Research Note

Functional Expression of the *DUR3* Gene in a Wine Yeast Strain to Minimize Ethyl Carbamate in Chardonnay Wine

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Abstract: During alcoholic fermentation *Saccharomyces cerevisiae* metabolizes arginine to ornithine and urea. Urea can be metabolized by wine yeasts; however, the presence of good nitrogen sources in grape must leads to transcriptional suppression of genes involved in urea import and metabolism. Urea is subsequently exported out of the cell where it spontaneously reacts with ethanol in wine to form the carcinogen ethyl carbamate (EC). Constitutive expression of *DUR1,2* in the wine yeast UC Davis 522 (Montrachet) leads to an 89% reduction in the EC content of Chardonnay wine. To reabsorb urea secreted into fermenting grape must by non-urea-degrading yeast, we constitutively expressed the *DUR3* gene under the control of the *S. cerevisiae PGK1* promoter and terminator signals and integrated this linear cassette into the *TRP1* locus of *S. cerevisiae* strain 522. The urea-importing strain 522^{DUR3} reduced EC by 81% in Chardonnay wine and was shown to be approximately four times as effective as the urea-degrading strain $522^{DUR1,2}$ at reducing EC in Chardonnay wine made from must with high endogenous urea levels.

Key words: wine, ethyl carbamate, *DUR3*, urea

Ethyl carbamate (EC) is known to induce mutations and tumors in a variety of test animals (Ingledew et al. 1987, Monteiro et al. 1989, Ough 1976, Schlatter and Lutz 1990) and is considered a probable human carcinogen by the World Health Organization's International Agency for Research on Cancer. Urea and ethanol are the main precursors for the formation of EC in wine (Monteiro et al. 1989). In addition to urea, potentially reactive carbamyl compounds include citrulline and carbamyl phosphate. Both compounds are mainly derived from arginine metabolism by lactic acid bacteria during malolactic fermentation in wine (Ough et al. 1988a).

Under ambient conditions (wine storage), ethanol reacts with urea to form EC in a time- and temperature-dependent manner (Ingledew et al. 1987, Kodama et al. 1994, Ough et al. 1988b). Urea is produced by the arginase (*CAR1*) dependent breakdown of arginine to ornithine and urea (Cooper 1982). Arginine is one of the main amino acids in grape musts (Kliewer 1970). Urea is a poor nitrogen source for *S. cerevisiae* and, at high concentrations, is toxic to yeast cells, which preferentially export it to the fermenting medium (An and Ough 1993). *Saccharomyces cerevisiae* is capable of metabolizing urea by the action of the enzyme urea amidolyase, which is encoded by the *DUR1,2* gene (Genbauffe and Cooper 1986, 1991, Whitney et al. 1973); however, native copies of *DUR1,2* are transcriptionally silenced by nitrogen catabolite repression (NCR) in media with rich nitrogen supplies (Genbauffe and Cooper 1986). Consequently, if yeast cells are not starved for nitrogen in grape must, which forces them to import and degrade urea, then urea will diffuse out of the cell through the constitutively expressed passive urea permease encoded by *DUR4*, thus resulting in wines with high residual urea and increased EC levels.

Entry of urea into yeast cells is bimodal (Cooper and Sumrada 1975, Sumrada et al. 1976). A facilitated diffusion system (*DUR4*) brings urea into the cell in an energy-independent fashion when urea is present at concentrations greater than 0.5 mM. In addition, the NCR sensitive *DUR3* gene that encodes a 735 aa integral membrane protein uses an energy dependent sodium symporter that imports urea at low concentrations (14 μM K_m) (Cooper and Sumrada 1975, Sumrada et al. 1976). Moreover, *DUR3* is an important regulator of intracellular boron concentration (Nozawa et al. 2006); however, a clear physiological role for *DUR3* in terms of boron utilization has yet to be defined. Another important role for *DUR3* is in the uptake of polyamines: *DUR3* specifically facilitates the uptake of polyamines, which are important for general cell growth, concurrently with urea (Uemura et al. 2007). As urea is a poor nitrogen source, and does not normally occur in significant quantities outside

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A U.S. provisional patent has been filed (61/071,138) by H.J.J. van Vuuren and J.I. Husnik on the constitutive expression of DUR3 in wine yeast to limit ethyl carbamate production in wine.

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the cell, the main physiological role of *DUR3* may well be polyamine uptake; in fact, *DUR3* mRNA is repressed in the presence of large quantities of polyamines (Uemura et al. 2007). Polyamine uptake by DUR3p is post-translationally regulated by the serine/threonine kinase *PTK2;* the kinase positively regulates *DUR3* polyamine uptake via the phosphorylation of three cytoplasmic residues. Although *DUR3* polyamine activity and subsequent *PTK2* regulation has been preliminarily investigated in a laboratory yeast strain (Uemura et al. 2007), there are no known studies that have investigated the role of *DUR3/PTK2* mediated urea or polyamine uptake during alcoholic fermentation.

Recently, we developed a urea-degrading industrial wine yeast, $522^{DUR1,2}$ (previously reported as 522^{EC-}) capable of significantly reducing EC in Chardonnay wine (Coulon et al. 2006). When the *DUR1,2* ORF was placed under the control of the *S. cerevisiae PGK1* promoter and terminator signals, and a single copy of the construct was integrated into the *URA3* locus of the industrial strain UC Davis 522, Chardonnay wine produced by the functionally enhanced strain ($522^{DUR1,2}$) contained 89% less EC. Analysis of the genotype, phenotype, and transcriptome of $522^{DUR1,2}$ suggested that this yeast strain was substantially equivalent to its parent, thus making it suitable for commercialization (Coulon et al. 2006).

In an effort to further reduce EC in wine, we created functionally enhanced urea-importing yeast cells. We constitutively expressed the *DUR3* gene under the control of the *S. cerevisiae PGK1* promoter and terminator signals and integrated this linear cassette into the *TRP1* locus of *S. cerevisiae* strain 522. The urea-importing strain 522^{DUR3} reduced EC by 81% in Chardonnay wine, indicating that the urea-importing yeast strain is a viable alternative to the urea-degrading strain $522^{DUR1,2}$ for EC reduction in Chardonnay wine. The urea-importing strain 522^{DUR3} is especially useful in reducing EC in Chardonnay wine made from must with high endogenous urea. As compared to strain $522^{DUR1,2}$, strain 522^{DUR3} reduced EC approximately fourfold more efficiently in Chardonnay wine made from must spiked with 200 mg/L urea.

Materials and Methods

Strains and media. The four yeast strains used in this study were cultured according to standard methods (Ausubel et al. 1995) (Table 1). The industrial wine strain UC Davis 522 was used for isolation of genomic DNA and for the integration of the linear *trp1-PGK1*$_p$*-DUR3-PGK1*$_t$*-kanMX-trp1* cassette. YPD plates supplemented with 300 µg/mL G418 were used to select for positive *S. cerevisiae* transformants containing the *DUR3* expression cassette. *Escherichia coli* strain F- φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ- was used for molecular cloning and was grown aerobically at 37°C in LB media. *E. coli* transformants were selected for on solid LB media supplemented with 100 µg/mL ampicillin.

Construction and integration of the linear *DUR3* cassette. To place the *DUR3* gene under the control of the constitutive *PGK1* promoter and terminator signals, the *DUR3* ORF was PCR amplified using specific primers (Table 2) and cloned into the *Xho1* site of the linearized-SAP treated vector pHVX2 (Volschenk et al. 1997), resulting in the expression vector pHVX2D3. A *kanMX* marker was obtained from pUG6 (Guldener et al. 1996) by double digestion with *Xho1* and *Sal1*. Following digestion, the 1500 bp *kanMX* band was gel purified and ligated into the *Sal1* site of linearized-SAP treated pHVX2D3, resulting in the plasmid pHVXKD3.

Table 1 *Saccharomyces cerevisiae* yeast strains used in this study.

Strain	Description	Reference
522	Industrial wine yeast strain	UC Davis
$522^{DUR1,2}$	Wine yeast strain 522 with *DUR1,2* cassette integrated at *URA3* locus	Coulon et al. 2006
522^{DUR3}	Wine yeast strain 522 with *DUR3* cassette integrated at *TRP1* locus	This study
$522^{DUR1,2/DUR3}$	Wine yeast strain 522 with *DUR1,2* cassette integrated at *URA3* locus and *DUR3* cassette integrated at *TRP1* locus	This study

Table 2 Oligonucleotide primers used in this study.

Primer	Sequence (5'→ 3')
DUR3fwdXho1	AAAACTCGAGATGGGAGAATTTAAACCTCCGCTAC
DUR3revXho1	AAAACTCGAGCTAAATTATTTCATCAACTTGTCCGAAATGTG
BamH1Apa1TRP1ORFfwd	AAAAAAGGATCCAAAAAAGGGCCCATGTCTGTTATTAATTTCACAGG
BamH1Apa1TRP1ORFrev	AAAAAAGGATCCAAAAAAGGGCCCCTATTTCTTAGCATTTTTGACG
pHVXKlongfwd	CTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG
pHVXKlongrev	CTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG
DUR3probefwd	CAGCAGAAGAATTCACCACCGCCGGTAGATC
DUR3proberev	CAATCAGGTTAATAATTAATAAAATACCAGCGG
TRP1probefwd	TTAATTTCACAGGTAGTTCTGGTCCATTGG
TRP1proberev	CAATCCAAAAGTTCACCTGTCCCACCTGCTTCTG

In order to target the *PGK1*p-*DUR3*-*PGK1*t-*kanMX* cassette to the *TRP1* locus, the *TRP1* coding region was PCR amplified from 522 genomic DNA using the *TRP1* specific primers (Table 2) and cloned into the *BamH1* site of linearized-SAP treated pUC18 (Yanisch-Perron et al. 1985), resulting in the plasmid pUCTRP1. The *PGK1*p-*DUR3*-*PGK1*t-*kanMX* cassette located within pHVXKD3 was PCR amplified from pHVXKD3 plasmid DNA using cassette specific primers (Table 2) and blunt end cloned into the *EcoRV* site of linearized-SAP treated pUCTRP1, resulting in the *DUR3* cassette containing plasmid pUCMD.

For the LiAC/PEG transformation of *S. cerevisiae* strains (Gietz and Woods 2002), the 6536 bp *DUR3* cassette was cut from pUCMD using *Apa1* and gel purified. To transform *S. cerevisiae* strains 522 and $522^{DUR1,2}$, 250 ng of purified linear cassette was used. Following transformation, cells were left to recover in YPD at 30°C for 2 hr before plating on to YPD plates supplemented with 300 µg/mL G418. Plates were incubated at 30°C until colonies appeared.

Southern blotting of genomic DNA from functionally enhanced strains 522^{DUR3} and $522^{DUR1,2/DUR3}$ and the parental strain 522 was performed as described (Ausubel et al. 1995). Blots were probed with PCR-generated fragments specific for *DUR3* and *TRP1* (Table 2). The AlkPhos Direct Nucleic Acid Labeling and CDP-Star Detection system was used as recommended for probe detection (GE Healthcare, Piscataway, NJ).

Production of Chardonnay wine. Single colonies of functionally enhanced 522^{DUR3} and $522^{DUR1,2/DUR3}$ and the parental strain were inoculated into 5 mL YPD and grown overnight at 30°C on a rotary wheel. Cells were subcultured into 50 mL YPD (0.05 final OD_{600}) and grown overnight at 30°C in a water-shaker bath (180 rpm). Cells were harvested by centrifugation (5000 rpm, 4°C, 5 min) and washed once with 50 mL sterile water. Cell pellets were resuspended in 5 mL sterile water and OD_{600} measured. Cell suspensions were used to inoculate (0.1 final OD_{600}) sterile 250-mL bottles filled with either 200 mL unfiltered Chardonnay must or 200 mL unfiltered Chardonnay must supplemented with 200 mg/L urea. Bottles were aseptically sealed with sterilized (70% v/v ethanol) vapor locks filled with sterile water. Sealed bottles were incubated at 20°C, and weighed daily to monitor fermentation progress. Chardonnay grape juice (23.75 Brix, pH 3.41, ammonia 91.6 mg/L, FAN 309.6 mg/L) was obtained from Calona Vineyards, Okanagan Valley, Canada.

Analysis of ethanol in Chardonnay wine. Wine samples were analyzed on an Agilent 1100 series liquid chromatograph (LC) running Chemstation Rev A.09.03 [1417] software (Agilent Technologies, Santa Clara, CA). The LC was fitted with a Supelcogel C-610H main column (column temp. 50°C, 30 cm x 7.8 mm i.d.; Supelco, Park Bellefonte, PA) that was protected by a Supelguard C-610H (5 cm x 4.6 mm i.d.; Supelco) guard column. A 10 µL sample was run isocratically with 0.1% (v/v) H_3PO_4/H_20 buffer at a flow rate of 0.75 mL/min. Ethanol was eluted from the column at ~19 min and was detected by a refractive index detector running in positive mode. The concentration of ethanol was determined automatically by Chemstation software as based on an ethanol standard curve.

Quantification of EC in wine. The content of EC in Chardonnay wine was quantified by solid-phase microextraction and gas chromatograph-mass spectrometry (GC-MS) as previously described (Coulon et al. 2006).

Results and Discussion

Characterization of the linear DUR3 cassette. During fermentation the *DUR3* gene is subject to transcriptional repression by NCR, which results in the inability of *S. cerevisiae* to reabsorb excreted urea in the presence of good nitrogen sources (ElBerry et al. 1993, Hofman-Bang 1999). This inability to metabolize excreted urea is a contributing factor in the production of wines with high residual urea and, in turn, high EC (An and Ough 1993, Kitamoto et al. 1991, Monteiro et al. 1989). In order to facilitate constitutive expression of *DUR3* in industrial yeast strains, a linear *DUR3* expression cassette with a positive selection marker was constructed (Figure 1). While it was suitable to use a *kanMX* positive selection marker for this study, it will be necessary to construct an antibiotic resistance-free cassette similar to that used in the construction of $522^{DUR1,2}$ (Coulon et al. 2006) should *DUR3* strains be developed for commercialization.

Single-strand sequencing revealed that the *DUR3* cassette contained the desired DNA fragments in the correct order and orientation (data not shown). Furthermore, in silico assembly of the *DUR3* coding region in the cassette revealed that the *DUR3* ORF was identical in amino acid sequence and length to that published on the *Saccharomyces* Genome Database (www.yeastgenome.org).

To constitutively express *DUR3* in industrial *S. cerevisiae* strains, the linear *DUR3* cassette was transformed into the wine yeast strains 522 and $522^{DUR1,2}$. We obtained two functionally enhanced wine yeast strains (522^{DUR3} and $522^{DUR1,2/DUR3}$); each of the two recombinant strains were confirmed by Southern blot to contain a single copy of the ~6.5 kb linear *DUR3* cassette integrated into one of their *TRP1* loci (Figure 2). Blotting also confirmed that the diploid strains 522^{DUR3} and $522^{DUR1,2/DUR3}$ retained a non-disrupted *TRP1* locus, thus maintaining their tryptophan prototrophy and wild-type phenotype (Figure 2).

Reduction of EC in Chardonnay wines. In order to assess the reduction of EC by functionally enhanced yeast strains, Chardonnay wine was made with the parental strain 522 and the functionally enhanced strains $522^{DUR1,2}$, 522^{DUR3}, and $522^{DUR1,2/DUR3}$ and the EC content quantified by GC-MS at the end of fermentation. Fermentation profiles

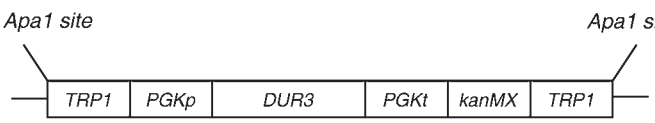

Figure 1 Schematic representation of the linear *DUR3* cassette.

are presented (Figure 3), and the final amount of ethanol produced by the functionally enhanced and control strains is shown (Table 3). All data indicate a high degree of similarity between the parental strains and their functionally improved counterparts.

In agreement with previous results (Coulon et al. 2006), $522^{DUR1,2}$ was highly efficient at reduction of EC in Chardonnay wine (81.5%, Table 4). During winemaking, 522^{DUR3} and $522^{DUR1,2/DUR3}$ reduced EC by 83% and 81%, respectively, indicating that 522^{DUR3} is as capable as $522^{DUR1,2}$ at reducing EC and that constitutive co-expression of *DUR1,2* and *DUR3* does not result in synergistic EC reduction. The observed equivalency in EC reduction between 522^{DUR3} and $522^{DUR1,2}$ is likely a function of the need for yeast cells to degrade urea once it is internalized, as urea is toxic to cells at high concentrations. Indeed, constitutive expression of *DUR3* in a functionally enhanced sake yeast strain resulted a four-fold induction of *DUR1,2*, as shown by quantitative reverse transcriptase PCR (M. Dahabieh, unpublished data, 2008). The highly efficacious reduction of EC by 522^{DUR3} and $522^{DUR1,2/DUR3}$ observed in this study are important because they validate the application of *DUR3* constitutive expression for the reduction of EC in grape wine. Given the ability for yeast with the integrated DUR3 cassette to

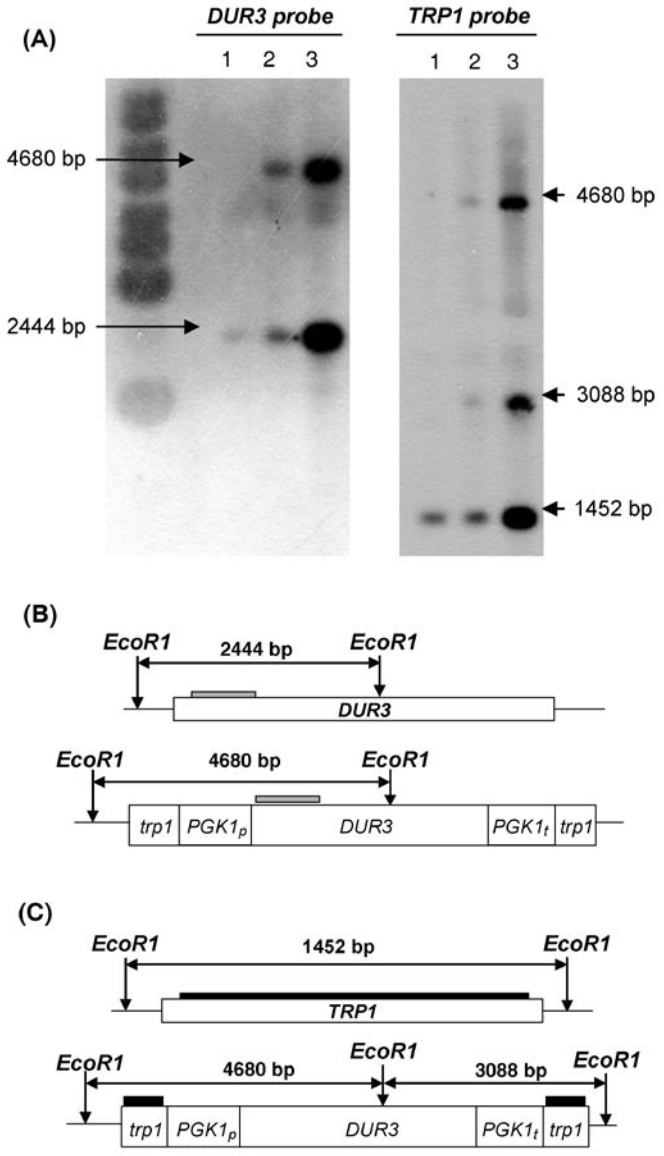

Figure 2 Integration of the *DUR3* cassette into the *TRP1* locus of 522^{DUR3} and $522^{DUR1,2/DUR3}$ confirmed by Southern blot analysis. (**A**) Approximately 1 µg of *EcoR1* digested genomic DNA from 522 (lane 1), 522^{DUR3} (lane 2), and $522^{DUR1,2/DUR3}$ (lane 3) was probed with either *DUR3* or *TRP1*. (**B**) Schematic representation of the integrated and wild-type *DUR3* genes. Grey boxes indicate the area of the *DUR3* gene used as a probe. (**C**) Schematic representation of the integrated and wild-type *TRP1* loci. Black boxes indicate the area of the *TRP1* gene used as a probe.

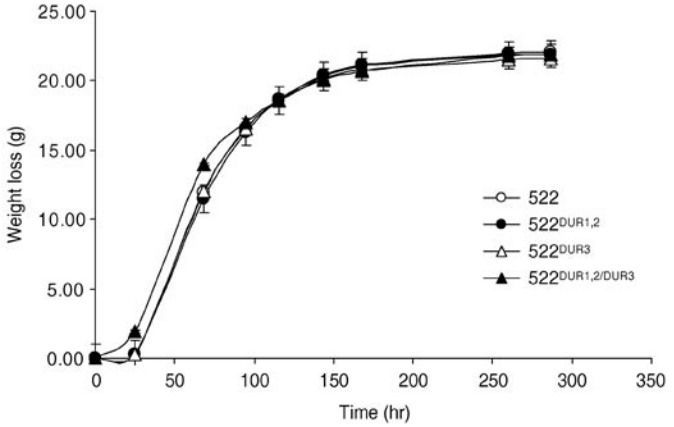

Figure 3 Fermentation profiles (weight loss) of wine yeast strains 522, $522^{DUR1,2}$, 522^{DUR3}, and $522^{DUR1,2/DUR3}$ in Chardonnay wine. Wine was produced from unfiltered Calona Chardonnay must inoculated to 0.1 final OD_{600} and incubated to completion (~300 hr) at 20°C. Fermentations were conducted in triplicate and data averaged; error bars indicate one standard deviation.

Table 3 Ethanol (% v/v) produced by four wine yeast strains in Chardonnay wine, measured at the end of fermentation. Data analyzed for statistical significance ($p \leq 0.05$) using two-factor ANOVA analysis; none of the values was significantly different from the parental strain.

	522	$522^{DUR1,2}$	522^{DUR3}	$522^{DUR1,2/DUR3}$
Replicate 1	13.65	13.71	13.74	13.54
Replicate 2	13.60	13.65	13.71	13.62
Replicate 3	13.71	13.66	13.55	13.58
Ethanol average (n = 3)	13.65	13.67	13.67	13.58
Std dev	0.06	0.03	0.10	0.04

Table 4 Reduction of EC (µg/L) in Chardonnay wine produced by wine yeast strains 522, $522^{DUR1,2}$, 522^{DUR3}, and $522^{DUR1,2/DUR3}$ from unfiltered Chardonnay must. Triplicate fermentations were incubated to completion (~300 hr) at 20°C.

	522	$522^{DUR1,2}$	522^{DUR3}	$522^{DUR1,2/DUR3}$
Replicate 1	199.3	31.32	28.76	36.63
Replicate 2	169.95	37.32	29.89	38.61
Replicate 3	177.61	32.66	34.56	31.3
Average (n = 3)	182.29	33.77	31.07	35.51
Std dev	15.22	3.15	3.07	3.78
% Reduction	--	81.48	82.96	80.52

Table 5 Reduction of EC (µg/L) in Chardonnay wine produced by wine yeast strains 522, $522^{DUR1,2}$, 522^{DUR3}, and $522^{DUR1,2/DUR3}$ from unfiltered Chardonnay must supplemented with 200 mg/L urea. Triplicate fermentations were incubated to completion (~300 hr) at 20°C.

	522	$522^{DUR1,2}$	522^{DUR3}	$522^{DUR1,2/DUR3}$
Replicate 1	2175.94	1816.00	259.30	512.21
Replicate 2	2212.23	1827.88	381.79	345.94
Replicate 3	2250.69	1566.26	183.40	456.75
Average (n = 3)	2212.95	1736.71	274.83	438.30
Std dev	37.38	147.73	100.11	84.66
% Reduction	--	21.52	87.58	80.19

import urea constitutively, we examined the efficiency of EC reduction in high-urea Chardonnay must. As expected, supplementation of the must with 200 mg/L of urea produced wine with ~100-fold higher EC content irrespective of the fermenting yeast strain (Table 4, Table 5); however, EC reduction was significantly enhanced in wine produced by urea-importing yeast (522^{DUR3}) versus wine produced by urea-degrading yeast ($522^{DUR1,2}$) (87.6% vs. 21.5% reduction). Therefore, the urea-importing yeasts created in this study (522^{DUR3} and $522^{DUR1,2/DUR3}$) are superior to urea-degrading yeast ($522^{DUR1,2}$) for reduction of EC in wines derived from musts with high endogenous urea. Consistent with nonsupplemented must, fermentation of high-urea must by the urea-importing and degrading strain $522^{DUR1,2/DUR3}$ offered no synergistic advantage for EC reduction (Table 5).

Conclusion

DUR3 constitutive expression is an important, valuable, and alternative strategy for EC reduction in wines, especially those made from high-urea musts. All of the *DUR3* expressing strains were substantially equivalent to parental strains in terms of fermentation rate and ethanol production. As the functionally enhanced strains can be created without the use of antibiotic resistance markers, such strains will not be considered as transgenic.

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