YEASTS PROTEIN EXTRACTS: SUSTAINABLE STRATEGY FOR WINE PROTEIN STABILIZATION

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1. Introduction

During wine production and storage, several defects can appear. These can be due to unwanted microbiological activity or unstable levels of some compounds, resulting from an unbalanced grape chemical composition or inadequate winemaking practices and storage conditions (Cosme et al. 2021). Several approaches can be adopted to remove the wine defects, but the best strategies are to prevent their appearance by several fining agents or additives, and technologies with different performances and impacts on wine quality.
The fining agents can be classified into several groups: (a) mineral, (b) animal, (c) vegetable-derived products, (d) macromolecules, and (e) mixed formulations (Fernandes et al., 2015). Animal fining agents shows high efficiency, but they can induce allergenic concerns on the consumers' health (Deckwart et al., 2014), and therefore if animal-derived proteins are detected on final wines must be declared on the respective bottle label (Regulation (EU) No 579/2012). Moreover, also the mineral fining agents showed several drawbacks despite their effectiveness. An example is the bentonite, the most common enological adjuvant used against wine protein instability. It is already described that bentonite can decreases aroma compounds and consequently it compromises the wine final quality (Armada et al., 2007). Other problems involved with this mineral product include long settling times, the associated manual handling requirements and the environmental costs for disposal of its waste (Lucchetta et al., 2013).
In order to overcome those concerns, biological and more environmental-friendly alternatives should be preferred and, in the last decades, several research activities have been focused on several innovative fining agents. One of them is represented by the Yeast Derived Products (YDP), obtained from wine and grape endogenous yeasts (Gaspar et al., 2019). There are many variants of YDPs which can be obtained from distinct components of yeasts including cytoplasm, cell wall and vacuole (Lochbühler et al., 2015). Depending on the production method they can be categorized into distinct types: inactive yeasts (obtained by thermal inactivation and drying of the yeasts), yeast autolysates (thermal inactivation followed by an incubation step allowing enzymatic activities and cell wall degradation), yeast hulls or walls (yeast walls without cytoplasmic content), and yeast extracts (the soluble extract of the cytoplasmic content, after elimination of the cell walls) (Pozo-Bayón et al., 2009).
Yeast protein extract (YPE) is obtained mainly from the cytoplasm of yeasts (*Saccharomyces spp)* cells by applying physical methods after an extraction process that limits protein hydrolysis. The extraction process is defined by an OIV resolution, that indicate also some specification of the final product. For example, the total protein content of YPEs must be greater than 50% of the dry product and at least 50% of the total proteins must have molecular weights higher than 15 kDa (OIV, 2012). Yeast proteins have flocculating properties allowing musts and wines clarification and colloidal stabilization and YPEs are already authorized for fining operations in musts and wines with a maximal dosage limit of 60 g/hL for red wines, and 30 g/hL for musts, white and rosè wines.
Proteins in YPEs have variable molecular weights and electrical charges depending on several factors. Moreover, beyond the presence of proteins, YPEs appear as very complex mixtures and their composition and the further activity are influenced by the yeast strains, extraction processes and/or drying processes (Francisco et al., 2021). Some authors highlighted that some yeast proteins showed an isoelectric point (1.4-2.4) below common wine pH, that means they have negative electric charge (Noriega-Dominguez et al., 2010) and consequently could interact with positive charged haze-related proteins.

In view of this, several experimental trials were carried out on two different unstable white wines (Pinot gris and Lugana). In the first step, the chemical properties of an YPE and its effectiveness against wine protein instability were evaluated. In the second step, it was studied the effect of dosage (from 5 to 60 g/hL) and treatment time (from 2 and 10 hours) of the same yeast protein extract. A qualitative analysis of YPE was carried out by the determination of zeta potential with electrophoretic light scattering (ELS). Instead, the effect of YPE addition at different dosages and times was evaluated considering several analytical parameters: turbidity, protein stability tests, and haze-related protein content, determined by HPLC analysis.

2. Material and methods

2.1. Reagents and solvents

Ethanol, methanol, and acetonitrile were of analytical grade (purity > 99%) and purchased from Sigma Aldrich Co. (Milan, Italy). The chemical used which include trifluoroacetic was of analytical grade and purchased from Sigma Aldrich Co. (Milan, Italy). The yeast protein extract for enological uses were purchased from Ever S.r.l. (Pramaggiore, Italy).

2.2. Wine treatments

Two white wines (Pinot gris and Lugana) were considered. Two dosages of the yeast protein extract (25 and 50 g/hL) were adopted on Pinot Gris wine to preliminary evaluate its effectiveness against the initial protein instability.
Moreover, the addition of the yeast protein extract was studied also on Lugana wine, at different dosages (5, 10, 20, 30, 40, 50, and 60 g/hL) and treatment times (2, 4, 6, 8, and 10 hours). At the end of each treatment time the wine was filtered at 0.45 µm and stored in dark flasks at room temperature until analytical determinations. All the treatments were done in triplicate.

2.3. Analytical methods

2.3.1. *Dynamic light scattering (DLS) measurement*

Dynamic light scattering measurements were carried out with a Nicomp 380 ZLS Nanoparticle Size Analyzer (Particle Sizing Systems, Santa Barbara, CA) equipped with a 10 mW He–Ne laser at a wavelength of 633 nm. Measurement occurred at 90° from the incident beam and gave an estimation of the particle mean diameter distribution, expressed in nanometers (nm).
DLS measurements were performed at 20°C for a period of 5 min. All assays were performed in triplicate.

2.3.2. Heat stability test (HT)

The untreated and treated wines were filtered through 0.45 µm filters and 10 mL were sealed in test tubes with screw caps. The tubes were heated at 80°C for 30 min and immediately cooled at room temperature (Gabrielli et al., 2016; McRae et al., 2018). The formula used to obtain this result is the difference between the turbidity after heat exposure and the initial turbidity, considering the data with a difference of ≥5.5 as unstable wine (Moreno-Arribas & Polo, 2009):

$$HT = NTUH - NTU1$$

where *HT* is the heat test, *NTUH* is the value of turbidity after heat treatment, and *NTU1* is the initial turbidity value.

2.3.3. Protein charge neutralization test (PCN)

The protein charge neutralization (PCN) test is a commercial rapid specific method (Protocheck ®) for the evaluation of protein instability (Celotti & Martellozzo, 2006). Each sample was filtered in a 0.45 µm syringe filter and added to the tubes containing a liquid solution of anionic compounds, which reacted with the wine proteins. The turbidity was measured initially and after 60 seconds of mixture agitation, and repeated 5 times. The results were then calculated with the following equation:

$$PCN = NTU2 - (NTU1 / 1.5)$$

where *PC* is Protein Charge Neutralization (PCN) value, *NTU1* is the initial turbidity value, and *NTU2* is the value of turbidity after PCN test.

2.3.4. PR-proteins determination by high performance liquid chromatography (HPLC)

Wine proteins were precipitated from 4 mL of wine sample, adding 20 mL of ethanol (96% v/v). Subsequently, 10 mL of the obtained solution was subjected to centrifugation at 3000 rpm, the ethanol was completely removed, and the proteins were dissolved in 1 mL of milli-Q water. HPLC analysis was performed on an LC-2010 AHT liquid chromatographic system (Shimadzu, Kyoto, Japan), equipped with an integrated autosampler and UV–Vis detector. Compound separation was achieved with a 4.6 × 250 mm Vydac C8 column (Altech, Milan, Italy), coupled with a 4.6 × 5 mm precolumn (Altech, Milan, Italy) with the same stationary phase, and thermostated at 35 °C. The mobile phase was composed of 83% (v/v) solvent A (0.1% trifluoroacetic acid in 8% acetonitrile solution) and 17% (v/v) solvent B (0.1% trifluoroacetic acid in 80% acetonitrile solution). A linear gradient was set as follows: sol- vent B was increased from 17% to 49% in the first 7 min, from 49% to 57%from7to15min, from57%to65%from15to16min, from65%to 81% from 16 to 30 min, and then held at 81% for 5 min before re- equilibrating the column in the starting conditions for an additional 6 min. The injection volume was 100 µL and the flow rate was set to 1 mL/ min.
The peaks were detected at 210 nm and qualitative analysis was carried out as reported in literature (Marangon et al., 2009): peaks with a retention time between 9 and 12 min were assigned to the TL protein classes, whereas peaks eluted from 18.5 and 24.5 min were assumed to be chitinases. Protein quantification was done through a calibration curve of Bovine Serum Albumin (BSA) at different concentration (50-1000 ppm).

2.3.5. Statistical analysis

All experiments and analysis were performed in triplicate and results are expressed as mean ± standard deviation. The Minitab 17 software (Minitab Inc., State College, PA, USA) was used for statistical analysis with one-way analysis of variance (ANOVA, with Tukey's HSD multiple comparison), and the level of significance was set at $p < 0.05$.

3. Results and discussion

3.1. Preliminary trials

The chemical properties of yeast protein extract were evaluated by the **ξ** potential measurements, that indicate the electrical charge at the surface of the hydrodynamic shear surrounding the colloidal particles. The electrical charge measurement is fundamental to understand if the YPE could interact with the haze-related proteins, which have positive charge.

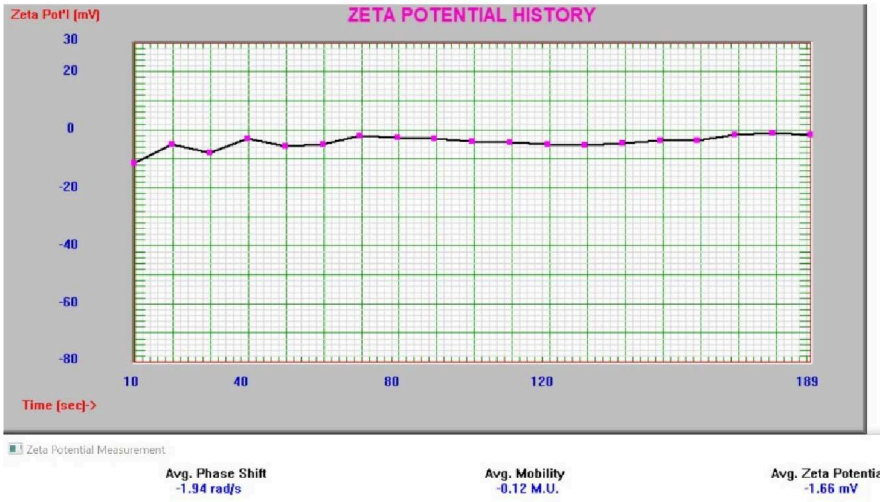

Figure 1. ξ potential measurement of a YPE solution (1g/L) in a tartaric buffer (pH 3.2).

The ξ potential of the yeast protein extract was reported in **Figure 1**. The YPE solubilized in a tartaric buffer (pH 3.2) showed a negative ξ potential (-1.66 mV), confirming its net negative charge, as reported by other authors (Noriega-Dominguez et al., 2010). The quality control of YPEs, focused on their chemical properties, is fundamental due to their complexity and heterogeneity, and ξ potential could represent a useful tool for this purpose.
In view of the quality analysis by DLS measurements, the YPE was added at an unstable Pinot Gris wine with two different dosage (25 and 50 g/hL). The results of protein charge neutralization (PCN) test were reported in **Figure 2**.

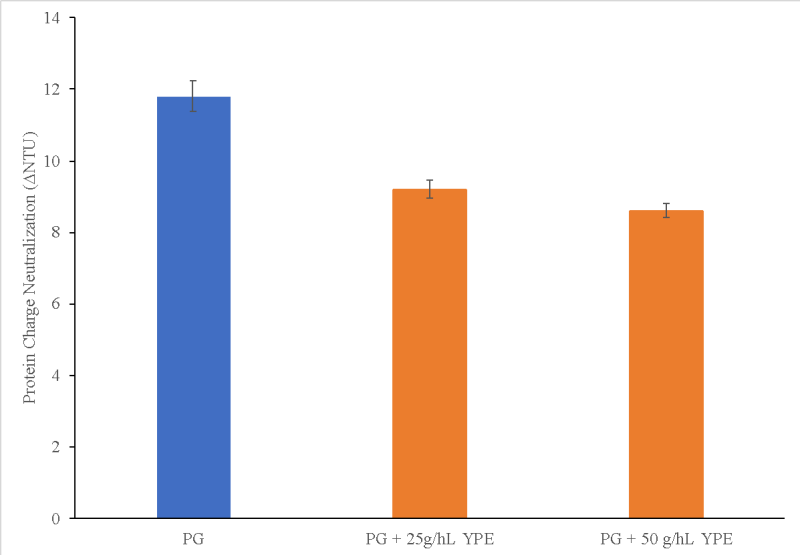

Figure 2. Protein charge neutralization test results of unstable Pinot gris before and after YPE addition at two different dosages (25 and 50 g/hL).

The YPE addition significantly decreased the PCN test, from 11.80 ± 0.43 to 9.20 ± 0.25 (YPE 25 g/hL), and 8.61 ± 0.19 (YPE 50 g/hL). The yeast protein extract allowed a 22% decrease of PCN already at the minimum dosage, and 27% at the maximum one.
The preliminary trials pointed out the effectiveness of YPE against wine haze-related proteins, but a preliminary qualitative analyze is mandatory to estimate its surface electric charge due to complexity and heterogeneity of yeast protein extracts (Francisco et al., 2021).

3.2. Effect of dosage and treatment time

In view of the promising results obtained from preliminary trials, a more detailed investigation on the YPE effect on wine protein stability was performed to take useful information on the interaction mechanism between YPE and PR-proteins. An unstable Lugana wine, as indicated by heat test (33.92 ± 1.08) and PCN test (46.16 ± 0.99), was considered. The effect of different dosages (from 5 to 60 g/hL) and treatment times (from 2 to 10 hours) were studied on several analytical parameters related to protein stability (HT and PCN) and on protein content, determined by HPLC analysis.
The Heat test (HT) and Protein Charge Neutralization test (PCN) results of Lugana wine after YPE addition with different dosages and treatment time were reported in **Figure 3** and **4**, respectively.

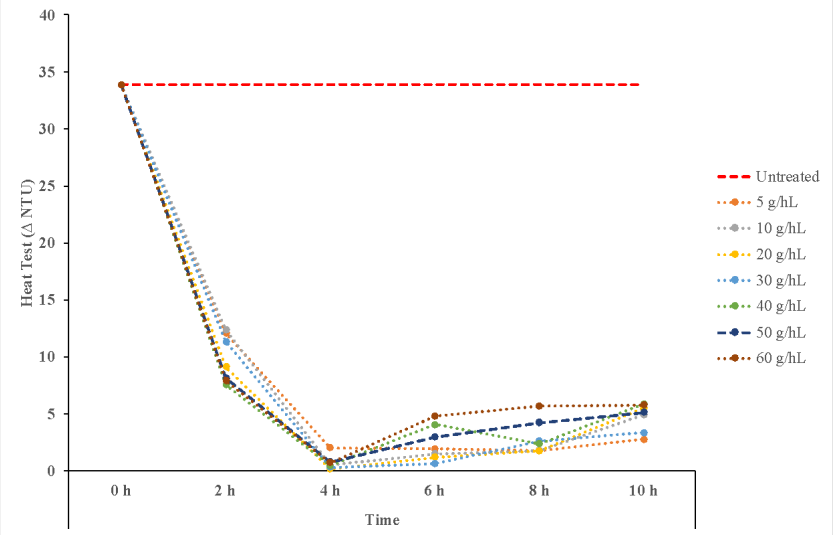

Figure 3. Heat test (HT) results of Lugana wine after YPE addition with different dosages (5, 10, 20, 30, 40, 50, 60 g/hL) and treatment times (2,4,6,8 and 10 h).

As depicted in **Figure 3**, a rapid decrease of HT values was observed in the first four hours of treatment from 33.92 ± 1.08 to 0.75 ± 0.36, highlighting an immediate effect of YPE. Remarkable is the consequent increase of HT for treatment time longer than 4 hours. The HT value increased up to 4.80 ± 1.21 at 10 hours, highlighting an increase in wine protein instability. The results trend could indicate a reversible bond between yeast proteins and PR-proteins, and so the treatment time becomes fundamental factor to consider for the optimization of fining treatments with YPEs.
Moreover, the increase of dosages didn't allow a significant effect on HT test, at 4 hours the minimum YPE quantity was enough to achieve an acceptable protein stability.
The same trend and phenomena were also observed for the Protein Charge Neutralization (PCN) Test (**Figure 4**). The results show a rapid decrease in PCN values during the first four treatment hours, independently of the dosage level, and a consequent increase wine instability for longer times. Also, the PCN results could indicate a potential reversible bond between the YPE and PR-proteins, and the treatment time represents the most important process parameter.

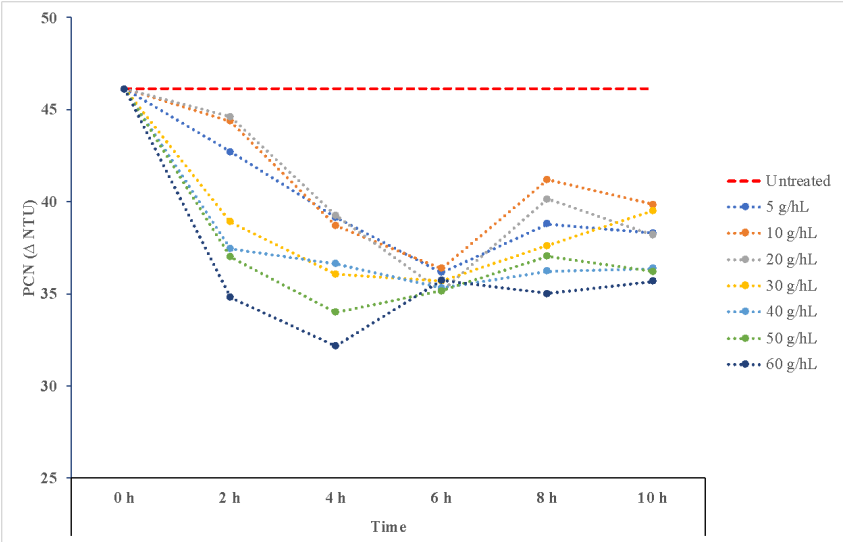

Figure 4. Protein Charge Neutralization test (PCN) results of Lugana wine after YPE addition with different dosages (5, 10, 20, 30, 40, 50, 60 g/hL) and treatment times (2,4,6,8 and 10 h).

Instead of HT test, PCN test allowed to pointed out a significant effect also of dosage. As reported in **Figure 4**, the increase of dosage from 5 to 60 g/hL induced an increase of YPE effectiveness.

The best result was obtained at 4 hours with 60 g/hL of YPE, which decreased the PCN test from 46.16 ± 0.99 to 32.20 ± 0.25, corresponding to a 30% decrease.
Comparing **Figures 3** and **4**, the two stability indices didn't highlight the same stabilization effect due to YPE addition. The heat test showed that YPE addition after 4 hours allowed nearly the complete stabilization of wine, as indicated by the experimental values below the unit.
Instead, the PCN test measurements showed a significant decrease, but the magnitude of the minimum values indicates a further instability of white wine. Generally, several analytical methodologies can be adopted to evaluate the wine protein instability and each one is based on different forced precipitation mechanisms. For instance, the heat test is based on the thermal unfolding of the pathogen-related proteins and the PCN test on the solubility decrease of wine proteins by their interaction with an electrolytic mixture (Cosme et al., 2020).

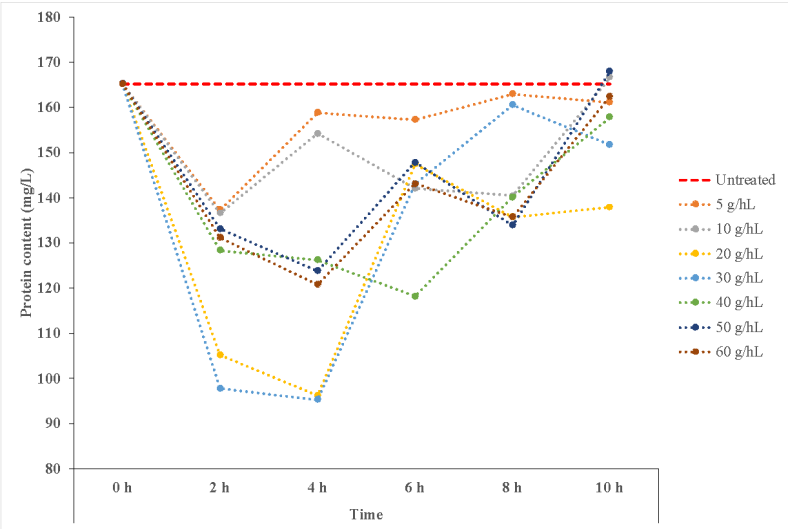

Figure 5. Total Content of PR-Proteins (mg/L) of Lugana wine after YPE addition with different dosages (5, 10, 20, 30, 40, 50, 60 g/hL) and treatment times (2,4,6,8 and 10 h).

More information on possible interaction mechanisms between YPE and PR-protein along treatment can be obtained by the PR-protein quantification with an HPLC method. In **Figure 5** the results of PR-proteins content (mg/L) were reported.
The trend of protein content is similar to stability indices (HT and PCN test) ones. The experimental results showed a decrease in the first period of fining treatment, and an almost increase at times above 6 h. The samples after 10 hours, expected at 20 g/hL, showed a final protein content similar to the Lugana wine before YPE addition (165.25 ± 4.18 mg/L).
However, the YPE addition, after 4 hours and at 20 or 30 g/hL, allowed a 42 % decrease of PR-protein content, from 165.25 ± 4.18 mg/L to 95.35 ± 6.70 mg/L.
The almost increase of PR-proteins content at longer treatment times could indicate a reversible interaction with YPEs, as pointed out by the stability indices. The process time becomes a fundamental factor to be considered to ensure the YPE effectiveness against PR-proteins and the partial stabilization of white wines.

4. Conclusions

The yeast proteins extracts (YPEs) are already approved by OIV for the treatment of musts and wines. The YPEs are constituted of complex and heterogeneous mixtures, and their chemical properties can be extremely variable. Several authors reported interesting electric properties of YPE, that can induce possible specific interaction with haze-related wine proteins and a possible use of YPEs as an alternative and more sustainable fining agent against wine protein instabilities.
Appropriate analytical methods are fundamental to evaluating their chemical properties and specific effectiveness. The **ξ** potential determinations highlighted a negative electric charge of chosen YPE and its potential to interact with PR-proteins. The preliminary trials of YPE addition at

25 and 50 g/hL on unstable Pinot Gris wine allowed a significant decrease of protein instability, measured by the Protein Charge Neutralization test, respectively 22 and 27%.
The experimental trials on an unstable Lugana wine, performed with different dosages and treatment times, confirmed the effectiveness of YPE and the results showed potential reversible interaction with haze-related proteins. The YPE addition is significant for times above 4-6 hours, and its effects disappear for longer times.
The best results were obtained at 20-30 g/hL of YPE concentration and 4 hours, which induced a 42 % decrease of initial PR-protein concentration (from 165.25 ± 4.18 mg/L to 95.35 ± 6.70 mg/L).
The yeast protein extract increased the protein stability of white wines, but it can't allow their complete stabilization. The addition of YPE could be considered a combined treatment with conventional ones, aimed to decrease the amount of conventional fining agents (e.g bentonite) and mitigate the environmental impact of their disposal. The YPE could represent an alternative treatment focused on the main goals of the circular economy and on more sustainable enology.

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5. References

Armada, L., and Falque, E. (2007). Repercussion of the clarification treatment agents before the alcoholic fermentation on volatile composition of white wines. *Eur. Food Res. Technol.* 225, 553–558. doi: 10.1007/s00217-006-0453-3

Celotti, E., & Martellozzo, E. (2006). New analytical approach to unstable protein evaluation in musts and wines. In First international symposium:" Macromolecules of grape and wine". Reims-France, 18-21 May 2006, Abstract book pag. 72. Proceedings, pag. 183-189

Cosme, F.; Fernandes, C.; Ribeiro, T.; Filipe-Ribeiro, L.; Nunes, F.M. White Wine Protein Instability: Mechanism, Quality Control and Technological Alternatives for Wine Stabilisation—An Overview. Beverages 2020, 6, 19. https://doi.org/10.3390/beverages6010019

Deckwart M., Carstens, C., Webber-Witt, M., Schafer, V., Eichhorn, L., Schroter, F., et al. (2014). Impact of wine manufacturing practice on the occurrence of fining agents with allergenic potential. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 31, 1805–1817. doi: 10.1080/19440049.2014.963700

F. Cosme, L. Filipe-Ribeiro, and F. M. Nunes, "Wine Stabilisation: An Overview of Defects and Treatments," Chemistry and Biochemistry of Winemaking, Wine Stabilization and Aging, Feb. 2021, doi: 10.5772/intechopen.95245.

Fernandes JP, Neto R, Centeno F, De Fátima Teixeira M and Gomes AC (2015) Unveiling the potential of novel yeast protein extracts in white wines clarification and stabilization. Front. Chem. 3:20. doi: 10.3389/fchem.2015.00020

Francisco T., Pérez-Gregorio R., Soares S., Mateus N., Centeno F., de Fátima Teixeira M., de Freitas V. (2021) Understanding the molecular interactions between a yeast protein extract and phenolic compounds, Food Research International, Volume 143, 110261, SSN 0963-9969, doi: 10.1016/j.foodres.2021.110261.

Gabrielli, M., Fracassetti, D., & Tirelli, A. (2016). Release of phenolic compounds from cork stoppers and its effect on protein-haze. *Food Control, 62*, 330–336. https://doi.org/10.1016/j.foodcont.2015.11.008

Gaspar LM, Machado A, Coutinho R, Sousa S, Santos R, Xavier A, Figueiredo M, Teixeira MdF, Centeno F and Simões J (2019) Development of Potential Yeast Protein Extracts for Red Wine Clarification and Stabilization. Front. Microbiol. 10:2310. doi: 10.3389/fmicb.2019.02310

Lochbühler, B., Manteau, S., Morge, C., Caillet, M.-M., Charpentier, C., Schnell, S., ... Rauhut, D. (2015). Yeast protein extracts: An alternative fining agent for red wines. *European Food Research and Technology, 240*(4), 689–699.

Lucchetta, M., Pocock, K. F., Waters, E. J., and Marangon, M. (2013). Use of zirconium dioxide during fermentation as an alternative to protein fin- ing with bentonite for white wines. *Am. J. Enol. Vitic.* 64, 400–404. doi: 10.5344/ajev.2013.12143

McRae, J. M., Barricklow, V., Pocock, K. F., & Smith, P. A. (2018). Predicting protein haze formation in white wines: Predicting wine protein haze. Australian Journal of Grape and Wine Research, 24(4), 504–511. https://doi.org/10.1111/ajgw.12354

Noriega-Domínguez, M. J., Durán, D. S., Vírseda, P., & Marín-Arroyo, M. R. (2010). Non-animal proteins as clarifying agents for red wines. OENO One, 44(3), 179–189. https://doi.org/10.20870/oeno-one.2010.44.3.1472

OIV (2012). Monograph on yeast protein extracts (YPE). Resolution OIV-OENO 452-2012. OIV, Paris, France

Pozo-Bayón, Á, Andújar-Ortiz, I., and Moreno-Arribas, M. V. (2009). Scientific evidences beyond the application of inactive dry yeast preparations in winemaking. *Food Res. Int.* 42, 754–761. doi: 10.1016/j.foodres.2009.03.004

Regulation (EU) No 579/2012 of 29 June 2012 amending Regulation (EC) No 607/2009 laying down certain detailed rules for the implementation of Council Regulation (EC) No 479/2008 as regards protected designations of origin and geographical indications, traditional terms, labelling and presentation of certain wine sector products. (2012). Official Journal, L 171, 4-7. ELI: http://data.europa.eu/eli/reg_impl/2012/579/oj