Washington State Grape and Wine Research Program
Washington State Wine Commission Research Grant Program

ANNUAL PROGRESS/FINAL REPORT FORMAT
2024-25 Funding Cycle

1. **Summary**: Experimental protease enzyme was added to Sauvignon blanc pre- (settled via small- and industrial-scale methods) and post-fermentation to evaluate the experimental enzymes viability as a substitute for bentonite fining to remove heat unstable proteins. Under the limited conditions we evaluated the experimental proteases surpassed bentonite for the removal of heat unstable proteins and could be used pre- and post-fermentation with similar effect.

2. **Final Report**: Final report for short-term project with limited scope and cost.

3. **Project Title**: Evaluate the impact of protease enzymes on heat stability of white wine

4. **Principal Investigator/Cooperator(s)**:
James Harbertson and Madelyn Calderon, Washington State University359 University Drive, Richland, WA 99354, (509) 372-7506 and jfharbertson@wsu.edu.
Vinzyme Cooperators: Youtian Cui (YoutianCui@vinzymes.com), Patrick Nichols (PatrickNichols@vinzymes.com). 2860 W Covell Blvd. Suite 1 Davis, CA 95616
Industry Cooperator: Katie Nelson Saint Michelle Wine Estates (Katie.Nelson@smwe.com)

5. **Objective(s) and Experiments Conducted to Meet Stated Objective(s)**:

Objective 1. Evaluate viability of protease enzyme addition timing with raw juice. Experiment #1 consists of a control and a protease enzyme treatment added prior to fermentation. The enzyme was added when the juice was delivered to three separate fermentors and three control tanks which have no enzyme added. Both sets were cold settled for three days, racked and then fermented. Enzyme was also added post-fermentation to determine if ethanol prevents its ability to digest proteins.

Objective 2. Compare enzyme to conventional bentonite additions with juice that has been settled and treated via industry methods. Experiment #2 consists of commercially settled juice (no protease enzyme) which was treated with either nothing, bentonite or protease enzyme (two treatment doses) prior to the onset of fermentation. Enzyme was also added post-fermentation to determine if ethanol prevents its ability to digest proteins. Each treatment or control was done in triplicate.

Experimental designs are summarized in figure 1 for objective 1 and figure 2 for objective 2. Please note that there is a considerable amount overlap between these experiments and that the major distinction is the inclusion of raw juice in experiment 1 and the inclusion of bentonite and two enzyme dosages (25 and 50 mg/L) in experiment 2. Experimental protease enzyme was donated by Vinzyme located in Davis, California. The post-fermentation treatments were not in original design of experiment but were added due to interest from representatives from Vinzyme company. Due to scale of experiment, we were able to split wine from control kegs to accommodate this request.

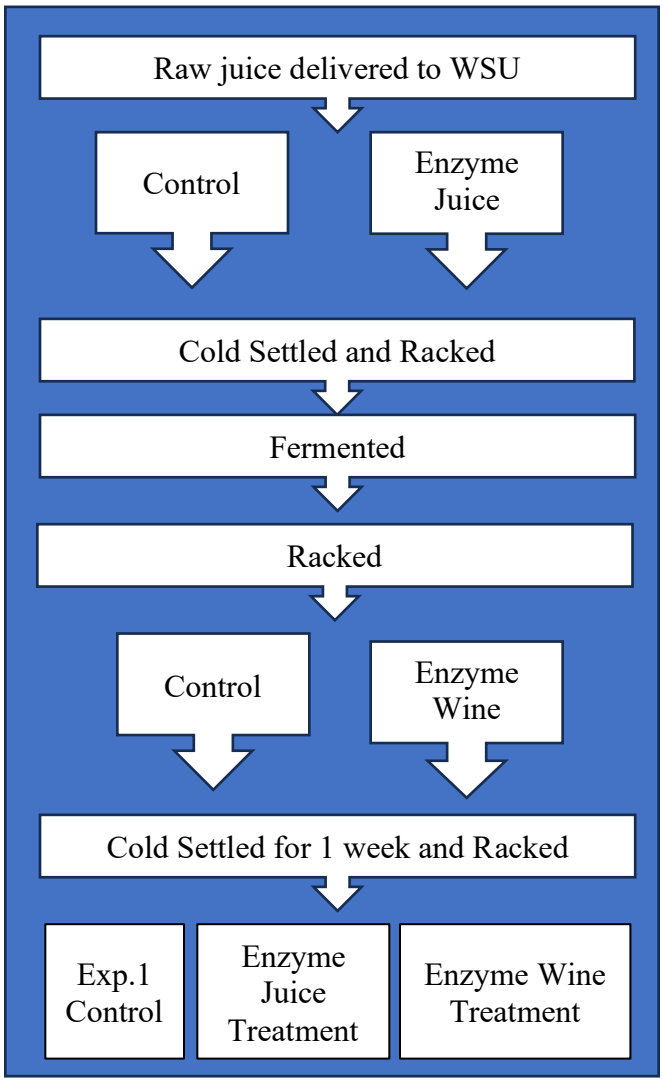

Figure 1 Experiment 1design: Evaluate viability of protease enzyme addition timing.

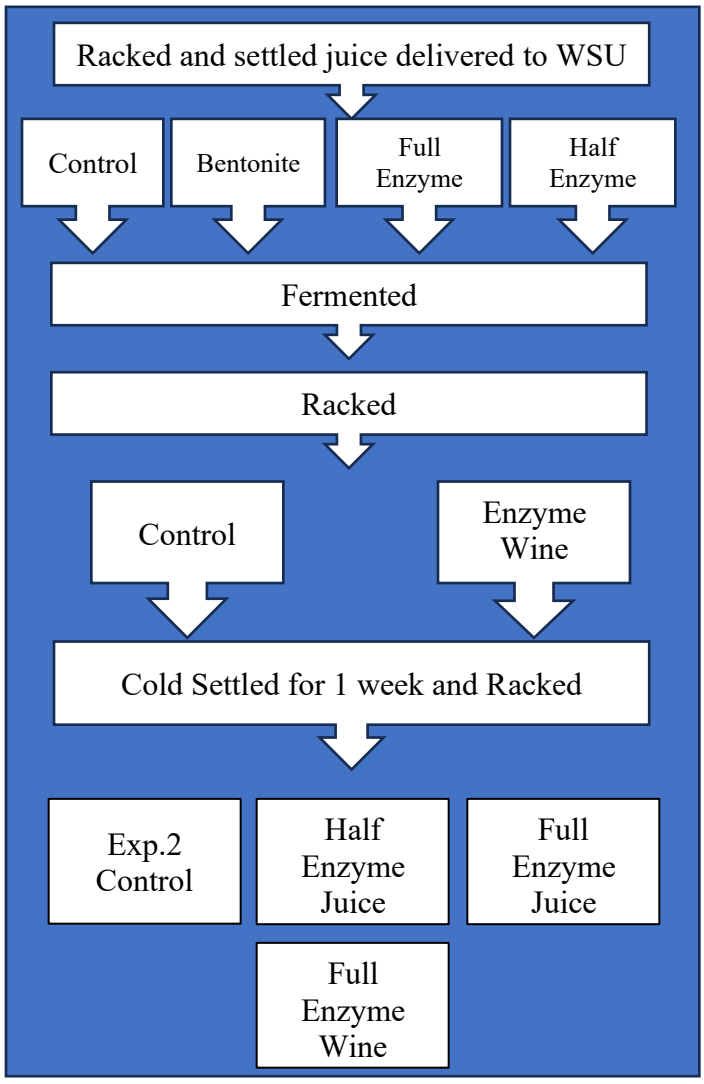

Figure 2 Experiment 2 design: Comparison of protease and bentonite treatments.

6. **Summary of Major Research Accomplishments and Results by Objective**

Evaluate viability of protease enzyme addition timing summary. Raw Sauvignon blanc juice was obtained from Saint Michelle Wine Estates during the fall of 2024. And cold settled at 5°C for three days. Sauvignon blanc juice with the following basic chemistry: Soluble Solids 18.9 Brix, titratable acidity 6.7 g/L tartaric acid equivalents, pH 3.37, yeast available nitrogen 171 mg/L isoleucine equivalents, malic acid 2.34 g/L). Fermentations were conducted with an inoculum containing VIN 13 yeast and Goferm Sterol Flash (both 300 mg/L). Yeast available nitrogen was adjusted to 225 mg/L with combination of diammonium phosphate and Fermaid O. Finished wine chemistry can be found in table 2. No significant differences were found between treatments other than residual sugar. The enzyme added post-fermentation had significantly greater concentration of residual sugar but is still considered dry (≤ 2.5 g/L). Heat stability tests (heat to 80°C) were performed throughout the experiment and figure 3 summarizes two of the most recent measurements. Significant differences were observed between the enzyme treatments and the control though no differences were observed for the timing of the enzyme addition. The enzyme reduced the amount of turbidity caused by heating by 75% regardless of when it was added. This shows that the enzyme can be added pre- or post-fermentation without any impact on its ability to digest heat instable proteins. The major concern alleviated here was that the protease may digest the proteins in the yeast cell walls and not just the proteins in the juice or wine and slow or prevent fermentation. The enzyme also proved rugged enough to avoid denaturation by ethanol

during the winemaking treatment. This provides winemakers with a very flexible time frame to add the enzyme. A juice treatment that can be done without risking oxidation has a lot of alluring possibilities particularly given that if the enzyme can replace or even reduce bentonite usage (which can't be recycled or regenerated) it would be a huge improvement to the wine industries efforts to utilize fewer wasteful practices.

Treatment	pH	TA (g/L)	Alcohol % (v/v)	Malic Acid (g/L)	Acetic Acid (g/L)	Glu + Fru (g/L)	Lactic Acid (g/L)	Free SO_2 mg/L	Total SO_2 mg/L
Control	3.33	5.48	11.40	2.02	0.05	0.17 b	0.01	18.0	60.7
Juice Enzyme	3.36	5.56	11.35	2.05	0.05	0.18 b	0.01	19.0	62.7
Wine Enzyme	3.38	5.68	11.36	2.02	0.03	0.83 a	0.01	18.0	59.3
p-value	0.080	0.281	0.592	0.910	0.689	***<0.0001***	1.000	0.670	0.689

Table 1 Sauvignon blanc wine composition that was treated with no protease enzyme (control), and protease enzyme added pre- (Juice enzyme) and post-fermentation (wine enzyme). Values shown are averaged from three winemaking replicates and duplicate analytical values. Letters denote differences utilizing Tukey post hoc-comparison of means with p-values from 1-way ANOVA shown.

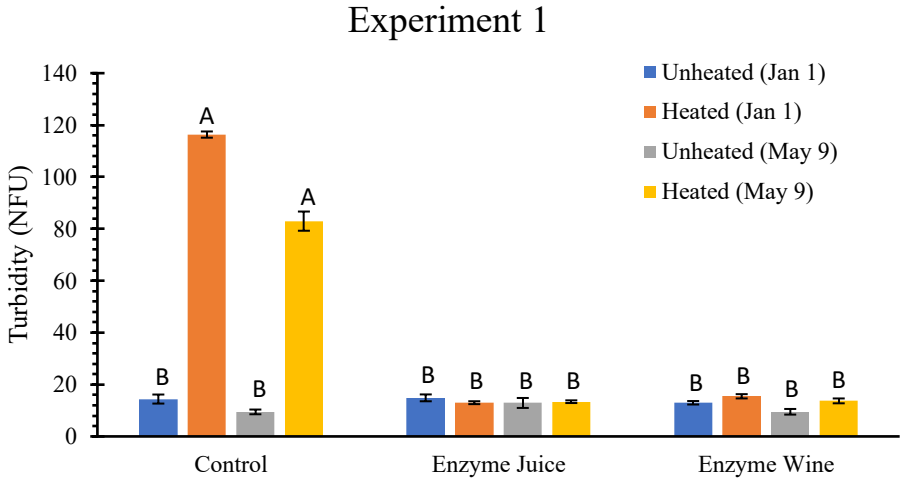

Figure 3 Evaluation of heat stability of Sauvignon blanc wines on two dates (Jan 1 and May 1, 2025) by comparing unheated and heated samples. Control wines had no fining agents added and protease was added pre-(enzyme juice) and post-fermentation (enzyme wine). Turbidity is measured in nephelometric formazin units (NFU). Letters denote differences utilizing Tukey post hoc-comparison of means with p-values after 1-way ANOVA ($p \leq 0.05$).

Compare enzyme to conventional bentonite additions with juice that has been settled and treated via industry methods. Commercial settled juice without bentonite or enzyme addition was utilized for the second experiment. Though this seems a minor distinction, small-scale winemaking has been called into question over the years over small-scale experiments. In this scenario, our cooperator wanted to ensure that the results of the experiment would be comparable to industry treatments. In any case cold settled Sauvignon blanc juice was obtained from Saint Michelle Wine Estates during the fall of 2024. Sauvignon blanc juice with the following basic chemistry: Soluble Solids 19.5 Brix, titratable acidity 5.8 g/L tartaric acid equivalents, pH 3.34, yeast available nitrogen 215 mg/L isoleucine equivalents, malic acid 2.32 g/L). The same winemaking protocol was followed as described in experiment 1 sans the cold settling period. Figure 4 summarizes the heat stability treatment data. Like the first experiment the enzyme treatments very clearly reduced the amount of turbidity during the heat stability test. The half-dosage (juice enzyme treatment is not significantly different from the full enzyme dosage or the wine treatment which suggests further experiments to optimize the treatment dosage are

necessary. The bentonite treatment was not significantly different from the control and still contained a significant portion of heat instable proteins. It is important to note that bentonite effectively works like an ion-exchange system. The phyllosilicate clay material has an effective neutral charge with the addition of positively charged sodium ions. When bentonite is added to juice positively charged proteins (and other positively charged ions) exchange with the sodium and can be racked and removed (as bentonite is only partially water soluble and forms a slurry in the juice or wine and is not completely dissolved in the juice). Though this mechanism is widely understood, what is sometimes overlooked is that proteins shift their overall charge based on their amino acid composition (which have varied pKa's). As a result of the protein amino acid make-up, the net charge of the protein is dependent upon the liquid pH. Our results show that the proteins in this Sauvignon blanc juice at ~pH 3.3 don't interact with bentonite and form a haze when heated. It is possible that if the juice pH was shifted so that the proteins that cause the heat instability were positively charged then the bentonite addition have been effective. However, this would require pH adjustments to ensure the efficacy of a fining agent instead of other winemaking considerations which is impractical.

	pH	TA (g/L)	Alcohol % (v/v)	Malic Acid (g/L)	Lactic Acid (g/L)	Acetic Acid (g/L)	Glu + Fru (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)
Control	3.36 c	5.53	11.31	2.25 ab	0.01	0.04	0.27 c	13.0	53.0
Juice Enzyme Half	3.41 a	5.93	11.27	2.11 abc	0.01	0.12	0.57 b	12.3	65.7
Juice Enzyme Full	3.39 ab	5.45	11.30	2.05 c	0.01	0.06	0.81 a	12.7	58.0
Bentonite	3.38 abc	5.61	11.32	2.06 bc	0.01	0.08	0.19 c	13.7	55.3
Wine Enzyme	3.37 bc	5.79	11.36	2.27 a	0.01	0.03	0.25 c	13.3	54.0
p-value	***0.04***	0.41	0.65	***0.01***	1.00	0.09	***0.0001***	0.1	0.1

Table 2 Sauvignon blanc wine composition that was treated with no protease enzyme (control), bentonite (0.25 g/L) and protease enzyme added pre-at two doses (Juice enzyme half 25 mg/L and full 50 mg/L) and post-fermentation (wine enzyme same as full juice dose). Values shown are averaged from three winemaking replicates and duplicate analytical values. Letters denote differences using Tukey-HSD post hoc comparison of means test with 1-way ANOVA p-value shown.

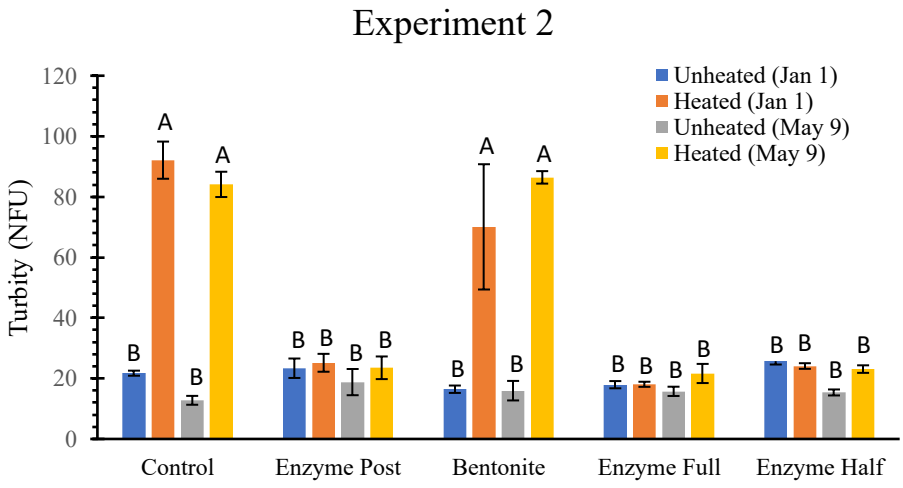

Figure 4 Evaluation of heat stability of Sauvignon blanc wines on two dates (Jan 1 and May 1, 2025) by comparing unheated and heated samples. Control wines had no fining agents added and protease was added pre-(enzyme half 25 mg/L and enzyme full 50 mg/L) and post-fermentation (enzyme wine). Bentonite was also added pre-fermentation at 0.25 g/L. Turbidity is measured in nephelometric formazin units (NFU). Letters denote differences using Tukey-HSD post hoc comparison of means test after 1-way ANOVA (p ≤0.05).

The impacts of the enzyme on residual sugar are inconsistent between the experiments which suggest that other factors may be at play here (temperature, stirring etc.). As in the first experiment all the wines are dry (≤ 2.5 g/L) so the enzyme didn't alter the ability of the yeast to carry out the fermentation. It is important to note that although that none of the wines from either experiments after aging or protease treatment were sufficiently low enough turbidity to allow for bottling and either filtration, or fining would be necessary. Due to the limited scope and time frame of the current project further research is necessary to understand whether the remaining proteins or peptides that are causing the residual turbidity in the protease treated wines would be simple to remove. Samples for proteomic analysis are being sent to Vinzyme to investigate. We are also hopeful that we can get confirmation of removal of thaumatin and chitinase pathogenesis related proteins, though the improvement of the heat stability of the wine proteins is possibly enough to convince most winemakers of the enzyme's efficacy.

7. **Outreach and Education Efforts - Presentations of Research**: None thus far, our hope is to present the project to the Washington Wine Technical Group or the Washington Winegrape Growers and potentially ASEV during one of their meetings. The work is limited, so it is not clear if there is enough data to support a publication.

8. **Research Success Statements**: This research shows the viability of protease enzyme treatments to remove heat instable proteins for white wine production. The enzyme treatment worked in both juice and wine scenarios which should improve its adoption by winemakers. The enzyme dosage used in this experiment although a small fraction of what was used for bentonite could be optimized (the half-dose was as effective as the full dose). The enzyme treatment is not enough to lower the turbidity for bottling; however, it does remove heat stability proteins that are not removed using conventional bentonite additions. Although the desire to compare industry and small-scale cold settling protocols is slightly obscured by differing Sauvignon blanc juice compositions the results of the experiment show comparable results for the controls and enzyme treatments which should alleviate any further questions about the validity of small-scale experiments. Sensory was not part of the original experimental plan however bench testing suggests very small but subtle impacts. The second experiment which contains both the enzyme and bentonite treatments will likely be evaluated though we anticipate the results will show that the wines are alike.

9. **Funds Status**: Funds were spent to support the winemaking and analysis of the wines produced for the experiment. Some funds were used to ship samples to Vinzyme for analysis.