow rate was 1 mL/min, and the injection volume was 20 μL. The concentrations were calculated in relation to a calibration curve prepared with standard solutions of reduced GSH and CYS (0–400 μmol/L), whereas RPC were estimated using the equation reported in the reference method (Tirelli et al., 2010), using equation (1):

$$|RPC| = |pBQ| - (|Cys| + |GSH| + |pHQ| + |3MPA_HQ|) \quad (1)$$

where $|pBQ|$: concentration of *p*-benzoquinone used for derivatization; $|Cys|$: concentration of cysteine quantified chromatographically; $|GSH|$: concentration of glutathione quantified chromatographically; $|pHQ|$: concentration of underivatized hydroquinone quantified chromatographically; $|3MPA_HQ|$: concentration of S-3-mercaptopropionyl-hydroquinone quantified chromatographically.

2.5. Statistical analysis

The results obtained were means and standard deviations of three repeated trials. Homogeneity of variance was evaluated by Brown-Forsythe and Cochran, Hartley, Bartlett tests; two-way ANOVA was performed for evaluating the effects of temperature and shaking on all the chemical parameters analyzed. Tukey HSD test was also carried out for all the parameters evaluated and differences were considered significant at $p < 0.05$. All elaborations were carried out by the software Statistica for Windows Version 8.0 (StatSoft, Tulsa, OK, USA).

3. Results and discussion

3.1. Growth kinetics

The effect of carbon source and yeast extract concentration on the growth kinetics of *S. cerevisiae* and *H. uvarum* is reported in Fig. 1 (a and b), respectively. *S. cerevisiae* well grown in all the culture media used, without differences in cell density, neither in relation to the carbon source nor to yeast extract concentration. On the other hand, *H. uvarum* showed a lower growth kinetics when sucrose was used as the main carbon source, whereas in presence of glucose and fructose this strain reached the maximum value of cell density (O.D. = 1.2), similarly to *S. cerevisiae*. This comparable consumption of the two monosaccharides has been previously reported, with a more pronounced utilization and preference towards glucose or fructose that seemed to be strain dependent (Ciani & Fatichenti, 1999). Conversely, the inability of this strain to ferment sucrose may be due to the lack of invertase enzyme, as observed by Takaya et al. (Takaya et al., 2021) for a strain of *Hanseniaspora vineae*. This behavior in relation to this disaccharide is a useful information to consider, since beet or cane molasses are the most common substrates used to produce yeast biomass at industrial level, and they contain sucrose as the main sugar. The trend here observed for *H. uvarum* suggests that, at practical level, pre-treating the substrate to obtain inverted sucrose or adding substrates rich in monosaccharides (*i.e.*, glucose or fructose) as carbon sources may be necessary to promote a suitable growth rate and then the production of biomass, when strains lacking invertase enzymes are used as starting microorganisms.

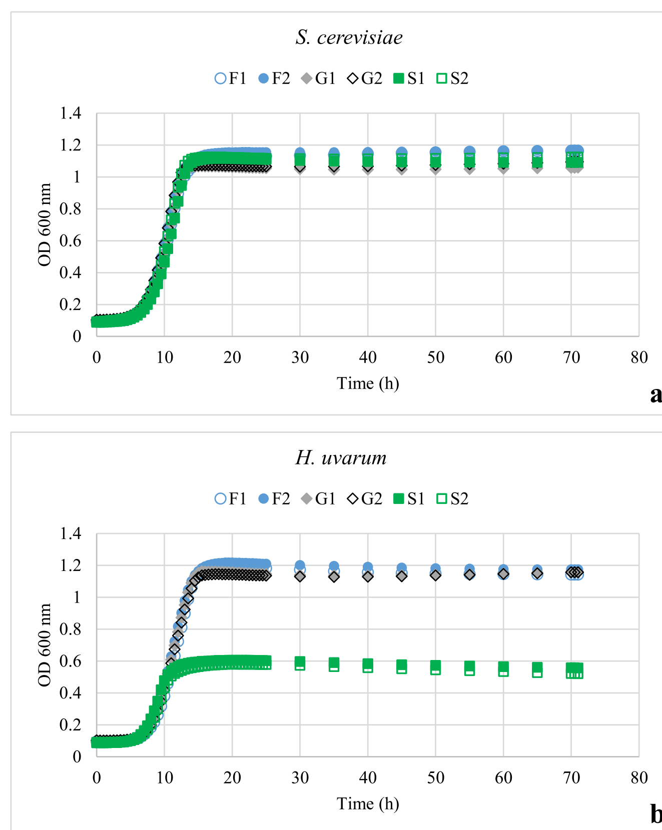

Fig. 1. Growth kinetics of *S. cerevisiae* (**a**) and *H. uvarum* (**b**) at 30°C in different culture media. **F1** (fructose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); **F2** (fructose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); **G1** (glucose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); **G2** (glucose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); **S1** (sucrose 20 g/L, yeast extracts 5 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L); **S2** (sucrose 20 g/L, yeast extracts 10 g/L, magnesium sulfate 2.5 g/L, peptone 10 g/L). Data are means of three repeated treatments.

3.2. Cell viability and biomass production

As stated above, the culture medium containing 20 g/L of glucose, 10 g/L of magnesium sulfate (2.5 g/L) and bacteriological peptone (10 g/L) was selected for biomass production. The results concerning cell viability and the amount of biomass produced after 24 h of growth are reported in Figs. 2 and 3, respectively, whereas the effect of temperature and shaking on the same parameters is reported in Table 1.

Starting from *S. cerevisiae*, the highest average value in terms of cell viability was observed under constant shaking at 22°C (8.0 log CFU/mL), even if no significant differences were observed among the different conditions applied (see Fig. 2). In the case of *H. uvarum*, the highest cell viability was found under constant shaking, with values of 8.4 log CFU/mL and 8.6 log CFU/mL at 30°C and 22°C respectively, thus resulting significantly different from static cultivation condition (7.6 log CFU/mL and 7.8 log CFU/mL at 22°C and 30°C, respectively). In general, it would be said that shaking seemed to be the parameter that mostly affected cell viability, whereas no effect of temperature was observed for both the strains (see Table 1).

Similarly, biomass production was significantly influenced by shaking (see Table 1), with the strains showing different behaviors. In particular, considering the results reported in Fig. 3, the highest production of biomass in *S. cerevisiae* was observed under static cultivation condition, reaching values of 10.3 g/L and 9.2 g/L of cell dry weight at 22°C and 30°C respectively; such values were significantly different from the quantity of biomass recovered when constant shaking was applied (3.9 g/L at 22°C and 5.2 g/L at 30°C). Conversely, in the case of *H. uvarum*, the highest biomass recovery was observed under constant shaking, with values of 3.2 g/L and 3.3 g/L of cell dry weight at 22°C and 30°C respectively, thus resulting significantly different from the biomass produced under static cultivation condition (about 1.6 g/L at both temperatures, see Fig. 3). Concerning cell viability and biomass production observed in the case of *H. uvarum*, the results were in line with those reported in literature, in which higher cell viability of NSY – ranging from 8 to 8.5 log CFU/mL - was obtained when the strains were grown under aerobic conditions (Lemos Junior et al., 2021), as well as low biomass production was observed in *Hanseniaspora guilliermondii* when static cultivation was performed (Albergaria et al., 2003). Concerning *S. cerevisiae*, the amount of biomass here detected was in line with the value reported by Perez-Torrado et al. (Pérez-Torrado et al.,

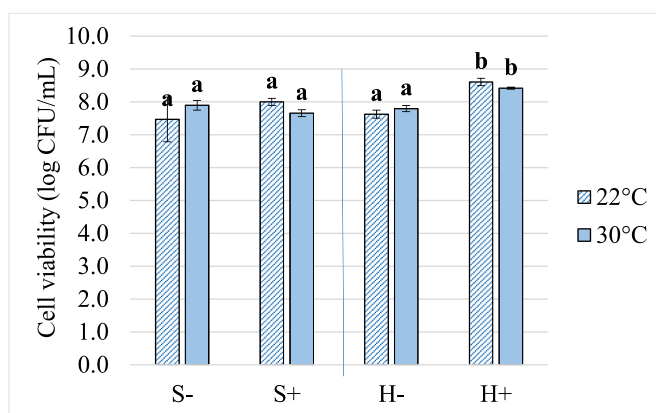

Fig. 2. Cell viability (in log CFU/mL) after 24 h of growth under different conditions. Data are means and standard deviations of three replicates. Different letters mark significant differences among the samples, as result of the interaction of the two factors (temperature x shaking), according to two-way ANOVA e Tukey HSD test at $p < 0.05$. Analysis was carried out separately for each strain. S: *S. cerevisiae*; H: *H. uvarum*; -: growth without shaking; +: growth under constant shaking (150 rpm).

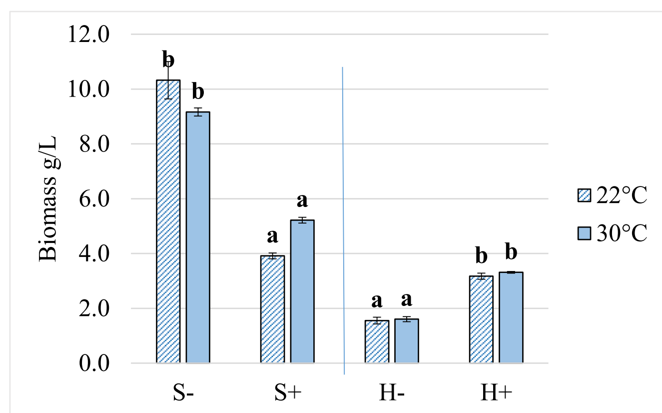

Fig. 3. Biomass production (in g/L) after 24 h of growth under different conditions. Data are means and standard deviations of three replicates. Different letters mark significant differences among the samples, as result of the interaction of the two factors (temperature x shaking), according to two-way ANOVA e Tukey HSD test at $p < 0.05$. Analysis was carried out separately for each strain. S: *S. cerevisiae*; H: *H. uvarum*; -: growth without shaking; +: growth under constant shaking (150 rpm).

2009), in which a final biomass recovery of 10 g/L was obtained when the strain was grown under fermentation condition. Other authors reported that, under static cultivation, the biomass production in *S. cerevisiae* was lower than that obtained by applying agitation (Lemos Junior et al., 2021) or under aerobic conditions (Schnierda et al., 2014), whereas de Deken (de Deken, 1966) observed a fast consumption of sugars by *Saccharomyces* spp. under anaerobic condition. The latter evidence, together with a hypothesized better adaptation to fermentation conditions of the commercial strain used, might have determined a higher cell growth rate in *S. cerevisiae*, thus possibly explaining the higher biomass production observed for this strain under the anaerobic conditions (static cultivation) tested in this study. Further investigations are needed to evaluate more in depth the effects of the metabolic changes occurring during the growth phase on the production of biomass by the two strains used in the present study, when different shaking conditions are applied. Nevertheless, the good cell viability and the production of a non-negligible amount of biomass obtained by the strain of *H. uvarum* used, especially under constant shaking conditions, makes this strain suitable for obtaining biomass that could be further used as starting material for the production of both fermentation starters and yeast derivatives.

3.3. *Release of amino acids after growth and enzyme-assisted lysis*

After the growth phase, a consumption of amino acids was observed for both the strains in all the conditions tested (see Table S1, Supplementary material). The concentration of amino acids (expressed in mg/g of cell dry weight) detected in autolyzed suspensions after enzyme-assisted lysis is reported in Fig. 4, whereas the effect of temperature and shaking on the same parameter is reported in Table 2. Like previously observed for cell viability and biomass production, the concentrations of amino acids released after lysis treatment were significantly affected by the agitation applied during the growth phase, with constant shaking being the most suitable condition (see Table 2). Considering the results reported in Fig. 4, the release of such compounds seemed to be also slightly affected by the temperature previously applied during biomass propagation. The content of amino acids was of 146 mg/g (at 22°C) and 96 mg/g (at 30°C) for *S. cerevisiae*, whereas mean values of 54 mg/g (at 22°C) and 50 mg/g (at 30°C) were observed for *H. uvarum*; only in the case of the former strain, statistical differences were observed among the treatments.

Under winemaking conditions, several yeast strains showed different

Table 1
Cell viability, biomass production and release of compounds of enological interest (polysaccharides, riboflavin, and glutathione) after the growth phase. For the latter, the concentrations are standardized per gram of starting biomass. Negative values indicate a decrease in concentration, in relation to the starting composition of the culture medium. For each strain, data represent the average values calculated considering the effect of temperature (T), shaking (S) and temperature x shaking (T x S). Data were analyzed by two-way ANOVA; when the differences were significant, means were separated by Tukey's HSD test ($p < 0.05$).

	S. cerevisiae								*H. uvarum*							
	T		S		T x S				T		S		T x S			
Parameter	22°C	30°C	–	+	S_22°C-	S_22 °C+	S_30°C-	S_30 °C+	22 °C	30 °C	–	+	H_22°C-	H_22 °C+	H_30°C-	H_30 °C+
Viable cells (log CFU/mL)	7.7	7.8	7.7	7.8	7.5	8.0	7.9	7.7	8.1	8.1	7.7 a	8.5 b	7.6 a	8.6 b	7.8 a	8.4 b
Biomass (g/L)	7.1	7.2	9.7 b	4.6 a	10.3 b	3.9 a	9.2 b	5.2 a	2.4	2.5	1.6 a	3.2 b	1.6 a	3.2 b	1.6 a	3.3 b
Polysaccharides (mg/g)	3 a	16 b	3 a	15 b	−3 a	8 b	10 b	22 c	−1 a	39 b	20	18	−9	7	49	30
Riboflavin (μg/g)	30 b	20 a	14 a	36 b	15 a	45 c	13 a	27 b	72 b	42 a	81 b	32 a	105 b	39 a	58 ab	26 a
GSH (μmol/g)	8.0 a	5.8 a	3.9 a	9.9 a	3.6 a	12.4 c	4.1 a	7.4 b	18.8	18.9	26.0 b	11.6 a	25.8	11.7	26.2	11.5

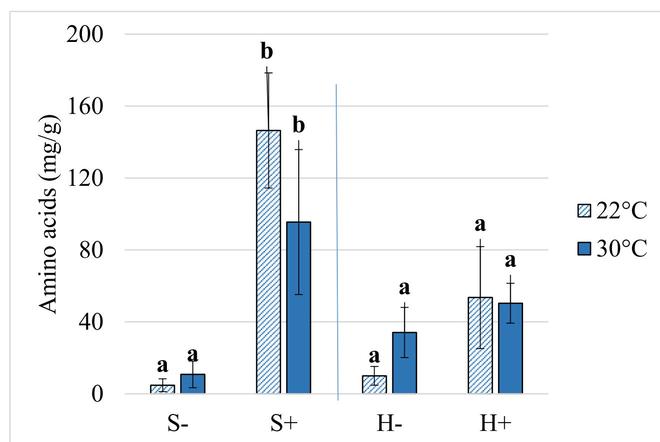

Fig. 4. Concentration of amino acids (in mg/g of cell dry weight) determined in autolyzed suspensions after enzyme-assisted lysis. Data are means and standard deviations of three replicates. Different letters mark significant differences among the samples, as result of the interaction of the two factors (temperature x shaking), according to two-ways ANOVA e Tukey HSD test at $p < 0.05$. Analysis was carried out separately for each strain. S: *S. cerevisiae*; H: *H. uvarum*; -: growth without shaking; +: growth under constant shaking (150 rpm).

preferences in terms of nitrogen sources and different utilization (Roca-Mesa et al., 2020); furthermore, nitrogen metabolisms may be affected by the growth conditions applied, consequentially influencing the content of intracellular amino acids. Junior et al. (Júnior et al., 2008) observed an improved nitrogen utilization when yeasts were grown under shaking conditions; moreover, performing the growth phase at lower temperatures seemed to increase the content of intracellular proteins (Pizarro et al., 2008). These previous findings support the results obtained in the present study, thus justifying the different behavior of the two strains in relation to the release of amino acids after lysis treatment, as well as the highest mean concentrations detected in autolyzed suspensions, that were obtained when both the strains were previously grown under constant shaking at low temperature (22°C). The results obtained also suggest that the application of constant shaking during biomass propagation may be an interesting tool for enhancing the concentration of amino acids in the resulting yeast derivatives. This may allow obtaining products, such as autolysates or extracts, that are naturally rich in amino acids, which represent important nutrients for the growth of several microorganisms. This kind of YDs might be then used in winemaking as fermentation enhancers, during both alcoholic and/or malolactic fermentation and during the rehydration/acclimatization phase of microbial starters.

3.4. *Release of polysaccharides after growth and enzyme-assisted lysis*

The concentrations of polysaccharides (expressed in mg/g of cell dry weight) determined in yeast suspensions after growth and after enzyme-assisted lysis are reported in Tables 1 and 2, respectively.

The data regarding the growth phase (see Table 1) showed that the release of polysaccharides was significantly influenced by temperature for both the yeasts, with amounts of 16 mg/g for *S. cerevisiae* and 39 mg/g for *H. uvarum* at 30°C. Regarding *S. cerevisiae*, shaking cultivation had a notable effect on the concentration of polysaccharides released after growth with a mean polysaccharides content of 15 mg/g, compared to 3 mg/g under static cultivation. In general, the highest production occurred at 30°C under shaking conditions, with a mean content of 22 mg/g. For *H. uvarum*, the highest mean concentrations were observed in suspensions obtained at 30°C, reaching values of 49 mg/g under static cultivation and of 30 mg/g under constant shaking, although no statistical differences were observed.

Conversely to the growth phase, the application of constant shaking

Table 2
Chemical composition of yeast suspensions after enzyme-assisted lysis. The concentrations are standardized per gram of starting biomass. For each strain, data represent the average values calculated considering the effect of temperature (T), shaking (S) and temperature x shaking (T x S). Data were analyzed by two-way ANOVA; when the differences were significant, means were separated by Tukey's HSD test ($p < 0.05$). T: effect of temperature; S: effect of shaking; T x S: effect of temperature x shaking. *nd: not detected.

	S. cerevisiae								*H. uvarum*							
	T		S		T x S				T		S		T x S			
Parameter	22°C	30°C	–	+	S_22°C-	S_22°C+	S_30°C-	S_30°C+	22°C	30°C	–	+	H_22°C-	H_22°C+	H_30°C-	H_30°C+
Amino acids (mg/g)	76	53	8 a	121 b	5 a	146 b	11 a	96 b	32	42	22 a	52 b	10	54	34	50
Polysaccharides(mg/g)	91 b	46 a	18 a	119 b	13 a	169 b	22 a	69 a	256	291	178 a	369 b	144 a	367 b	211 a	370 b
Riboflavin (μg/g)	35	30	6 a	59 b	4 a	66 b	9 a	52 b	66	61	58	68	55	78	62	59
GSH (μmol/g)	44.5	39.2	5.0 a	78.6 b	3.8 a	85.2 b	6.3 a	72.1 b	47.5	36.0	38.8	44.7	42.0	53.1	35.6	36.3
CYS (μmol/g)	22.4 b	16.6 a	3.9 a	35.1 b	3.0 a	41.8 c	4.7 a	28.4 b	22.8	19.5	26.1 b	16.2 a	26.0	19.7	26.2	12.7
RPC (μmol/g)	nd*	nd	nd	nd	nd	nd	nd	nd	199.0	244.1	nd a	443.1 b	nd a	398.0 b	nd a	488.2 b

during yeast biomass propagation significantly increased the release of polysaccharides in the respective autolyzed suspensions for both the strains (see Table 2). Furthermore, the low temperature used during biomass production also had a significant impact on the concentration of such compounds in the case of autolysates from *S. cerevisiae*. Considering these results, the conditions that enhanced the production of polysaccharides during the growth phase did not always result in the highest release of such compounds in the respective autolyzed suspensions. In the case of *S. cerevisiae,* autolyzed suspensions from the biomass previously obtained under constant shaking condition at low temperature (S_22°C+) were characterized by the highest content in polysaccharides (169 mg/g of cell dry weigh), thus resulting significantly different from all the other samples. A similar effect of the growth conditions on the release of polysaccharides was observed in *H. uvarum*; the highest mean concentration of polysaccharides was detected in autolyzed suspensions from the biomass previously obtained under constant shaking condition, with mean values of 370 mg/g at 30°C and 367 mg/g at 22°C, thus resulting significantly different from samples previously obtained under static cultivation (211 mg/g and 144 mg/g detected in H_30°C- and in H_22°C-, respectively). Nevertheless, it is worth noting that, by comparing the mean concentration of polysaccharides released by the two strains previously grown under the same conditions, *H. uvarum* had the ability to release a content of such compounds from two to ten times higher than that released by *S. cerevisiae* (*e.g.,* 22 mg/g in S_30°C- and 211 mg/g in H_30 °C, see Table 2).

These results are in line with those previously reported in literature about the ability of *Hanseniaspora* spp. strains to release a higher amount of polysaccharides compared to *Saccharomyces* spp. during alcoholic fermentation (Domizio et al., 2014; Romani et al., 2010). Furthermore, the content of polysaccharides tended to increase when the cells were not alive, probably due to a greater cell susceptibility and faster autolysis occurrence that characterizes this yeast strain (Giovani et al., 2012). This evidence may explain the tendential higher concentration of polysaccharides released after enzyme-induced lysis by *H. uvarum* compared to *S. cerevisiae,* in all the conditions tested here.

The effect of temperature on the production of mannans by *S. cerevisiae* during growth has been already reported, with an enhanced synthesis at lower temperatures (Liu et al., 2009); moreover, the dissolved oxygen seemed to enhance the production of cell wall mannans and chitin in *Pichia pastoris* during biomass propagation carried out in a bioreactor at 30°C (Farinha et al., 2022). Based on this scientific evidence, it could be hypothesized that, compared to static cultivation, the constant shaking here applied during biomass production might have dissolved the oxygen in the suspensions, thus potentially enhancing the synthesis of cell wall polysaccharides. This supposed higher production of such compounds during the growth phase might have positively influenced the release of polysaccharides after lysis treatment, thus explaining the higher concentration detected in autolyzed suspensions obtained from both the yeasts previously grown under constant shaking condition, and even at lower temperature, in the case of *S. cerevisiae*.

The great ability to release polysaccharides shown by the strain of *H. uvarum* tested in the present study is an interesting feature that makes it suitable for different winemaking applications. Firstly, this strain may be used as a fermentation starter, thereby becoming a consistent part of the fermentation lees. The ability of this strain to release high amounts of polysaccharides after lysis may consequentially improve wine quality and stability during the subsequent period of aging on lees. Secondly, the use of this strains, together with the production of biomass under constant shaking, may allow the production of derivatives naturally rich in polysaccharides. These products may then be used as wine quality enhancers during both alcoholic fermentation and wine aging on lees.

3.5. Release of riboflavin after growth and enzyme-assisted lysis

The amount of riboflavin (expressed in μg/g of biomass) released after growth and lysis is reported in Tables 1 and 2, respectively.

Temperature and shaking had a significant impact on the amount of extracellular riboflavin for both the strains during the growth phase (see Table 1). The highest mean values were detected when both the yeasts were grown at low temperature (30 μg/g and 72 μg/g, in S_22°C and H_22°C, respectively). However, the effect of shaking on the production of riboflavin during the growth phase differed between the two strains. Constant shaking enhanced the production of riboflavin in *S. cerevisiae* (36 μg/g), whereas the highest concentration for *H. uvarum* was found under static cultivation condition (81 μg/g). In general, the highest amount was found under static cultivation at 22°C for *H. uvarum* (105 μg/g), and under shaking condition at the same temperature for *S. cerevisiae* (45 μg/g).

Considering the chemical composition of autolyzed suspensions (see Table 2), only temperature significantly affected the release of riboflavin after lysis treatment in *S. cerevisiae*, with a concentration of 59 μg/g under constant shaking and of 6 μg/g under static cultivation. No differences were observed in the amount of riboflavin released by *H. uvarum*, in relation to the growth conditions previously performed (see Table 2).

The condition that enhanced the release of riboflavin during the growth phase of *S. cerevisiae* – *i.e.*, shaking at both temperatures - also determined the highest release of such molecule after lysis, with a concentration of 66 μg/g and 52 μg/g for S_22°C+ and S_30°C+, respectively. On the contrary, in the case of *H. uvarum*, the highest production of riboflavin during the growth phase (105 μg/g) which was observed under static cultivation and low temperature conditions, corresponded to the lowest release after lysis treatment (55 μg/g), at least in terms of mean values. Riboflavin is produced by yeasts during alcoholic fermentation with concentrations up to 170 μg/L, mainly dependent on the strains used (Fracassetti et al., 2017). Glucose and yeast extract seemed to be the best nutrient sources (Cheng et al., 2011), whereas temperature of 28°C and shaking speed of 170 rpm were the most suitable manufacturing parameters for enhancing the production of this vitamin in *Candida* spp. (Chi et al., 2008). The growth conditions applied in the present study were quite similar to those reported in literature concerning the microbial production of riboflavin, thus possibly explaining the amounts here detected. Nevertheless, no further studies have been reported in literature about how the manufacturing process may affect the production of riboflavin during yeast biomass propagation, *e.g.*, the comparative effect of low and high temperatures, or static cultivation and shaking, as here tested.

Over the course of the YDs manufacturing process, the production of riboflavin should be limited since this vitamin may be involved in light-induced reactions that determines the appearance of the so-called light-struck defects, particularly relevant for white wines (Daniela Fracassetti et al., 2019). However, according to the data obtained in the present study, if derivatives are added at a concentration of 200–400 mg/L (the normal dosage used for YDs), the amount of riboflavin released into the wine might be considered negligible, since it would be lower than the threshold level necessary to trigger the light-induced reactions (50 μg/L).

3.6. Release of antioxidant compounds

The concentrations of antioxidant compounds (expressed in μmol/g of cell dry weight) detected in yeast suspensions after growth and enzyme-assisted lysis are reported in Tables 1 and 2, respectively. Regarding the growth phase, only GSH was quantified, whereas CYS was also detected in autolyzed suspensions. The same analysis was also conducted on the insoluble fraction of the autolyzed suspensions due to the potential presence of reducing proteins containing-cysteine residues (RPC) that are linked to insoluble cell wall fractions.

The data related to the growth phase (see Table 1) show that the amount of GSH produced by *H. uvarum* significantly increased under static cultivation conditions (26 μmol/g), whereas the application of constant shaking determined the highest release of such molecule in

S. cerevisiae (9.9 μmol/g), even if no significant differences were observed (see Table 1). In general, the highest mean production of GSH after the growth phase was observed in the samples H_30°C- and H_22°C- (26.2 μmol/g and 25.8 μmol/g, respectively) and in S_22°C+ (12.4 μmol/g), the latter resulting significantly different from the other samples.

Similarly, the constant shaking applied during yeast biomass propagation significantly increased the amount of GSH released by *S. cerevisiae* after lysis treatment, with a content of 78.6 μmol/g; this growth condition also determined the highest concentration of GSH in autolyzed suspensions obtained from *H. uvarum* (44.7 μmol/g), even if no significant differences were observed (see Table 2).

Concerning CYS, a different behavior was observed for the two strains. Similarly to GSH release, constant shaking seemed to be the most suitable growth condition for enhancing the release of CYS after lysis in *S. cerevisiae*, whereas the application of static cultivation enhanced the concentration of this compound in autolysates from *H. uvarum*. Considering the effect of temperature, biomass production at 22°C increased the amount of CYS in autolyzed suspensions obtained from both the strains, with significant differences observed only for *S. cerevisiae* (see Table 2).

In general, it would be said that the condition that enhanced the production of GSH during the growth phase (constant shaking at low temperature) also determined the highest release of both GSH and CYS after lysis treatment, at least in the case of *S. cerevisiae.* The concentrations of GSH detected in autolyzed suspensions from *S. cerevisiae* were 72.1 μmol/g at 30°C and 85.2 μmol/g at 22°C under shaking condition; when the strain was previously grown under static cultivation, the content of GSH released was only 3.8 μmol/g at 22°C and 6.3 μmol/g at 30°C, thus resulting significantly different from the formers. Similarly, the highest release of CYS was found in autolysates deriving from biomass previously obtained under constant shaking, with mean content of 28.4 μmol/g at 30°C and 41.8 μmol/g at 22°C, the latter resulting significantly different from all the other samples.

It is interesting to note that *H. uvarum* released a non-negligible content of GSH, regardless of the growth conditions previously applied. The lowest average content of GSH was detected when this strain was incubated at 30°C during the growth phase (35.6 μmol/g in H_30°C- and 36.3 μmol/g in H_30°C+), whereas the production of intracellular GSH seemed to be enhanced at a temperature of 22°C, which determined the highest release of such compound in the respective autolyzed suspensions (42 μmol/g in H_22°C-, and 53.1 μmol/g in H_22°C+); however, no significant differences were observed among the treatments. Concerning CYS, the highest mean content - about 26.1 μmol/g - was detected in autolyzed suspensions deriving from biomass previously obtained under static cultivation condition but, also in this case, no significant differences were observed among the treatments. However, even if the concentrations of GSH and CYS released by *H. uvarum* were in some cases lower than those detected in *S. cerevisiae*, it is worth noting that, given the conditions here applied, RPC linked to the insoluble cell wall residues were only detected in autolyzed suspensions of *H. uvarum* obtained from biomass produced under constant shaking (see Table 2).

Several strains of NSY are characterized by a non-negligible production of intracellular GSH, with concentrations comparable or even higher than those produced by *S. cerevisiae* (Lemos Junior et al., 2021). Gamero-Sandemetrio et al. (Gamero-Sandemetrio et al., 2018) evaluated the performance of different NSY strains for producing active dry yeasts, observing that some strains of *Hanseniaspora* spp. showed a high activity of enzymes involved in GSH synthesis (Gamero-Sandemetrio et al., 2018). On the other hand, Torrelas et al. (Torrellas et al., 2020) observed that, during biomass propagation in batch fermentation at 30°C under agitation (180 rpm), the content of intracellular GSH released by different strains of *Hanseniaspora* spp. was lower than that produced by *S. cerevisiae*. This scientific evidence might explain, on one hand, the non-negligible production and release of GSH by *H. uvarum* - regardless of the growth conditions here applied - and, on the other hand, the lower content of this compound released by this strain in comparison to *S. cerevisiae*, when biomasses were previously obtained under the same growth conditions (*e.g.*, 36.3 μmol/g in H_30°C+ and 72.1 μmol/g in S_30°C+).

During biomass propagation, the consumption of sugars determines a metabolic change in *S. cerevisiae*, thereby enhancing the expression of genes involved in the GSH pathway (Pérez-Torrado et al., 2009); furthermore, the production of biomass at lower temperature seems to enhance the production of intracellular GSH in *C. utilis*, up to 2.5% w/w (Wei et al., 2003). Even if no information was provided about the metabolic changes that occurred during the biomass propagation process as performed in the present study, it might be reasonable to hypothesize a similar metabolism in the strain of *S. cerevisiae* used. This might also explain the higher concentration of GSH produced and then released into the suspensions deriving from biomass previously obtained under constant shaking conditions at low temperature (22°C); however, further investigations are needed to evaluate this aspect in depth.

4. Conclusions

The chemical composition of yeast derivatives may be strongly affected by some process parameters applied during yeast biomass propagation.

The application of shaking for producing biomass and above all, the use of *H. uvarum* as starting microorganism, are interesting and promising tools for increasing the concentrations of antioxidant compounds in yeast derivatives, possibly reducing the amount of sulfur dioxide to be added along the whole winemaking process. Furthermore, the presence of a non-negligible content of reducing proteins in the insoluble fraction of derivatives from *H. uvarum*, may allow to better protect wine against oxidation, and to favor the removal of mercaptans responsible for the appearance of "reduced" *off-flavors*, thanks to the formation of disulfide bounds. Furthermore, the YDs obtained from *H. uvarum* also showed high contents of polysaccharides, which might potentially contribute to enhance wine mouthfeel and colloidal stability.

Since few specifications are currently reported in literature about the production of biomass to be used for obtaining YDs for winemaking use, the results obtained in the present study may represent a first step for standardizing the manufacturing process and for managing the composition of these enological additives. This might allow obtaining products with specific composition and different winery applications, thus improving their use during both fermentation (as nutritional supplements) and aging on lees (as stabilizing agents and wine quality enhancers). In addition to this, the production of YDs naturally rich in antioxidants and polysaccharides might allow winemakers to improve wine quality and stability in shorter times, possibly reducing the amount of chemical additives (*i.e.*, sulfur dioxide), as well as the conventional period of wine aging. The next step of this research might be to scale-up the process, in order to evaluate its feasibility at large-scale level, also including the estimation of production times and costs, with the aim of standardizing the production chain of yeast derivatives.

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CRediT authorship contribution statement

Sabrina Voce: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Paolo Passaghe:** Writing – review & editing. **Piergiorgio**

Comuzzo: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing interest

The authors declare that they have no conflicts of interest regarding the topics discussed in the present paper.

Data availability

The data that has been used is confidential.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116276.

References

*These research papers are considered key references because they represent the basis for: a) choosing the most promising non-*Saccharomyces* spp. strain in terms of polysaccharides and glutathione production; b) evaluating the most suitable growth conditions for enhancing the production of glutathione and polysaccharides; c) determining antioxidant compounds also in the insoluble fraction of autolyzed yeast suspensions.

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