Research Note

Effect of Timing of Diammonium Phosphate Addition to Fermenting Grape Must on the Production of Ethyl Carbamate in Wine

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Abstract: Grape must is often supplemented with diammonium phosphate (DAP) to reduce stuck fermentations. The timed addition of DAP to grape must significantly affects the amount of ethyl carbamate (EC) produced in wine. This effect was found to be yeast strain dependent: Pasteur Red produced less EC when DAP was added at the onset of fermentation, whereas strain 522 produced less EC when DAP was added during the later stages of fermentation. EC production by strain EC1118 was less affected by the timing of DAP addition. The metabolically enhanced yeast strains Pasteur Red^{EC-}, 522^{EC-}, and EC1118^{EC-}, which constitutively express *DUR1,2*, significantly reduced the amount of EC produced regardless of the timing of DAP addition.

Key words: nitrogen supplementation, ethyl carbamate, urea, *Saccharomyces cerevisiae*

Ethyl carbamate (EC), also known as urethane, is a known carcinogen that occurs naturally in many fermented foods and beverages (Zimmerli and Schlatter 1991). In 1985, a study conducted by the Ontario Liquor Board (Canada) determined that a number of alcoholic beverages, especially dessert wines and spirits, contained substantially high quantities of EC (several hundreds µg/L) (Conacher et al. 1987). As a result, Canada has imposed limits of 30 µg/L EC and the United States has voluntary limits of 15 µg/L EC allowed in table wine (Coulon et al. 2006).

Stuck and sluggish fermentations are major enological problems resulting in increased vinification time and spoilage of wine (Blateyron and Sablayrolles 2001). Since nitrogen depletion is often the cause of problem fermentations (Salmon 1996), ammonium salts such as diammonium phosphate (DAP) are frequently added to grape must fermentations to alleviate nitrogen deficiency. However, there is little information on the timing of DAP addition and the effect that it has on EC production. The amount of urea secreted into wine depends on the nitrogen requirements of the wine yeasts and the nitrogen composition of the grape must (Ough et al. 1991).

Urea and ethanol are the main precursors of EC in wine (Monteiro et al. 1989, Ough et al. 1988a). The production of urea during fermentations is a direct consequence of the metabolism of arginine (Monteiro and Bisson 1991), one of the most abundant amino acids in grape must. Arginine is degraded into ornithine and urea by arginase encoded by the *CAR1* gene (Sumrada and Cooper 1984). Since urea becomes toxic to yeast cells at higher concentrations, yeast cells secrete urea into the wine where it spontaneously reacts with ethanol in a time- and temperature-dependent manner to produce EC (Monteiro et al. 1989). However, when better nitrogen sources are depleted, urea can be transported back into the cell and degraded to ammonia, which can be used for the synthesis of new nitrogenous compounds. This degradation is accomplished by urea amidolyase encoded by the *DUR1,2* gene (Genbauffe and Cooper 1986).

The addition of DAP to fermenting grape must results in the repression of genes that encode enzymes, which metabolize poorer nitrogen sources, a phenomenon called nitrogen catabolite repression (NCR) (Cooper 1982). *DUR1,2* is subject to NCR (Genbauffe and Cooper 1986), and the addition of DAP leads to repression of this gene resulting in the secretion of urea into wine, which may inevitably lead to the production of high levels of EC in the wine (Coulon et al. 2006). We have constitutively expressed *DUR1,2* in several strains of commercially available wine yeast. The metabolically enhanced Davis 522 strain reduced EC levels in Chardonnay wine up to 89% when compared with the parental strain (Coulon et al. 2006). Here we examined the impact that timed DAP supplementation had on EC production with three commercially available wine yeast strains and their metabolically enhanced counterparts in which *DUR1,2* was constitutively expressed.

Materials and Methods

Strains and media. *Saccharomyces cerevisiae* Pasteur Red was supplied by American Tartaric Products (Windsor, CA) in active dry form. UC Davis 522 (Montrachet) and EC1118 (Prise de Mousse) were purchased from Lallemand

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(Montreal, Canada) in active dry form. *Saccharomyces cerevisiae* strain 522^{EC-} was created previously (Coulon et al. 2006). Briefly, *DUR1,2* was amplified from *S. cerevisiae TCY1* and cloned into plasmid pHVX2 between the *PGK1* promoter and terminator sequence. This *DUR1,2/PGK1* expression cassette, flanked by *URA3* sequences, was integrated into the *URA3*-locus of *S. cerevisiae* 522. Pasteur Red^{EC-} and EC1118^{EC-} were metabolically enhanced in the University of British Columbia (UBC) Wine Research Centre according to the methods described previously (Coulon et al. 2006). All *Saccharomyces* strains were maintained on YPD agar at 4°C. Calona Chardonnay must was obtained during the 2004 growing season from Calona Vineyards, Okanagan Valley, Canada (total sugar 26.0%, pH 3.4, ammonia 65.9 mg/L, FAN 295.6 mg/L).

Fermentations. Fermentations were conducted in triplicate at 20°C in 200 mL volumes as described previously (Coulon et al. 2006). Carbon dioxide loss was recorded to monitor fermentation rate. At 0, 24, 36, 48, and 121 hr after inoculation, 101.6 mg sterile DAP was injected into the Chardonnay must through the rubber stopper containing the vapor lock using a sterile 12.7 cm needle and 3 mL syringe, increasing the YAN from 361.5 to 500 mg/L. After the DAP was added, the flasks were mixed thoroughly, and 1 mL samples were immediately taken. Samples were filter sterilized and stored at -20°C for future ethanol analysis.

Quantification of ethanol. The ethanol content of the wine was analyzed by HPLC on a SupelcoGel C-61OH 30 cm x 7.8 mm column (Sigma-Aldrich, St. Louis, MO) at 50°C. The method for the ethanol analysis consisted of a 23 min isocratic run of 0.1% phosphoric acid at 0.75 mL/min. Peak monitoring was performed with an Agilent G1362A refractive index detector (Agilent, Santa Clara, CA) with positive polarity and an optical unit temperature of 35°C. Technical triplicates were performed for all samples analyzed.

Quantification of EC in wine by GC-MS. The fermentations were deemed complete after 10 days, at which time the wine was centrifuged (1,000 x *g*, 10 min, 20°C) to remove any residual yeast. Urea and ethanol spontaneously react in a time- and temperature-dependent manner to produce EC. To mimic the natural aging process of wine and to maximize the EC concentration, the filtered wine samples were placed at 70°C for 48 hr. Gas chromatography-mass spectrometry (GC-MS) analysis of EC was performed in triplicate using an Agilent 6890N GC interfaced to a 5973N mass selective detector (Coulon et al. 2006).

DNA sequencing of *DUR1,2* promoter. Sequencing was performed at the UBC Nucleic Acid Protein Services Unit using a PRISM 377 sequencing and AmpliTaq FS Dyodeoxy Terminator Cycle sequencing chemistry (Applied Biosystems, Foster City, CA). The *DUR1,2* promoter sequences were PCR amplified using iProof DNA polymerase (Bio-Rad, Foster City, CA). All sequences were compared to the published sequences available on *Saccharomyces* Genome Database (www.yeastgenome.org).

Results and Discussion

Results confirm that different wine yeast strains produce varying amounts of EC in wine, as reported elsewhere (Ough et al. 1991). Pasteur Red, 522, and EC1118 produced wines containing 205 (SD 26.99) µg/L, 89 (SD 19.27) µg/L, and 31 (SD 2.96) µg/L EC, respectively, in Chardonnay must that was not supplemented with DAP (Figure 1). As expected, the three metabolically enhanced strains Pasteur Red^{EC-}, 522^{EC-}, and EC1118^{EC-} produced wines with 74%, 64%, and 22% less EC than their parental strains, respectively, when Chardonnay must was not supplemented with DAP (Figure 1).

Since the nitrogen content of the grape must has a profound impact on the final EC concentration in the wine (Ough et al. 1988b), our objective was to quantify the effect

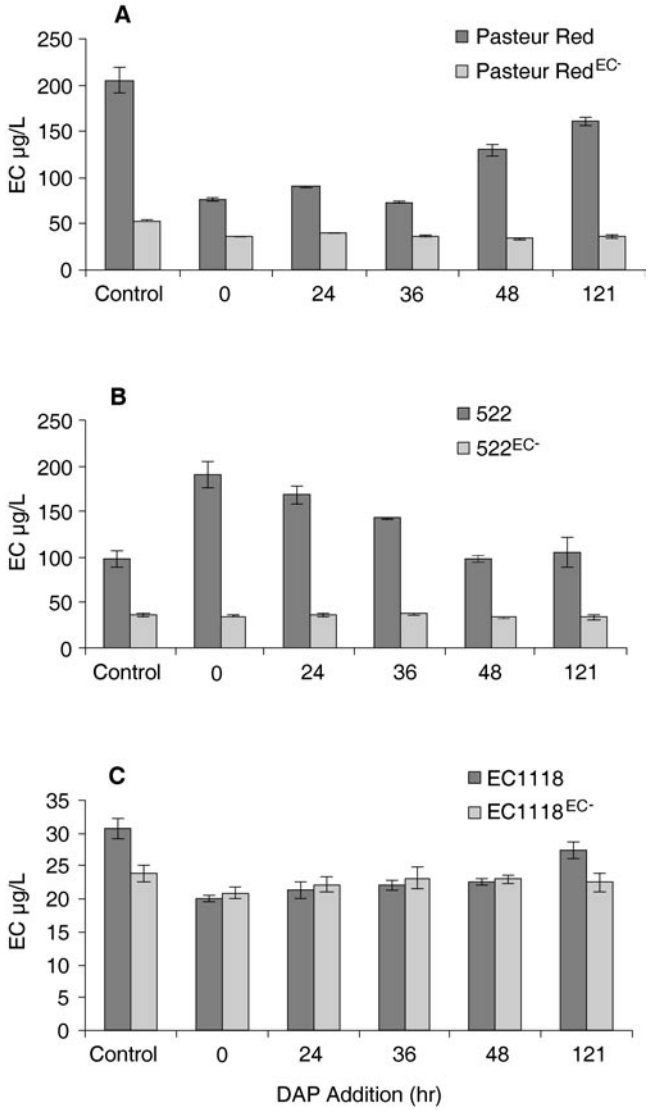

Figure 1 Effect of timed DAP additions on EC formation by three metabolically enhanced wine yeast strains and their respective parental strains. At 0, 24, 36, 48, and 121 hr after inoculation, 101.6 mg of sterile DAP was anaerobically injected into each fermentation. Before inoculation with yeast (0 hr) the YAN of Chardonnay juice was increased from 361.5 to 500 mg/L with DAP. No DAP was added to the control fermentation. EC was measured at the end of fermentation for all samples.

that timed DAP additions might have on EC production. Increasing the YAN of Chardonnay grape must from 361.5 to 500 mg/L with DAP dramatically changed the levels of EC produced in wine fermented with Pasteur Red, 522, and EC1118 (Figure 1). However, the addition of DAP at different time points during the fermentation did not affect the fermentation rate of these yeast strains compared with the control, which was not supplemented with DAP (Figure 2). The addition of DAP at the beginning of the fermentation (0 hr) resulted in a 48% increase in EC levels in wine produced with 522; however, we observed a decrease of 63% and 35% in EC levels in wine produced with Pasteur Red and EC1118, respectively (Figure 1). Moreover, the timing of DAP addition significantly impacted the final EC concentration in the wine; two distinct patterns were found when DAP was added to Chardonnay fermentations at 0, 24, 36, 48, and 121 hr after inoculation (Figures 1). Pasteur Red produces less EC when DAP is added at early time points but produces progressively more EC when DAP is added at later time points. Interestingly, the reverse trend is seen with 522; this strain produces high levels of EC when DAP is added at the early time points but significantly less EC when DAP is added at later time points. EC production by EC1118 was not affected significantly by timed DAP additions; in fact, EC1118 behaved similarly to the functionally enhanced yeast strains in which *DUR1,2* was constitutively expressed.

The ethanol produced by the six yeast strains was fairly similar during the later stages of fermentation when DAP was added (Table 1), indicating that the varying trends of EC production seen between the parental strains is not due to a variation in fermentation rate. The timing of DAP additions did not affect EC production by the metabolically enhanced (EC-) strains; little EC was produced and there

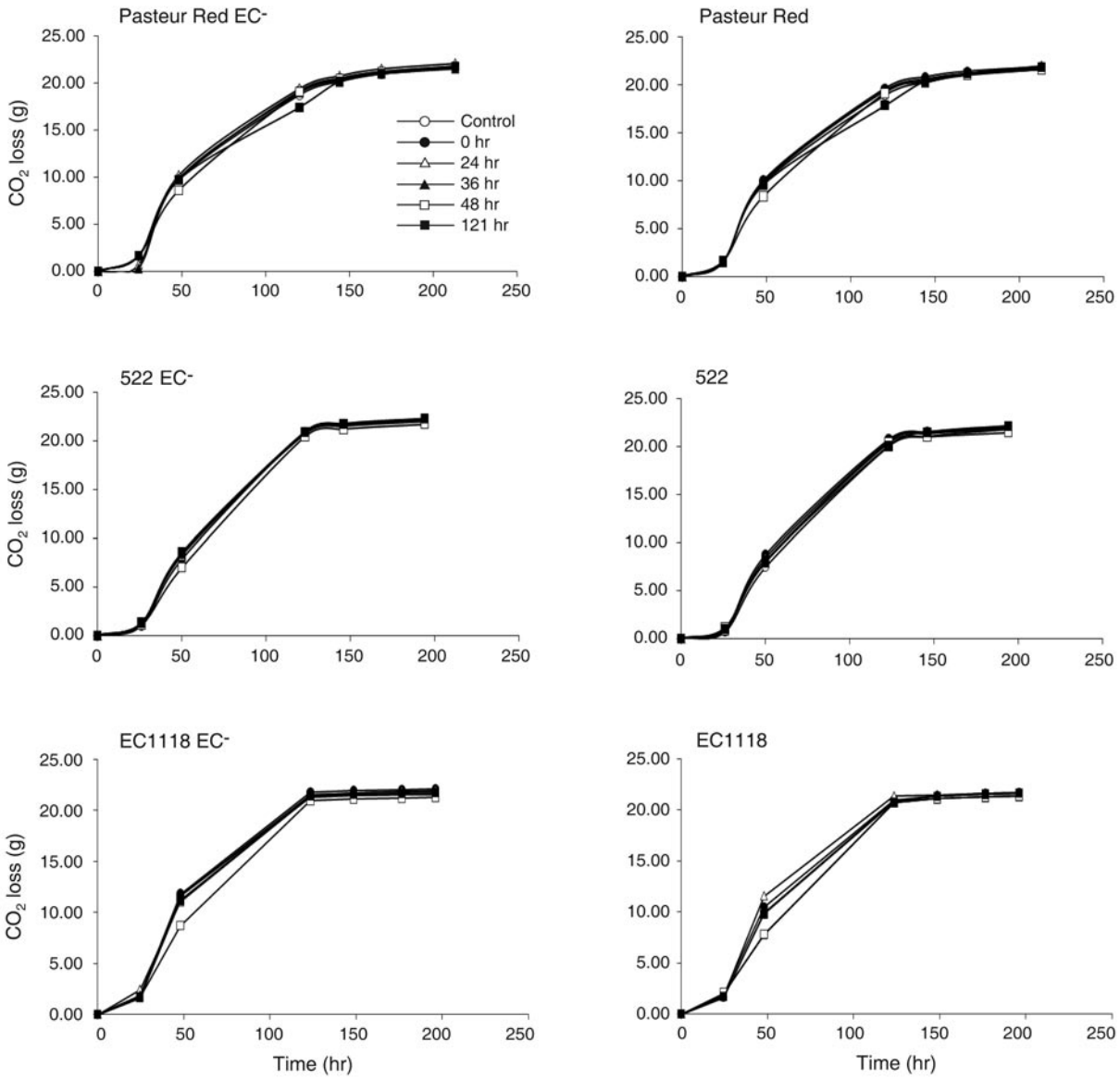

Figure 2 Comparison of the fermentation rates of three metabolically enhanced wine yeast strains and their respective parental strains. All strains were fermented in triplicate in Calona Chardonnay juice at 20°C.

were only minor differences in the amount of EC produced (Figure 1). This result was expected for the metabolically enhanced (EC-) strains, as *DUR1,2* is constitutively expressed and degrades urea before it is secreted into the wine, minimizing EC production. Fermentation rates among all six yeast strains were similar, especially during the later stages of fermentation, indicating that the different trends in EC production observed during DAP supplementation were probably not a result of different growth rates of the yeast (Figure 2, Table 1).

The varying amounts of EC produced and the differing trends of EC production in the same Chardonnay grape must suggest that there is significant variation in the regulation of genes involved in urea metabolism in the different wine yeasts used in this study. To determine if expression of the *DUR1,2* gene is differentially regulated in the three parental strains, the promoter sequences of *DUR1,2* were analyzed (Figure 3). With the exception of a single nucleotide change at position -422 in Pasteur Red, which falls between the nitrogen catabolite repression upstream activation sequence (UAS_{NTR}) and the gene-specific upstream induction sequences (UIS), the three parental strains have identical *DUR1,2* promoter sequences, strongly suggesting that the expression pattern of *DUR1,2* should be the same among the three strains. These results indicate that the varying levels of urea production observed between different wine yeast strains was not caused by variable *DUR1,2* expression patterns but was likely caused by varying regulation of one or more genes involved in urea metabolism. Urea metabolism in *Saccharomyces* is a complex process, involving multiple pathways comprised of many enzymes and transport systems. Urea is produced from the degradation of arginine, the most predominant amino acid in grape must. Arginine is transported into the cell through

Table 1 Ethanol concentration (% v/v) at specific time points when DAP was added to the fermentations. Fermentations were performed in triplicate; standard deviation is presented in brackets. Control fermentation is excluded as no DAP was added.

Strain	0 hr	24 hr	36 hr	48 hr	121 hr
Pasteur Red	0.00	1.40 (0.08)	3.62 (0.07)	6.19 (0.06)	11.66 (0.29)
Pasteur Red^{EC-}	0.00	1.46 (0.02)	3.71 (0.07)	6.20 (0.27)	11.71 (0.07)
522	0.00	0.70 (0.02)	1.62 (0.26)	5.06 (0.10)	12.72 (0.15)
522^{EC-}	0.00	0.67 (0.12)	1.78 (0.22)	4.25 (0.53)	12.95 (0.09)
EC1118	0.00	1.47 (0.04)	4.65 (0.05)	6.34 (0.01)	13.06 (0.09)
EC1118^{EC-}	0.00	1.42 (0.04)	5.13 (0.05)	7.06 (0.07)	13.21 (0.06)

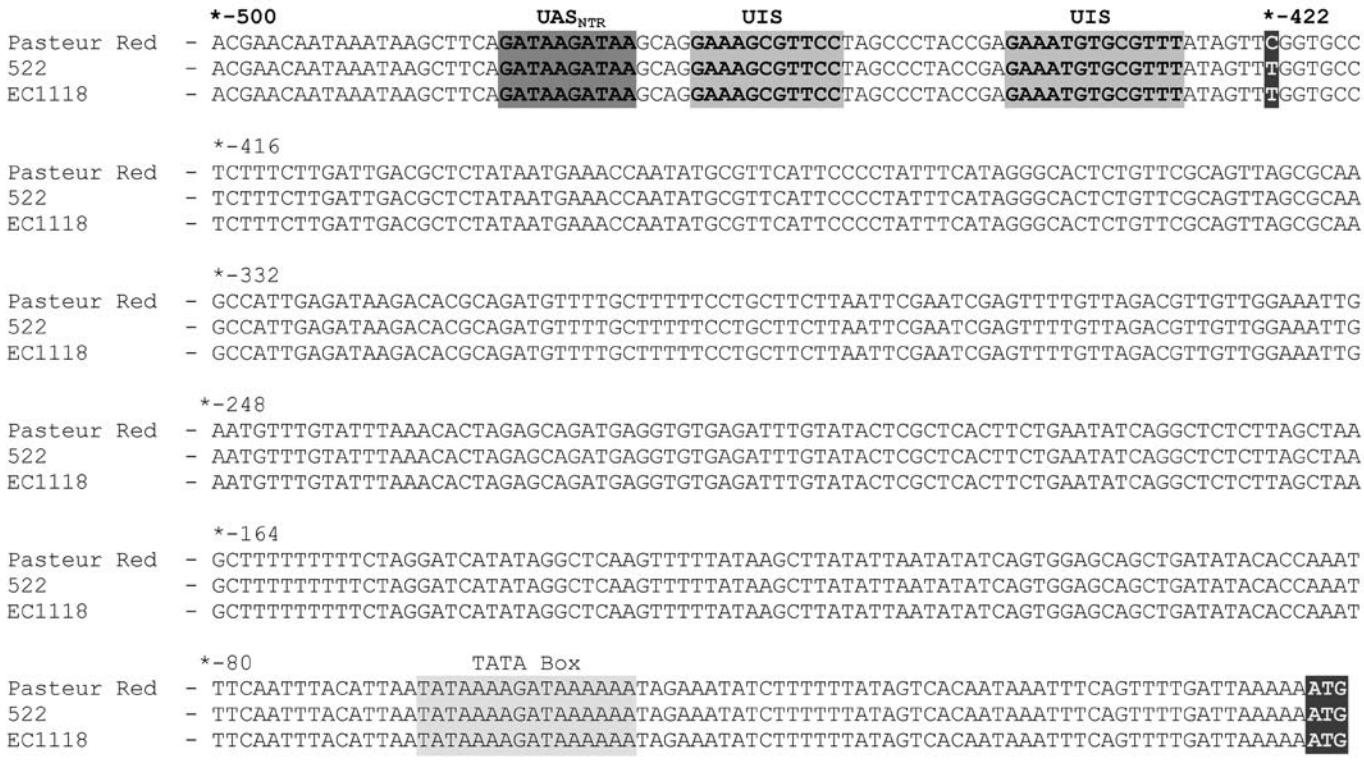

Figure 3 Sequence comparison of the promoter region of *DUR1,2* for three industrial wine yeast strains. With the exception of a single nucleotide change at position -422 in EC1118, the three yeast strains have identical *DUR1,2* promoters. UAS_{NTR} indicates a nitrogen catabolite repression upstream activation sequence and UIS indicates an upstream induction sequence.

Gap1p or the arginine specific permease Can1p and is then degraded into ornithine and urea by the arginase Car1p (Coulon et al. 2006). Urea can also be formed in *Saccharomyces* from the degradation of allantoin or allantoate. Allantoin and allantoate are transported into the cell through Dal4p and Dal5p, respectively, and are converted into urea through the allantoin pathway by the actions of allantoinase (Dal1p), allantoicase (Dal2p), and ureidoglycolate hydrolase (Dal3p) (Chisholm and Cooper 1982). Urea can be used as a nitrogen source by *Saccharomyces*; however, in high concentrations urea becomes toxic to the cell. In order to deal with high intracellular concentrations, urea is transported out of the yeast cell by urea permease encoded by *DUR4* (Chisholm and Cooper 1982). When all of the more favorable nitrogen sources are depleted, *Saccharomyces* can transport urea back into the cell by urea permease, encoded by *DUR3*, where it can be metabolized into ammonia and carbon dioxide by the action of Dur1,2p (Coulon et al. 2006). Considering the complexity of urea metabolism, variation in regulation of one or more of the enzymes or transport systems could affect the amount of urea present in the wine and ultimately affect the final EC concentration in wine.

The initial nitrogen content of the grape must may also affect the pattern of EC production. Our results indicate that more research is required on each individual commercial yeast strain to establish a DAP addition regime in grape musts with varying nitrogen composition. The timing of DAP additions did not affect EC production by the functionally enhanced yeast strains Pasteur Red^{EC-}, 522^{EC-}, and EC1118^{EC-}. EC1118^{EC-} produced slightly less EC, whereas Pasteur Red^{EC-} and 522^{EC-} produced significantly less EC than their respective parental strains.

Conclusions

The timing of DAP additions to a Chardonnay grape must significantly affected the production of EC in the resulting wines. Pasteur Red produced less EC when DAP was added at the onset of fermentation. In contrast, *S. cerevisiae* 522 produced less EC when DAP additions occurred later during fermentation. Ethyl carbamate production in wine made with EC1118 was less affected by the timing of DAP addition. The metabolically enhanced wine yeasts significantly reduced the amount of EC produced in the Chardonnay wine. The use of these metabolically enhanced wine strains may simplify the winemaking process, allowing winemakers to supplement their grape musts at any time without the risk of increasing harmful levels of EC in their product.

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