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Multienzyme engineering in the cellar: Versatile biotechnological potential of pectolytic-based clarification enzymes in rosé winemaking

Ilaria Prezioso [a], Gabriele Fioschi [a], Maria Tufariello [b], Luigi Sanarica [c], Ignazio Zara [d], Francesco Grieco [b], Alessia Di Fraia [e], Gabriella Fiorentino [e], Vito Michele Paradiso [a,*]

[a] Department of Biological and Environmental Sciences and Technologies, University of Salento, S.P. 6, Lecce-Monteroni, I-73100, Lecce, Italy
[b] National Research Council - Institute of Sciences of Food Production, via Prov. Lecce-Monteroni, 73100, Lecce, Italy
[c] Enolife S.r.l., Viale Delle Industrie, 74020, Montemesola, Italy
[d] I.I.S.S. Basile Caramia–Gigante, Via Cisternino, 284, 70010, Locorotondo, Italy
[e] Department of Biology, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, 80126, Napoli, Italy

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ABSTRACT

Commercial pectolytic enzymes are widely used in winemaking for must clarification, but their side activities remain scarcely explored. The present study evaluated three commercial clarification enzymes (E1, E2, E3) for their collateral activities and their technological impact in both laboratory-scale and pilot-scale rosé winemaking. Enzymatic assays revealed that E1 exhibited the broadest and most intense accessory activity profile, including significant hemicellulolytic and glycosidase activities. Laboratory trials highlighted beneficial side effects, including increased yeast assimilable nitrogen and decreased calcium ions, an impact of potential interest for calcium tartrate stabilization. Pilot-scale rosé winemaking with Bombino nero and Primitivo grapes demonstrated that clarification enzymes can significantly modulate rosé wine composition, affecting colour indices and volatile profiles in a cultivar-dependent manner. Commercial pectolytic preparations can be considered versatile enzymatic cocktails that can be tailored to grape properties to optimise wine complexity and stability, moving beyond basic clarification toward multienzyme engineering.

1. Introduction

The use of enzymes has become a common practice in winemaking over the last decades. Several enzymatic activities warrant attention for potential applications in wine production, including pectolytic activities, glycosidases, proteases, and glucanases (Benucci et al., 2022; Claus & Mojsov, 2018; Fia et al., 2016; Sui et al., 2020). Pectolytic enzymes are currently the most widely used in winemaking and the cheapest among those commercially available. Their application is mainly aimed at either grape must clarification for white and rosé wine production or at improving colour/phenolic extraction during maceration in red winemaking (Claus & Mojsov, 2018; Osete-Alcaraz et al., 2022). The target of these enzymatic activities is, in fact, the grape cell wall, whose deconstruction improves the sedimentation of suspended solids during must clarification and the extraction of phenolic compounds contained in vacuoles or directly integrated into the cell wall (Ribéreau-Gayon et al., 2006). Pectolytic cocktails are therefore optimised for their target and commercialised specifically as either maceration enzymes or clarification enzymes.

Although characterised primarily by pectolytic activities, commercial enzymatic clarification preparations are usually cocktails of different activities, including β-glycosidases, proteases and esterases (Claus & Mojsov, 2018; Fia et al., 2014). Enologists are aware of some drawbacks regarding side activities in pectolytic preparations. As an example, cinnamoyl esterase activities can impact white wine quality by releasing free cinnamic acids, which *Saccharomyces cerevisiae* converts into undesirable volatile vinyl-phenols (Canal-Llaubères, 2010). On the other hand, the effect of side activities of pectolytic cocktails is not necessarily detrimental to wine quality. In fact, Fia et al. (2016) howed that, besides vinyl phenols, the levels of other volatile compounds (alcohols, acids, esters) could also be affected by enzyme use.

Osete-Alcaraz et al. (2022) recently highlighted the opportunity to revisit paradigms for the use of pectolytic enzymes in oenology to achieve optimal results with appropriate use of this powerful biotechnological tool. In particular, the Authors reported that the use of pectolytic-based clarification enzymes resulted in better chromatic

* Corresponding author.
E-mail address: vito.paradiso@unisalento.it (V.M. Paradiso).

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characteristics in red winemaking compared to traditional maceration enzymes, probably due to the creation of an extraction gradient induced by the precipitation of phenolic compounds bound to the precipitated lees.

As regards rosé winemaking, research on biotechnological approaches to improve the chemical and sensory profile of wines has mainly focused on the use of non-*Saccharomyces* yeasts to leverage their rich enzyme profile and induce enzyme-driven aroma release(Maicas & Mateo, 2023; Tolosa & Maicas Prieto, 2019). Strains of *Torulaspora delbrueckii*, *Lachancea thermotolerans*, and *Metschnikowia pulcherrima* have shown the ability to modulate fermentation-derived volatiles as well as grape-derived ones (Muñoz-Redondo et al., 2021; Paradiso et al., 2022). Very limited research has been conducted on the use of pectolytic enzymes in rosé winemaking, though enzyme technology is widely used (Bayindirli, 2010; Claus & Mojsov, 2018). The application of a commercial enzyme with cellulase and hemicellulase activities before pellicular maceration improved colour features of Monastrell rosé wines, while aroma was improved by the application after fermentation of a commercial pectolytic and cellulosolytic preparation with a declared β-glucosidase side activity (Salinas et al., 2003). Recently, the application of pectolytic enzymes before pellicular maceration was related to improved sensory evaluation in Slovak Cabernet Sauvignon rosé wines (Selnekovic et al., 2025). The addition of a pectolytic enzyme with arabinase side-activity to Shiraz rosé must was evaluated by Kassara et al. (2019), who reported that the enzyme affected colloidal particles and presumably increased polysaccharide solubility. However, no must clarification was performed. To the best of our knowledge, no data is available in the literature regarding the impact of enzyme activities applied during must clarification in rosé winemaking.

The present investigation is therefore aimed at contributing to a revisited paradigm in the use of pectolytic enzymes in rosé must clarification, highlighting other potentially relevant activities, in view of an exploitation of commercial pectolytic preparations as versatile biotechnological tools. To this end, side effects and side activities of three commercial pectolytic-based clarification enzymes were evaluated on different polysaccharides and glycosides at varying pH values. The tested enzymes were then compared in the rosé winemaking of grapes from two different cultivars, to highlight different behaviours.

2. Materials and methods

2.1. Materials

Three clarification enzymes were provided by Enolife s.r.l. (Montemesola, Italy): one powder sample (E1) and two liquid samples (E2 and E3). The technical sheet provided by the manufacturer reported the following enzymatic activities:

- E1: pectinlyase min 50 U g^{-1}; pectinmethyl esterase min 350 U g^{-1}; polygalacturonase min 1750 U g^{-1};
- E2: pectinlyase min 72 U g^{-1}; pectinmethyl esterase min 561 U g^{-1}; polygalacturonase min 880 U g^{-1};
- E3: pectinlyase min 300 U g^{-1}.

The *S. cerevisiae* strain LF13V was provided by Enolife s.r.l. (Montemesola, Apulia, Italy). Diammonium phosphate (DAP) and yeast derivative (Aminoarom®) provided by Enolife s.r.l. (Montemesola, Apulia, Italy). The wine model solution (12 % ethanol, 5 g L^{-1} tartaric acid, pH 3.4) was prepared using reagent-grade chemicals.

2.2. Analysis of side activities in pectolytic-based clarification enzymes

The three clarification enzyme mixtures were tested for their side activities using different synthetic substrates. The powder sample was initially dissolved in 50 mmol L^{-1} sodium phosphate buffer (pH 7) prior to analysis, whereas the pH of the liquid samples was measured using a pH meter. Protein concentration was quantified according to the Bradford assay, using a standard calibration curve constructed with bovine serum albumin (BSA) (Bradford, 1976). For the powder sample, 10 mg were dissolved in 40 μL of 50 mmol L^{-1} sodium phosphate buffer (pH 7) before the assay. The protein concentration in the three pectolytic formulations was the following: E1, 1.48 mg g^{-1}; E2, 3.9 mg g^{-1}; E3, 1.33 mg g^{-1}.

2.2.1. DNS assay

Hydrolytic activity was assessed using the 3,5-dinitrosalicylic acid (DNS) assay with a panel of plant-derived polysaccharide substrates, including, besides pectin, xylan, carboxymethyl cellulose (CMC), konjac, lichenan, galactomannan, and arabinogalactan. The colorimetric method measures the release of reducing sugars resulting from the internal cleavage of glycosidic bonds (Gusakov et al., 2011). Reaction mixtures (total volume: 40 μL), containing 1 % (w/v) substrate and 50 mmol L^{-1} sodium citrate buffer (pH 3, 4, 5), were pre-incubated at 25 °C for 10 min. Subsequently, 1 μL of enzyme sample (E1, E2, or E3) was added, and the reactions were incubated for 30 min before being quenched on ice. For detection, 40 μL of each reaction mixture was transferred to a 96-well microplate, and 160 μL of DNS reagent was added. Plates were incubated at 100 °C for 20 min using a thermoblock (Eppendorf, Hamburg, Germany), followed by cooling at 4 °C for 45 min. Absorbance was measured at 540 nm using a multiwell plate reader (Synergy H4, Biotek, Agilent, Santa Clara, CA, USA) (Carbonaro et al., 2024). The data reported are the average of three experiments performed in triplicate.

The amount of reducing sugars (μmol) was quantified using a calibration curve generated from standard solutions containing 0, 0.2, 0.4, 0.6, 0.8, and 1 μmol of D-(+)-glucose monohydrate and D-(+)-xylose.

2.2.2. pNP assay

Side glycosidase activities were evaluated using selected p-nitrophenyl (pNP)-glycosides as chromogenic substrates to cover a broad range of glycosidic linkages. The following substrates were tested: pNP-β-D-xylopyranoside, pNP-α-D-glucopyranoside, pNP-β-D-glucopyranoside, pNP-α-D-galactopyranoside, pNP-β-D-galactopyranoside, pNP-α-L-arabinofuranoside, pNP-α-L-fucopyranoside, pNP—β-L-fucopyranoside pNP-α-L-rhamnopyranoside, pNP-α-D-mannopyranoside, and pNP-β-D-mannopyranoside. Reactions were carried out at 25 °C by adding 2 μL of the E1, E2, or E3 to a 100 μL reaction mixture containing 10 mmol L^{-1} substrate and 50 mmol L^{-1} sodium citrate buffer at pH 3, 4, or 5. After 15 min of incubation, reactions were stopped with 100 μL of 1 mol L^{-1} sodium carbonate. Absorbance was measured at 405 nm using a multiwell plate reader (Synergy H4, Biotek, Agilent, Santa Clara, CA, USA). Direct quantification of pNP-OH formation enabled assessment of the enzymes' ability to cleave glycosidic bonds at the non-reducing ends of substrates. The activities were expressed in International Units (U), corresponding to the quantity of enzyme(s) able to release 1 μmol of pNP-OH in 1 min under the conditions described (extinction coefficient, 1850 m^2 mol^{-1}). The experiments were carried out with three technical and biological replicates.

2.3. Laboratory-scale clarification trials

Vitis vinifera L., cv. *Bombino nero* grapes were harvested at technological maturity and phytosanitary health in a vineyard located in Locorotondo (Bari, Apulia, Italy), placed in boxes, transported to the laboratory, and immediately processed. Grapes were manually crushed and pressed with a benchtop vertical screw press, yielding about 50 % of the must weight. The obtained must was added with 15 g hL^{-1} of potassium metabisulfite. Aliquots of 100 mL were treated in 100 mL cylinders for laboratory scale clarification trials. The enzymes were added at the amounts suggested by the manufacturer: 3 g hL^{-1} for E1 (as a powder, previously dissolved in water at 1:10 w/v ratio), 10 g hL^{-1} for E2 and E3 (in liquid form). No addition was made to the control must. A

gentle mixing was applied after the addition to distribute the enzymes in the musts. Static clarification was carried out at 7 °C for 24 h. The laboratory scale clarification trial was carried out at least in triplicate, in comparison with control must clarified without the addition of any enzyme. The turbidity of the clarified juice was determined by a turbidimeter (Hanna Instruments, Woonsocket, RI, USA). Clarified juices and lees were then analysed. Glucose and fructose (g L^{-1}) and total dry extract (g L^{-1}) were determined by Fourier transform infrared spectroscopy (FTIR) by a WineScan™ FT 120 (FOSS®, Hillerød, Denmark). Titratable acidity (g tartaric acid L^{-1}) was determined by titration, and pH was measured with a pH meter. The amount of Ca^{2+} was determined by the Arsenazo III iron-chromogen reaction using a Hyperlab Smart enzymatic analyzer (Steroglass s.r.l., Perugia, Italy) (Fioschi et al., 2024). Lees were centrifuged before all analyses except turbidity.

2.4. Pilot-scale winemaking

Grapes from *Vitis vinifera* (L.) cv. *Bombino nero* and *Primitivo* were harvested at technological maturity and phytosanitary health in a vineyard located in Locorotondo (Bari, Apulia, Italy), placed in boxes, transported to the winery, and immediately processed. Bombino nero and Primitivo rosé wines were produced using the three enzymes during must clarification, while control wines were obtained without any clarification enzyme addition. Each wine was produced in triplicate. The winemaking protocol was the same as that reported in Paradiso et al. (2022). Grapes were crushed and destemmed. After 2-h skin contact at room temperature (20 °C ± 2 °C) and pressing, juice was sulfited (7.5 g hL^{-1} SO_2 added as potassium metabisulfite), divided into three aliquots, each one treated with one of the three pectolytic enzymes. The enzymes were added at the amounts suggested by the manufacturer: 3 g hL^{-1} for E1 (as a powder, previously dissolved in water at a 1:10 w/v ratio), 10 g hL^{-1} for E2 and E3 (in liquid form). No addition was made to the control must. A gentle mixing was applied after the addition to distribute the enzymes in the musts. Static clarification was carried out in jacketed stainless steel tanks at 7 °C for 24 h. Each juice aliquot was further divided into three 50 L stainless steel tanks in order to carry out three independent fermentation processes. Diammonium phosphate (DAP, 30 g hL^{-1}) and yeast derivative (30 g hL^{-1}) were added to the clarified juice before inoculation with the yeast starter (dry active yeast reactivated in water, 1 • 10^{10} CFU g^{-1}; 20 g hL^{-1}). Fermentation was carried out at 16 °C ± 1 °C using plate heat exchangers and temperature probes connected to a central cooling station. After fermentation, wines were racked into glass jars, sulphited (25 mg L^{-1} SO_2 added as potassium metabisulfite), and bottled until analyses.

2.4.1. Wine analysis

Colour indices were determined using the modified Somers assay described by Mercurio et al. (2007). The following indices were measured: color density corrected for SO_2 (C.D. corr.), Hue, total phenols (TP), total anthocyanins (T.A.), anthocyanin ionization (A.I.), anthocyanin ionization corrected for SO_2 (A.I. corr), SO_2 resistant pigments (RP), chemical age (chem age), chemical age 2 (chem age 2). Volatile compounds were analysed according to Tufariello et al. (2012).

2.5. Statistical analysis

One-way analysis of variance (ANOVA) followed by the post hoc Tukey's HSD test was performed on the data from laboratory-scale clarification trials. As regards pilot-scale winemaking, both one-way analysis of variance (ANOVA) to compare enzymes within each cultivar and two-way ANOVA with interactions to compare cultivars and enzymes and to evaluate different enzyme behaviours in the two cultivars were performed, followed by the post-hoc Tukey's HSD test. ANOVA robustness was checked based on the following conditions: equality of variances (Levene's test) and normality of residuals (Q-Q plot). When the conditions were not all satisfied, one-factor or two-factor non-parametric analysis (Kruskal-Wallis test) was carried out, followed by Dunn's post-hoc test.The analyses were carried out with JASP (JASP Team, 2024).

Hierarchical clustering with heatmap visualization (data autoscaling, Euclidean distances, Ward's clustering algorithm) was performed using Metaboanalyst 6.0 (Xia Lab, 2024). Datagraph software ver. 5.2 was used for other Figures.

3. Results and discussion

3.1. Analysis of side activities in pectolytic-based clarification enzymes

A representative panel of the main structural components of the vegetable/yeast cell walls was selected as substrates, including xylan, CMC, konjac, glucomannan, glucan, galactomannan, and arabinogalactan (Fig. 1). Enzymatic assays were conducted at three pH values (3.0, 4.0, and 5.0) to assess the influence of pH on enzymatic activity. The activities of the commercial preparations are related to 1 mg of enzyme mixture. Pectolytic activity was in the range ≈115–130 U mg^{-1} for E1, ≈10 U mg^{-1} for E2, ≈25–30 U mg^{-1} for E3. E1 also showed high levels of hydrolytic activities towards the other polysaccharides under examination (i.e., xylan, carboxymethylcellulose, konjac polysaccharides, glucan, galactomannan and arabinogalactan) with values ranging ≈ 50–110 U mg^{-1} across the entire tested pH range, with the exception of arabinogalactan at pH 5. Enzyme E2 showed the lowest activities per mg of enzyme mixture, with values of a few U mg^{-1} and some minima near zero for glucan at pH 4, galactomannan at pH 3 and arabinogalactan at pH 5. Finally, enzyme E3 showed intermediate levels of activity towards other polysaccharides, mainly in th ranhe ≈ 4–20, except for almost zero activity towards arabinogalactan at pH 5. Among the tested formulations, enzyme E1 exhibited the broadest and more intense activity profile across all substrates, whereas E2 showed only minimal activity. E3 displayed relevant activity on most substrates, even though it was much lower compared to E1.

As shown in Fig. 1, pH 4.0 generally represented the most favourable condition for the accessory activities analysed. Endogenous grape enzymatic activity typically requires a high pH and is generally reduced or inhibited under the acidic conditions found in wine (Pérez-Martín et al., 2012; Ruiz et al., 2019). However, in the present study, the enzyme E1 activity at pH 3 was found to be comparable to, or only slightly lower than, that at pH 4. This suggests that E1 remains active and exerts significant enzymatic function even under the acidic conditions characteristic of wine.

These results are consistent with findings reported for non-*Saccharomyces* yeast strains used in winemaking processes (Tufariello et al., 2021), which displayed complex enzymatic profiles, including xylanase, cellulase, and galactomannanase activities. Although these studies refer to crude microbial extracts, they provide a useful comparative framework for interpreting our data. Similarly, commercial enzymatic preparations derived from microbial fermentations may exhibit comparable functional compositions, thus effectively acting as enzymatic cocktails. The coexistence of pectinolytic and hemicellulolytic activities observed in our samples suggests a potential synergistic action on the must matrix, by enhancing the clarification efficiency and the release of structural and grape-derived volatile compounds relevant to winemaking. All tested enzymes, and in particular enzyme E1, showed relevant β-glucanase activity, reaching almost 80 U mg^{-1} at pH 3 and 75 U mg^{-1} at pH 4. This side activity could be of interest as it could be tested to improve either the clarification in musts derived from grapes affected by *Botrytis* spp. (Jadhav & Gupta, 2016) or the release of yeast lysis-derived products (Rodriguez-Nogales et al., 2012; Torresi et al., 2014). As well, the high arabinogalactan activity of the enzyme E1 at pH 3 and 4 (≈60 and 80 U mg^{-1}, respectively) could impact the role of this significant pectic fraction, acting on astringency perception (Kuhlman et al., 2024; Lei et al., 2023). The xylanase activity, instead, has been reported to increase free-flow wine yield and to impact the volatile profile of the

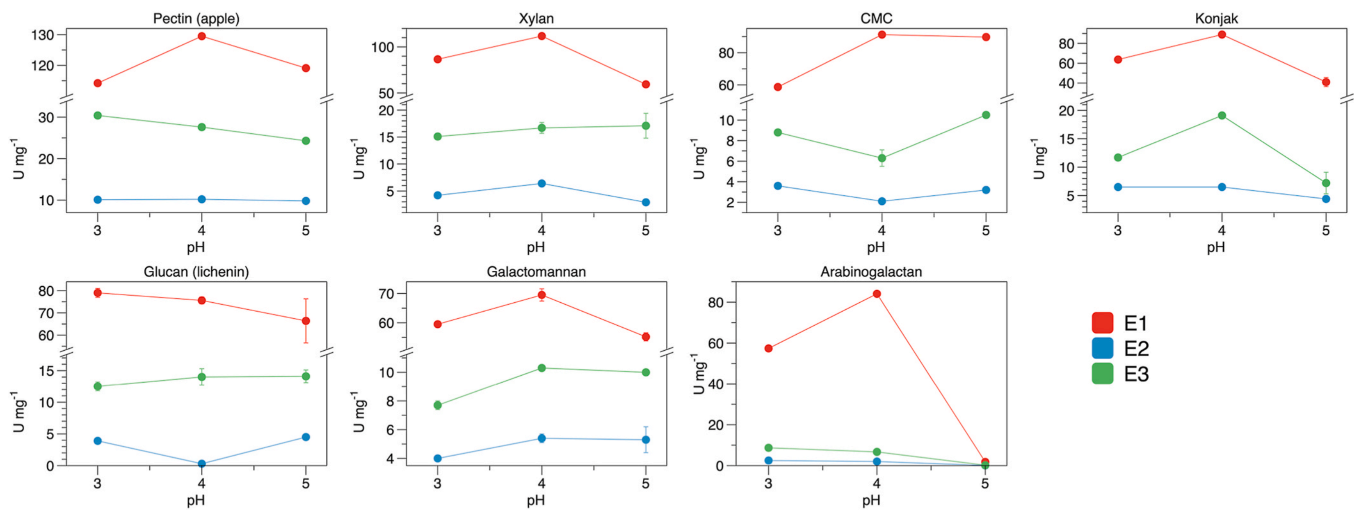

Fig. 1. Hydrolytic activity (expressed as U mg^{-1} protein) of E1, E2 and E3 commercial pectolytic enzymes towards structural and non-structural polysaccharides evaluated at pH 3, 4 and 5.

produced wine (Louw et al., 2006).

To further investigate glycosidic bond specificity, additional assays were performed using *p*-nitrophenyl (pNP) substrates at pH 3, 4, and 5. The evaluation of this aspect is extremely important in winemaking since different enzymes are usually required to release glycosylated aroma precursors. As shown in Fig. 2, the highest glycosidase activity in the pectolytic preparations was α-arabinofuranosidase, reaching values around 5 and 10 U mg^{-1} in E2 and E1 preparations, respectively. In particular, in E1 the α-arabinofuranosidase activity was comparable at pH 3 and 4 (9.88 and 10.12 U mg^{-1}, respectively), with a decline at pH 5 (6.16 U mg^{-1}). The α-arabinofuranosidase activity was predominant at pH 3 and 4 across all preparations. This activity can play a role in grape aroma release, since terpene di-glycosides are found in grapes, whose disaccharide moieties include 6-*O*-α-l-rhamnopyranosyl-β-d-glucopyranose, 6-*O*-α-l-arabinofuranosyl-β-d-glucopyranose, 6-*O*-β-d-xylopyranosyl-β-d-glucopyranose and 6-*O*-β-d-apiofuranosyl-β-d-glucopyranose (Belda et al., 2017; Gunata et al., 1985; Thakur et al., 2019; Voirin et al., 1990). The liberation of the terpene from such glycosides requires a two-step hydrolysis, providing at first the intervention of exoglycosidases that cleave the terminal sugar residue (β-xylopyranosidase, α-arabinofuranosidase, α-rhamnopyranosidase, β-apiofuranosidase); the second step consists of the intervention of β-glucopyranosidase that remove the residual sugar moiety from the aglycone (Belda et al., 2017; Gunata et al., 1988; Rodríguez-Nogales et al., 2024; Ugliano, 2009). Interestingly, enzyme E1 presented the highest activities of β-xylopyranosidase (almost 1.0 U mg^{-1} at all pH values, compared to levels below 0.2 U mg^{-1} in the other enzymatic preparations) and β-glucopyranosidase (0.73 U mg^{-1} at pH 4). The β-glucopyranosidase activity is particularly important in winemaking because of its role in the hydrolysis of monoglycosides and di-glycosides aromas in wine grapes (Martínez et al., 2025). The α-rhamnopyranosidase activity was relatively lower in all enzymes at all pH values. Notably, it was detected exclusively in E1 at all pH values and in E3 at pH 5 (out of the pH range of musts), and it is strongly related to the modulation and the enhancement of wine aroma (Yadav et al., 2010).

The β-galactosidase activity was also consistently detected in all samples, whereas α-galactosidase activity was similar in E1 and E3 but markedly lower in E2. The β-galactosidase showed in E1 its highest activity at pH 3 (1.87 U mg^{-1}) and the lowest at pH 4 (1.20 U mg^{-1}), while showing more constant activity across the pH range in the other two enzyme cocktails (around 1.5 U mg^{-1} in E2 and 1.0 U mg^{-1} in E3). The α-galactosidase was relatively lower, in the range 0.7–0.8 U mg^{-1} in E1 and E3, about 0.3 U mg^{-1} in E2. Both galactosidase activities have been previously associated with grape softening during ripening, suggesting a role in the hydrolysis of wall polysaccharides (Fuentes et al., 2018; Gao et al., 2019), even though no significant effect was found in Monstraell red wines obtained using β-galactosidase (Apolinar-Valiente et al., 2014). However, red winemaking implies more extended maceration than rosé winemaking, so analogous outcomes cannot necessarily be inferred.

The α- and β-fucopyranosidase activities were negligible, as well as the α-mannosidase.

The β-D-mannosidase activity was more relevant and present only in E1 and in E3 at pH 4. This activity, as well as the α-arabinofuranosidase, is, however, involved in hemicellulose hydrolysis (Aulitto et al., 2019; Juturu & Wu, 2013; Moreira & Filho, 2008; Thakur et al., 2019). Therefore, a contribution to the deconstruction of wall polysaccharides can be hypothesized.

E1 displayed, therefore, the most comprehensive and active enzymatic profile, while E2 and E3, although marketed for clarification, also showed some relevant glycosidic activities, suggesting broader functional potential. These findings are also consistent with previous reports (Fia et al., 2014; Guérin et al., 2009), which similar activity profiles in enzymes used for juice processing and oenological applications.

The detection of α-rhamnosidase and β-mannosidase in E1, and to a lesser extent in E3, further expands the functional spectrum of these preparations. These findings indicate their potential use in biotechnological applications targeting the degradation of complex glycans and the release of varietal aroma precursors (Fernández-Pacheco et al., 2021), thus compensating for the absence of these enzymatic activities in grape and fermentation yeast enzymes.

Overall, these results highlight the enzymatic richness of commercial pectinolytic preparations, which still maintain their complexity within the wine-typical pH range (3–4). The accessory activity profiles observed suggest a technological potential that extends beyond clarification, encompassing the release of grape-derived volatile compounds and structural modification of the plant matrix under the acidic conditions typical of must and wine. A deeper understanding and strategic exploitation of these activities might open new application perspectives in oenology and beyond.

3.2. Effects of clarifying enzymes on must quality in laboratory-scale clarification trials

Fig. 3 reports some quality parameters and post-hoc comparisons for clarified musts and lees (cv. Bombino nero) derived from laboratory-scale clarification trials. The full results and their statistical analysis are reported in Supplementary Table S1. Must clarification was

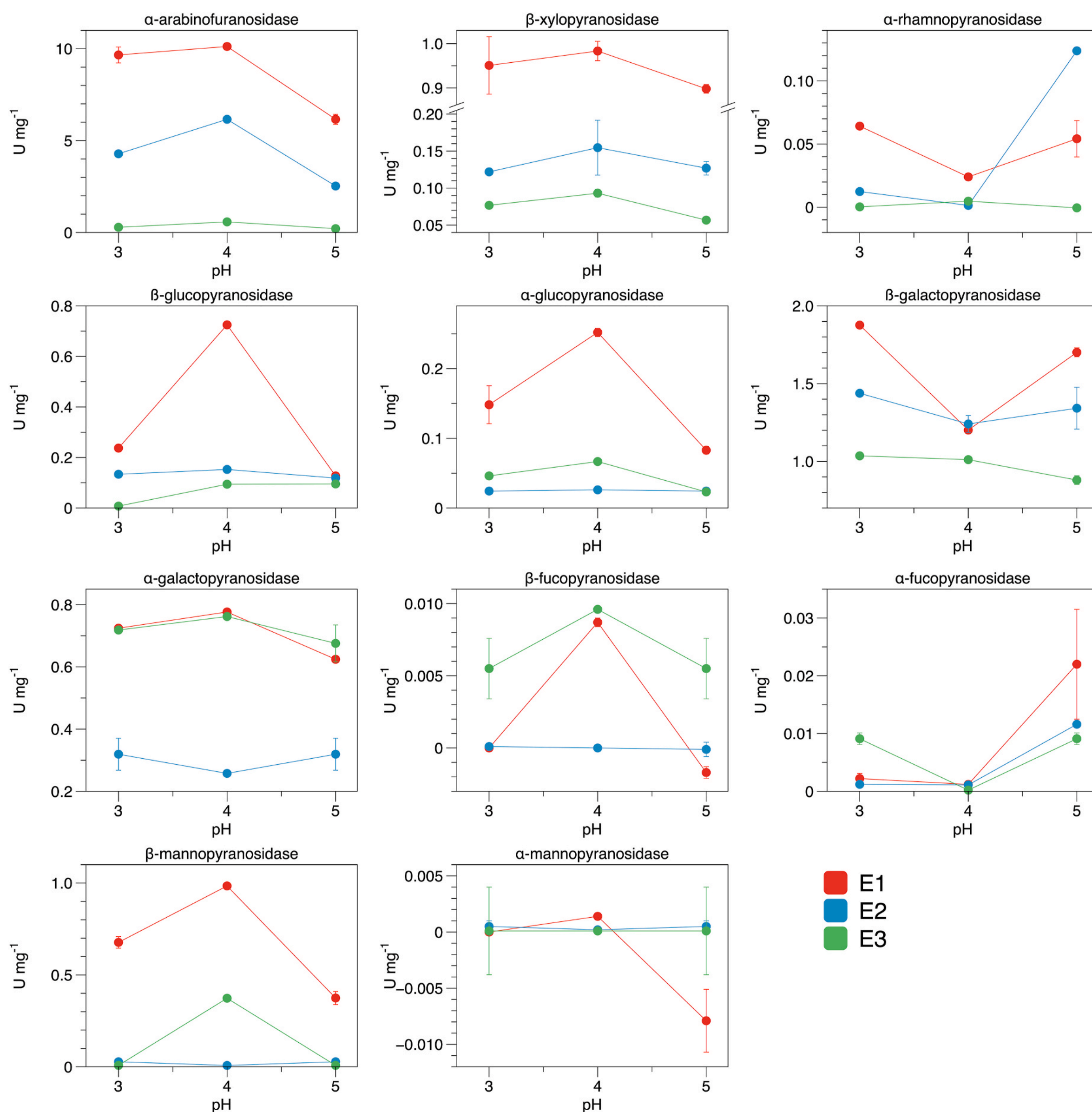

Fig. 2. Glycosidic bond specificities of side activities(expressed as U mg^{-1} protein) of E1, E2 and E3 commercial enzymes evaluated at pH 3, 4 and 5.

dramatically improved, as expected, by the enzymatic treatments, resulting in slightly but significantly different final turbidity levels (Fig. 3/I). In particular, enzyme E1, which showed the highest activity towards structural polysaccharides, resulted in the lowest final turbidity levels. The enzymatic treatment of crushed grapes resulted in a significant increase in reducing sugars (about 7 g L^{-1}) and total extract (Fig. 3/II and 3/III, respectively) compared to the control must. Literature reports increases, not in all cases significant, of reducing sugars as a consequence of the hydrolysis of polysaccharides (Chakraborty et al., 2023; El Darra et al., 2016; Masino et al., 2008; Wang et al., 2023). The enzyme E2 also determined an increase in sugars in the lees sediment compared to the control. In contrast with the findings of Masino et al. (2008), a slight but significant decrease in titratable acidity was also observed (Fig. 3/IV). The cause of such a decrease should be investigated. Slight differences were also observed in the titratable acidity of lees. Another significant effect of enzymatic clarification concerned the yeast assimilable nitrogen (YAN) content (Fig. 3/V). In musts treated with enzymes, YAN increased by 14 %–18 % compared to the control must. Although the levels achieved cannot be considered sufficient for optimal yeast nutrition, this increase may still be relevant for the subsequent fermentation process. The literature reports inconsistent findings on this topic, with studies showing increases (Scutaraşu et al., 2023), decreases (Burin et al., 2016), or no significant effect (Fia et al., 2016). The tested enzymes differed significantly in their impact on YAN content, with enzyme E1 producing the greatest increase, despite being applied at the lowest concentration (mg protein hL^{-1} must). Interestingly, a significant decrease in calcium ions was also observed in musts clarified with E1 and E3 enzymes (Fig. 3/VI), which also showed the highest activities toward pectin. Previous research has highlighted the ability of pectic polymers (polyuronic acids, rhamnogalacturonans) to bind calcium ions (De Fátima & Marques, 2014, pp. 1–11; McKinnon et al., 1996; Pellerin et al., 2013). We hypothesize that the observed decrease in calcium levels is related to the release of calcium-binding pectic fragments, though measurement of these fragments is required to confirm this hypothesis. This potential side effect of clarifying enzymes is indeed worth attention. In fact, calcium tartrate instability in white and rosé wines has emerged as a growing concern in recent years, primarily due to the impact of global warming on wine composition (AWRI, 2018; Fioschi et al., 2024; Philipp et al., 2022). Nowadays, there are few tools for stabilizing calcium tartrate, with either limited efficacy

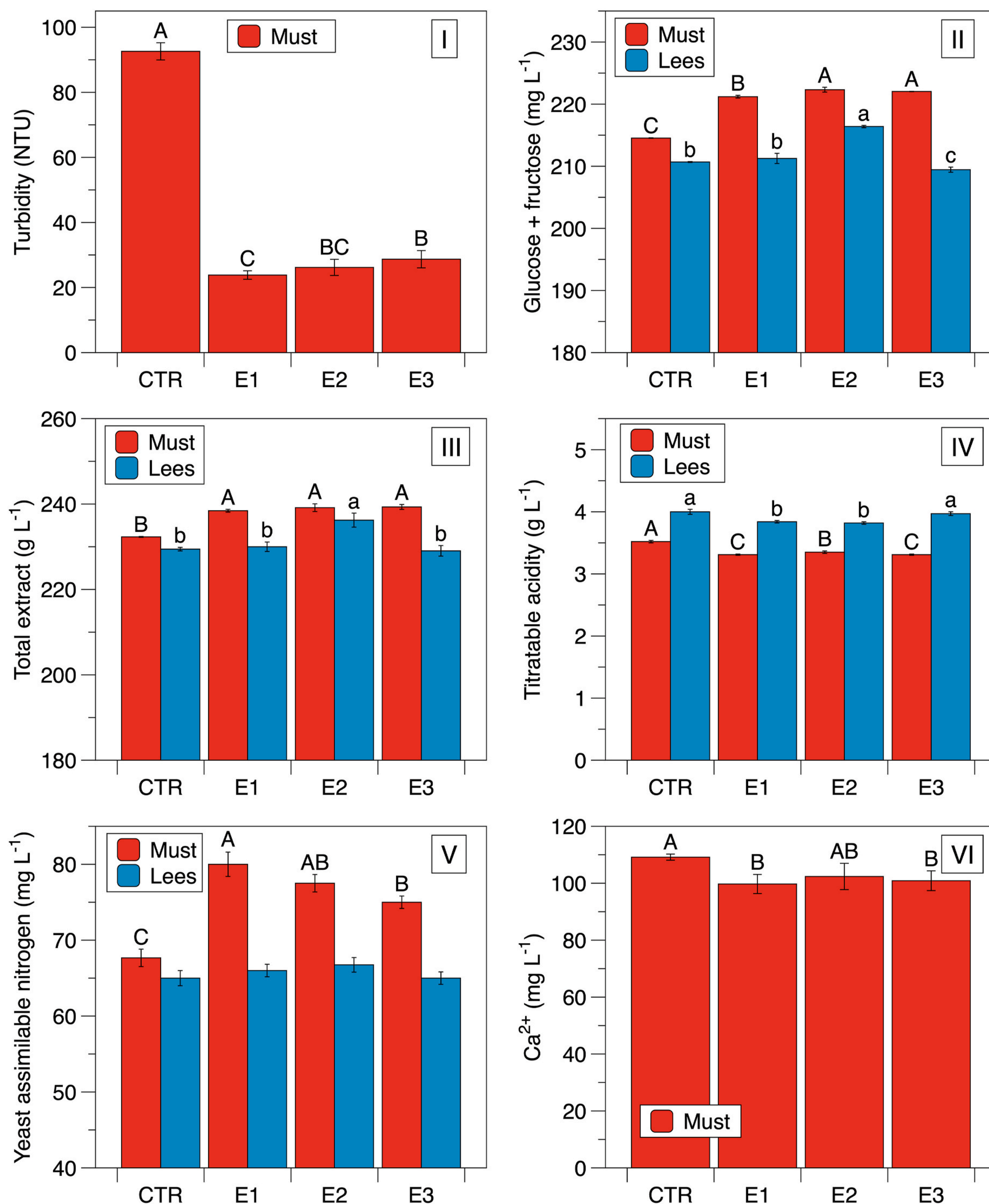

Fig. 3. Bar plots (means ± standard deviations) of the quality parameters of musts and lees after must clarification using different enzymes. The results of post-hoc Tukey's HSD test for multiple comparisons following one-way analysis of variance (ANOVA) are reported in labels. Different letters indicate a significant difference at $p < 0.05$. Capital letters refer to musts, lowercase letters refer to lees.

or high cost (Fioschi et al., 2024; Philipp et al., 2022). Together with pH and tartaric acid content, the calcium content is one of the factors determining the final stability of calcium tartrate (AWRI, 2018). Even though we observed a decrease in calcium content insufficient to suggest a practical impact on calcium tartrate stability, further research on the clarification stage and on pectolytic-based clarification enzymes, even applied before pressing, could provide a potential biotechnological approach to calcium ion removal and calcium tartrate stabilization, warranting further investigation.

3.3. Side effects of clarifying enzymes on wine properties in pilot scale winemaking

Three independent pilot-scale vinifications were carried out using three different enzymes on grapes from two different cultivars (Bombino nero and Primitivo). The cultivars were chosen as representatives of typical Apulian cultivars, differing in skin thickness and hardness, with Bombino nero having high skin thickness and hardness, and Primitivo having very low.

The analytical data related to the rosé wines and the corresponding

results of statistical analysis are reported in Supplementary materials (Tables S2 and S3). Fig. 4 reports, instead, the heatmap deriving from the hierarchical clustering analysis, including data of color indices and volatile profile of the wines, together with the ANOVA/Kruskal-Wallis significance.

As expected, both volatile compounds and color parameters clearly clustered, differentiating the two grape cultivars. As regards color, higher color density and related color indices (total phenols, total anthocyanins, SO_2-resistant pigments, anthocyanin ionization corrected for SO_2 content) characterized the wines from Bombino nero. In contrast, wines from Primitivo presented higher hue and chemical age of color. Also, the volatile profile differentiated the wines obtained from the two cultivars.

The Primitivo rosé wines showed richer volatile profiles, with higher amounts of some relevant esters, including ethyl leucate (Antalick et al., 2015) and ethylphenyl acetate (clusters I and II in the Figure and Table S3), while Bombino nero wines showed higher levels of ethyl-2-hexenoate and ethyl-2-decenoate. Also, grape-derived volatile

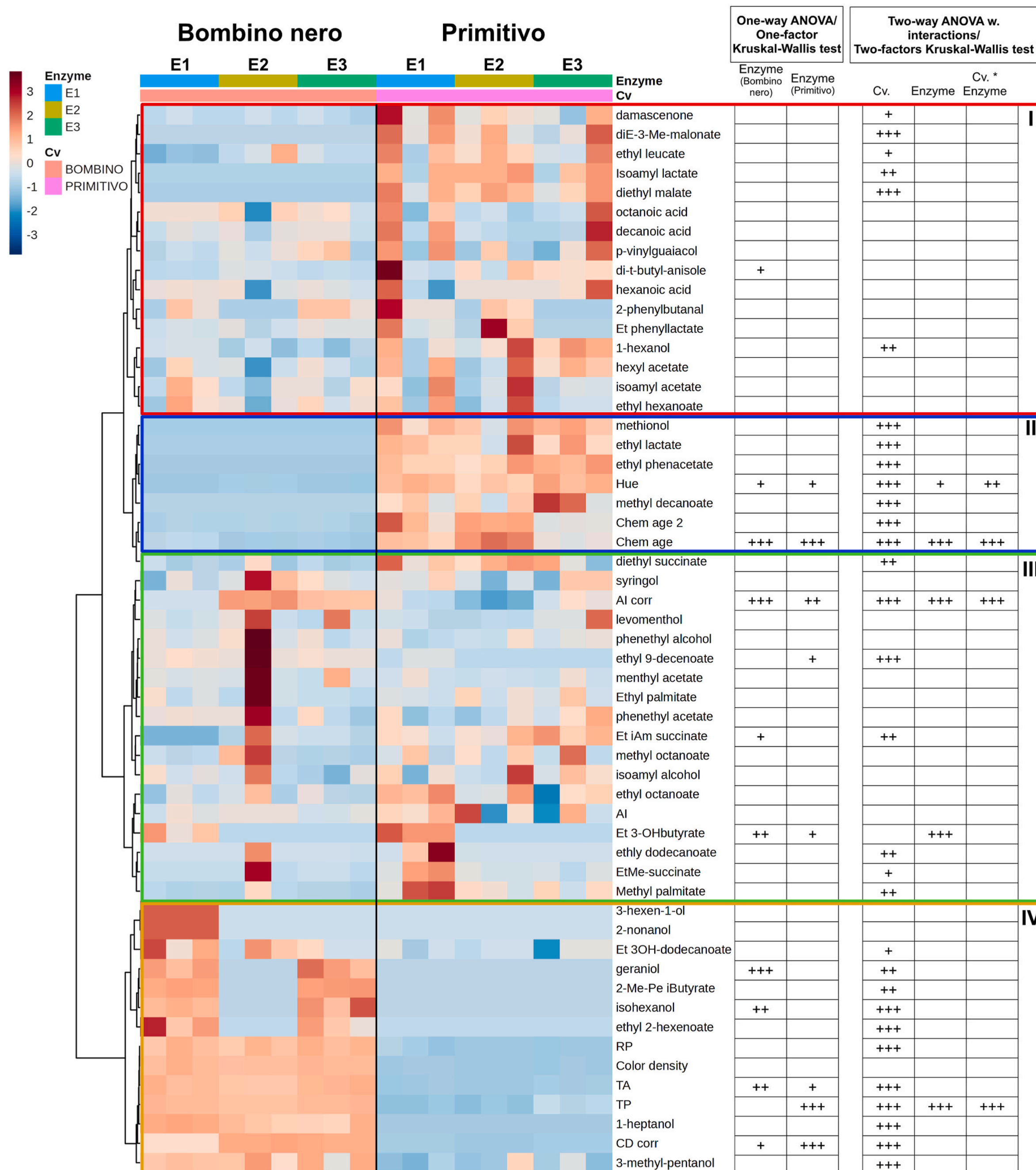

Fig. 4. Heatmap of volatile compounds and phenolic indices. TP, total phenols; T.A., total anthocyanins; A.I., anthocyanin ionization; A.I. corr, anthocyanin ionization corrected for SO_2; R.P., SO_2-resistant pigments; chem age, chemical age; chem age 2, chemical age 2; EtMe-succinate, ethyl methyl succinate; ethyl phenacetate, ethyl phenylacetate; phenethyl acetate, phenylethyl acetate; Et phenyllactate, ethyl phenyllactate; Et 3OH-dodecanoate, ethyl 3-hydroxy-dodecanoate; Et I.am succinate, ethyl isoamyl succinate; 2-Me-Pe iButyrate; 2-methyl-pentyl isobutyrate; DiE-3-Me-malonate, diethyl-3-methylmalate; Ethyl palmitate, ethyl hexadecanoate; Methyl palmitate, methyl hexadecanoate; Et 3-OHbutyrate, ethyl 3-hydroxybutyrate; phenethyl alcohol, phenylethyl alcohol; phenacetaldehyde, phenylacetaldehyde; 2-phenylbutanal, 2-phenylbutyraldehyde; di-t-butyl-anisole, di-tert-butyl-anisole. One-way ANOVA/One-factor Kruskal-Wallis test significance of Enzyme within each cultivar, as well as Two-way ANOVAwith interactions/Two-factors Kruskal-Wallis test significance of cultivar (Cv), enzyme and cultivar*enzyme interaction is reported in the table on the right (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$).

compounds differentiated Bombino nero and Primitivo wines, with the former showing the terpene geraniol in its volatile profile and the latter showing higher amounts of the norisoprenoid β-damascenone (clusters 1 and 4, respectively; Table S2). Regarding the impact of pectolytic enzymes and their interaction with the cultivar on phenolic indices and the volatile profile, several variables were affected by the enzymatic treatment. Cluster 1 included only parameters affected by the cultivar and predominantly characterizing Primitivo wines, except for di-tert-butyl-anisole, whose levels in Bombino nero increased when the E2 enzyme was applied. Cluster 2 included parameters clearly associated with Primitivo wines and, in some cases, affected by the pectolytic enzyme. In fact, Primitivo wines showed higher hue and chemical age than Bombino nero, which typically yields wines with low hue values (Suriano et al., 2015). These indices were significantly higher when using E3 (hue) and E2 (chemical age) in Primitivo wines, suggesting different extraction profiles of anthocyanins and other compounds involved in colored adducts with anthocyanins (Leborgne et al., 2022). On the other hand, enzymes E3 determined the lowest hue in Bombino nero wines, though the differences were relatively low. The different behaviour of the enzymes indicates the importance of adapting choices to the specific cultivar.

Cluster 3 included a group of volatile compounds and color indices that were only weakly associated with one of the specific cultivars under examination. This cluster included two parameters affected by the pectolytic enzyme used in must clarification. Anthocyanin ionization (corrected for SO_2 content), that is, the percentage of anthocyanins in the colored flavylium form, showed higher values in Bombino nero wines than in Primitivo and distinct values across the three enzymes. Enzyme E3 led to high A.I. values in both cultivars, while the other two enzymes showed opposite trends, with E1 performing better in Primitivo and E2 in Bombino nero. One volatile compound in this group was also affected by the adopted enzyme: ethyl-3-hydroxy-butyrate, also known as grape butyrate. Interestingly, this compound was found in wines of both cultivars only when enzyme E1 was applied for clarification. Considering that this compound has been reported to impart a fresh and fruity odor in cherry wines (Niu et al., 2011), further research should evaluate whether the clarification step could contribute amounts that are significant from a sensory point of view. Enzyme E1 also increased ethyl-9-decenoate levels in Primitivo wines, which showed lower levels of this ethyl ester than in Bombino nero wines.

Cluster 4 included compounds and parameters that characterized the Bombino nero wines. Total anthocyanins, total phenols, and color density (corrected for the SO_2 content) were higher in wines obtained with the enzyme E3. This was particularly evident in Primitivo wines, while the differences observed in Bombino nero wines were less marked. The greater amount of enzymatic preparation added, compared to E1, and the higher hydrolytic activities exerted compared to E2, may have played a determining role. Probably for the same reason, enzyme E1, although showing the highest enzymatic activity, exhibited lower overall performance in the extraction of phenols and color, due to the smaller quantity added as per the manufacturer's instructions. On the other hand, enzyme E1 had the strongest impact on the volatile compounds in cluster 4, which included compounds typically associated with *Bombino nero* wines. The alcohols 3-hexen-1-ol and 2-nonanol were detected only in Bombino nero wines obtained using enzyme E1 (Tables S2 and S3). Moreover, the compounds geraniol, 2-methyl-pentyl-isobutyrate, isohexanol and ethyl 2-hexenoate were detected only in Bombino nero wines obtained with the enzymes E1 and E3.

We suggest that the enhanced response of Bombino nero grapes to different enzymatic activities is related to the skin thickness and hardness of Bombino grapes (La Notte et al., 2012), known as quite higher compared to Primitivo grapes. No data are available in the literature on the terpene composition of Bombino nero grapes. Enzyme E1, though added at lower levels, could have increased the levels of some volatile compounds through higher side activities, including hydrolases and glycosidases. This result highlighted that selecting the appropriate enzymatic tool requires a careful evaluation of the specific properties of the processed grapes. At the same time, clarification enzymes can be considered multifunctional enzymatic cocktails with potentially broad and valuable effects that merit further exploration.

Variations in the volatile profile highlight the interaction of clarifying enzymes in the generation of the wine volatilome (Espejo, 2021). In particular, the impact on C_6 unsaturated compounds and their derivatives (3-hexen-1-ol and the ethyl ester of 2-hexenoate) is of interest, since C_6 unsaturated compounds have been reported as precursors of aroma-relevant thiols, whose conversion can be achieved in certain conditions during fermentation (Clark & Deed, 2018; Harsch et al., 2013). Therefore, the possible effect of clarifying enzymes on the release of endogenous enzymes of the lipoxygenase chain, and on their activity towards unsaturated fatty acids, deserves future investigation. The increased levels of 3-hydroxy acids also indicate another direction for future investigation: the possible enhancement of the activity of β-oxidation enzymes involved in fatty acid catabolism (Osorio et al., 2010). The interest in the variation of 3-hydroxy acids is justified by the sensory relevance of ethyl 3-hydroxybutyrate, which has been previously documented (Gracia-Moreno et al., 2015; Lytra et al., 2017; Pineau et al., 2009), even below its olfactory threshold, to the red-fruit aroma in red wines, due to a hyper-additive effect. However, the present investigation could not determine whether the observed changes influenced the wines' sensory profiles. The different effect of clarifying enzymes on geraniol, previously reported in literature (Armada et al., 2010; Scutaraşu et al., 2023), highlights that the extraction of grape-derived volatile compounds, such as terpenes, from skins can be improved by also selecting the appropriate clarifying pectolytic enzyme. Enzyme E1 seemed the most effective for extracting this terpene, either due to easier release from the skin or increased hydrolysis of precursors.

Based on these results, grape must clarification could be included among multienzyme engineering approaches, aiming at multiple side objectives (Chen et al., 2025; Osete-Alcaraz et al., 2022).

4. Conclusions

The results obtained during this study demonstrated that commercial pectolytic enzymes, typically used for must clarification, exert accessory enzymatic activities – especially glycosidase and hemicellulase activities – even at acidic pH, suggesting a remarkable and more versatile biotechnological potential. These enzymes may contribute to the release of grape-derived volatile compounds from glycosylated precursors and to the structural transformation of the must matrix, with potential benefits reflecting on the volatile profile and wine complexity, though requiring focused investigation.

Laboratory-scale tests confirmed their effectiveness in reducing turbidity and revealed additional effects of potential interest, including increases in yeast-assimilable nitrogen and a significant reduction in calcium ions.

Pilot-scale fermentations with Bombino nero and Primitivo grapes highlighted how these enzymes can modulate wine composition, depending on the properties of the processed grapes. Overall, these findings support the integrated and strategic use of clarification enzymes, viewing them not merely as clarification agents but as versatile tools for enhancing the volatile profiles, stability, and sensory quality of wines across different grape varieties. This underscores the importance of tailoring enzyme selection to the specific characteristics of each grape variety to maximize benefits, thereby positioning these preparations as key instruments for advanced enological management.

CRediT authorship contribution statement

Ilaria Prezioso: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Gabriele Fioschi:** Writing – review & editing, Visualization, Investigation, Data curation. **Maria Tufariello:** Writing – review & editing, Investigation, Data curation. **Luigi**

Sanarica: Writing – review & editing, Methodology, Conceptualization. **Ignazio Zara:** Resources, Investigation, Data curation. **Francesco Grieco:** Writing – review & editing, Methodology, Formal analysis. **Alessia Di Fraia:** Writing – original draft, Investigation, Formal analysis, Data curation. **Gabriella Fiorentino:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Vito Michele Paradiso:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vito Michele Paradiso reports that Enolife s.r.l. provided materials and technical support for the research. Luigi Sanarica reports a relationship with Enolife srl that includes: employment. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2026.108337.

Data availability

Data will be made available on request.

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