Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Functional enhancement of Sake yeast strains to minimize the production of ethyl carbamate in Sake wine

M.S. Dahabieh[1]*, J.I. Husnik[2] and H.J.J. van Vuuren[1,3]

1 Wine Research Centre, Faculty of Land and Food Systems, The University of British Columbia, Vancouver, BC, Canada
2 Phyterra Yeast Inc., Charlottetown, PE, Canada
3 Director of Wine Research Centre, The University of British Columbia, Vancouver, BC, Canada

Keywords
DUR1,2, *DUR3*, ethyl carbamate, fermentation, *Saccharomyces cerevisiae*, Sake, urea.

Correspondence
Hennie J.J. van Vuuren, Wine Research Centre, Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC V6T 1Z4, Canada.
E-mail: hjjvv@interchange.ubc.ca

***Present address**
M.S. Dahabieh, PhD Student, Department of Biochemistry and Molecular Biology, Life Sciences Centre, University of British Columbia, Vancouver, BC V6T 1Z3, Canada.

A U.S. Provisional patent has been filed (61/071,138) on the constitutive expression of *DUR3* in wine yeast to limit ethyl carbamate production in wine.

2009/2032: received 23 November 2009, revised 8 March 2010 and accepted 12 March 2010

doi:10.1111/j.1365-2672.2010.04723.x

Abstract

Aims: In fermented alcoholic beverages and particularly in Japanese Sake wine, the ubiquitous presence of the probable human carcinogen ethyl carbamate (EC) is a topic of significant concern. This study aims to develop novel methods for the reduction of EC in Sake wine.

Methods and Results: To reduce the high levels of EC in Sake wine, urea-degrading and urea-importing yeast strains were created by integrating linear cassettes containing either the respective *DUR1,2* or *DUR3* genes, under the control of the constitutively active *Saccharomyces cerevisiae PGK1* promoter, into the Sake yeast strains K7 and K9. The self-cloned, urea-degrading Sake strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$ produced Sake wine with 87 and 68% less EC, respectively, while the urea-importing Sake yeast strain $K7^{DUR3}$ reduced EC by 15%. All functionally enhanced yeast strains were shown to be substantially equivalent to their parental strains in terms of fermentation rate, ethanol production, phenotype and transcriptome.

Conclusions: Under the conditions tested, urea-degrading yeast (constitutive *DUR1,2* expression) are superior to urea-importing yeast (constitutive *DUR3* expression) for EC reduction in Sake wine, and constitutive co-expression of *DUR1,2* and *DUR3* does not yield synergistic EC reduction.

Significance and Impact of the Study: The self-cloned, substantially equivalent, urea-degrading Sake yeast strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$, which contain the integrated *DUR1,2* cassette, are capable of highly efficacious EC reduction during Sake brewing trials, are suitable for commercialization and are important tools for modern Sake makers in their efforts to reduce high EC levels in Sake wine.

Introduction

In fermented alcoholic beverages the ubiquitous presence of the probable human carcinogen ethyl carbamate (EC) has become a topic of significant concern (Ough 1976a,b; Ingledew *et al.* 1987; Canas *et al.* 1989; Schlatter and Lutz 1990). By itself EC is not carcinogenic; however, products of EC metabolism have been shown to covalently bind DNA thereby inducing mutations. Furthermore, EC has been shown to cause various cancers in a variety of test animals (Leithauser *et al.* 1990; Schlatter and Lutz 1990; Hubner *et al.* 1997; Beland *et al.* 2005). As it is found in most alcoholic beverages in the $\mu g\ l^{-1}$ range (Weber and Sharypov 2009), exposure to EC may be increased by the regular consumption of alcoholic beverages, and this may be a factor involved in human cellular mutagenesis and resultant tumorigenesis (Zimmerli and Schlatter 1991; Park *et al.* 2009). As a result, winemakers have been actively reducing EC levels in wines by both agricultural practices, wine-making practices and, more recently, molecular biological means (Kitamoto *et al.* 1991; Butzke and Bisson 1998; Yoshiuchi *et al.* 2000; Park *et al.* 2001;

Schehl *et al.* 2007). In addition to the presence of EC in table wines, Sake wine, which is a Japanese alcohol made from fermented rice, has some of the highest EC contents amongst fermented beverages; typical Sake wines range from 100 to 250 $\mu g\ l^{-1}$ EC because of a pasteurization process that all Sakes undergo prior to bottling (Canas *et al.* 1989).

The excretion of nonmetabolized urea by yeast cells into wine is the major factor involved in the formation of EC (Monteiro *et al.* 1989; Monteiro and Bisson 1991). Under ambient conditions (wine storage), ethanol and urea react, in a time- and temperature-dependent manner, to form EC (Ingledew *et al.* 1987; Ough *et al.* 1988b; Kodama *et al.* 1994).

Urea forms from the arginase (*CAR1*)-dependent breakdown of arginine to ornithine and urea (Cooper 1982). At high concentrations, urea is a toxic, poor nitrogen source for *Saccharomyces cerevisiae*, and is therefore exported to the surrounding medium (An and Ough 1993). Under the right conditions, *S. cerevisiae* is capable of metabolizing urea by means of the enzyme urea amidolyase (*DUR1,2* gene product); DUR1,2p is an ATP- and biotin-dependent bifunctional enzyme that degrades urea to ammonia and carbon dioxide in two steps via an allophanate intermediate (Whitney and Cooper 1972, 1973; Whitney *et al.* 1973; Genbauffe and Cooper 1986, 1991). Despite the ability of yeast to degrade urea (via DUR1,2p), most fermentations finish with residual urea because native copies of *DUR1,2* are transcriptionally silenced by nitrogen catabolite repression (NCR) in fermentations with rich nitrogen supplies (Genbauffe and Cooper 1986; Ough *et al.* 1988a,b, 1990; Coulon *et al.* 2006). As long as yeast cells are not starved for nitrogen, at which point they will import and metabolize urea, it will diffuse out of the cell through a constitutively expressed passive urea permease DUR4p, thus resulting in wines with high residual urea and high EC.

Yeast can import urea in one of two ways, depending on environmental urea concentration (Cooper and Sumrada 1975; Sumrada *et al.* 1976). At high extracellular concentrations (>0·5 mmol l^{-1}), urea enters the cell in an energy-independent fashion via a facilitated diffusion system encoded by the *DUR4* gene. At low concentrations (K_m = 14 μmol l^{-1}), urea is imported via a 735 amino acid, integral membrane, ATP-dependent sodium-urea symporter, which is encoded by the NCR-sensitive *DUR3* gene (Cooper and Sumrada 1975; Sumrada *et al.* 1976). In addition to its role as a urea importer, *DUR3* has been shown to regulate intracellular boron concentration (Nozawa *et al.* 2006); however, a clear physiological role for *DUR3* has yet to be defined. Further to its role in boron transport, *DUR3* has been shown to specifically facilitate the uptake of polyamines, which are important for cell growth, concurrently with urea (Uemura *et al.* 2007). In fact, excess polyamines causes repression of *DUR3* mRNA (Uemura *et al.* 2007), suggesting that the cell modulates *DUR3* expression in response to both urea and polyamines. Additionally, polyamine uptake by DUR3p is subject to post-translational regulation by the serine/threonine kinase PTK2p that positively regulates polyamine uptake via phosphorylation of three DUR3p cytoplasmic residues (Uemura *et al.* 2007). It is unknown whether urea uptake by DUR3p is also modulated by the activity of PTK2p. While one group has examined the polyamine uptake activity of *DUR3* and *PTK2* 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.

Given the need for efficacious solutions to the problem of EC in alcoholic beverages, our group recently developed a urea-degrading industrial wine yeast, $522^{DUR1,2}$ (previously reported as 522^{EC-}), which was shown to be capable of significantly reducing EC in Chardonnay wine by approx. 90% (Coulon *et al.* 2006). This yeast strain contained a single, stable, constitutively active copy of *DUR1,2*. Analysis of the genotype, phenotype and transcriptome of $522^{DUR1,2}$ suggested that the enhanced strain was substantially equivalent to its parent, thus making it suitable for commercialization (Coulon *et al.* 2006). Furthermore, because the urea-degrading strain contains no foreign DNA sequences, it is not classified as transgenic (Coulon *et al.* 2006).

In this study, we reduced EC in Sake wine by transforming two popular industrial Sake yeast strains (Kyokai No. 7 and Kyokai No. 9) with the linear *DUR1,2* cassette previously developed by our group (Coulon *et al.* 2006). We then demonstrated that the newly developed, self-cloned Sake yeast strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$ were highly capable of EC reduction during Sake-brewing trials. Analysis of the functionally improved strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$ indicated that said strains were substantially equivalent to their respective parent strains in terms of genotype, phenotype and transcriptome, thus making the yeast strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$ suitable for commercialization.

To further reduce EC in Sake, as well as to compare two methods of EC reduction, we sought to create novel urea-importing yeast strains through the constitutive expression of the active urea permease *DUR3*. Here, we provide the first comparison of efficacy between two different functionally enhanced yeast strains developed for the reduction of EC in Sake wine. Moreover, we provide the first attempt at reducing Sake wine EC through functional expression of multiple genes in *S. cerevisiae*.

Materials and methods

Strains and media

The *S. cerevisiae* Sake strains Kyokai No. 7 and Kyokai No. 9 were used for integration of the linear *ura3-PGK1$_p$-DUR1,2-PGK1$_t$-ura3* cassette (Coulon *et al.* 2006) as well as the linear *trp1-PGK1$_p$-DUR3-PGK1$_t$-kanMX-trp1* cassette (Dahabieh *et al.* 2009) (Fig. 1). Unless otherwise stated, all yeasts were cultured aerobically with shaking at 30°C either in liquid YPD medium (yeast extract, peptone, dextrose) or on YPD + 2% (w/v) agar plates. Chardonnay grape juice (23·75 Brix, pH 3·41, ammonia 91·6 mg l^{-1}, free amino nitrogen 309·6 mg l^{-1}) was obtained from Calona Vineyards, Okanagan Valley, Canada. Filter-sterilized (0·22 μm filter; Millipore, Billerica, MA) Chardonnay juice was used for total RNA isolation from fermenting yeast strains.

Integration of the linear *DUR1,2* cassette into the *URA3* locus of *Saccharomyces cerevisiae* K7 and K9

The linear *DUR1,2* cassette was purified from plasmid pJC3 by digestion with *Srf1*, and strains K7 and K9 were transformed using the co-transforming shuttle vector pUT332 as previously described (Coulon *et al.* 2006). Yeast colony PCR was used with cassette specific primers to detect the presence of the linear *DUR1,2* cassette integrated correctly into the yeast genome at the *URA3* locus. Southern blots to confirm correct integration and loss of antibiotic resistance marker genes were performed as previously described (Coulon *et al.* 2006). DNA sequencing of the integrated *DUR1,2* cassette in the genomes of K7^{DUR1,2} and K9^{DUR1,2} was performed as previously described (Coulon *et al.* 2006).

Construction and integration of the linear *DUR3* cassette into the *TRP1* locus of *Saccharomyces cerevisiae* K7 and K7^{DUR1,2}

To place the *DUR3* gene under the control of the constitutive *PGK1* promoter and terminator signals, a *trp1-PGK1$_p$-DUR3-PGK1$_t$-kanMX-trp1* linear cassette was constructed as previously described (Dahabieh *et al.* 2009). *Saccharomyces cerevisiae* strains K7 and K7^{DUR1,2} were transformed with the DUR3 cassette as previously described (Dahabieh *et al.* 2009). Southern blots of genomic DNA from functionally enhanced strains K7^{DUR1,2}, K7^{DUR3} and K7$^{DUR1,2/DUR3}$, as well as the parental strain K7, were probed with PCR-generated fragments specific for *DUR3* and *TRP1*. The AlkPhos™ Direct Nucleic Acid Labeling and CDP-Star Detection system was used as recommended for probe labelling and detection (GE Healthcare, Piscataway, NJ).

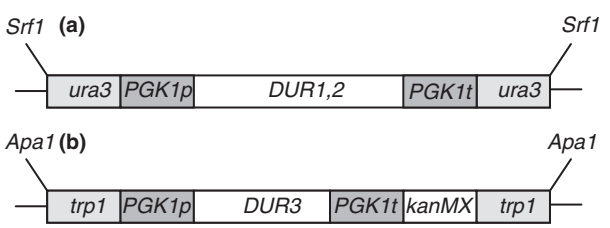

Figure 1 Schematic representations of the linear (a) *DUR1,2* and (b) *DUR3* cassettes.

Analysis of gene expression by qRT-PCR

Parental and functionally improved yeast strains were inoculated to a final OD_{600} = 0·1 in 200 ml Chardonnay must and were grown for 24 h at 20°C. Total RNA from 24-h fermentations was extracted using hot acidic phenol and was subsequently used for analysis by qRT-PCR and DNA microarray.

For qRT-PCR, total RNA was cleaned up post extraction using a total RNeasy Mini kit (Qiagen, Valencia, CA). Clean total RNA (1 μg) was used for cDNA synthesis (iScript™; Bio-Rad, Hercules, CA) according to the manufacturer's instructions. iTAQ™ SYBR® Green Supermix with ROX (Bio-Rad) was used in conjunction with an Applied Biosystems (Foster City, CA) 7500 Real Time PCR machine and gene specific primers to quantify the levels of gene expression relative to the housekeeping gene *ACT1*. *DUR1,2* expression was quantified with the primers DUR1,2RTfwd (5′-CTCTGGTCCAATGGACGCATA-3′) and DUR1,2RTrev (5′-GATGGATGGACCAGTCAA-CGTT-3′), while *DUR3* expression was quantified with the primers DUR3RTfwd (5′-GATCGGCCATGGTTGC-TACTT-3′) and DUR3RTrev (5′-GCGATAGTGTT-CATCCCGGTT-3′). *ACT1* expression was quantified with the primers ACT1RTfwd (5′-GTTTCCATCCAAGCCG-TTTTG-3′) and ACT1RTrev (5′-GCGTAAATTGGAACG-ACGTGAG-3′). Thermal cycling was performed as follows: 95°C for 3 min, 40 cycles of 95°C for 15 s followed by 60°C for 30 s followed by data collection. For each strain, gene expression was analysed by six independent amplifications, and the results were averaged. Relative quantification (RQ) data were analysed using the Applied Biosystems RQ Study software ver. 1.2.2.

Global gene expression analysis

Total RNA from strains K7, K7^{DUR1,2} and K7^{DUR3} was extracted after 24 h of fermentation in Chardonnay must. A 'GeneChip® One-Cycle Target Labeling and Control' kit (Affymetrix, Santa Clara, CA) was used according to manufacturer's instructions for synthesis and clean-up of cDNA

and for synthesis, cleanup and fragmentation of biotinylated cRNA from 10 μg of total RNA. Microarray analyses were performed in duplicate, each with independently grown cell cultures. Six oligonucleotide yeast genome arrays (YGS98; Affymetrix), two per strain, were used for hybridization of fragmented labelled cRNA. The preparation of hybridization solution, hybridization, washing, staining, scanning of the microarrays and data analysis were performed as previously described (Coulon *et al.* 2006). Array annotations were linked to their gene ontology annotations using the 'gene_association.sgd.tab' table (http://www.yeastgenome.org/gene_list.shtml). Reported changes in gene expression were cut-off at a minimum fourfold change as supported by previous analysis of global gene expression during alcoholic fermentation (Marks *et al.* 2008). Microarray data were deposited in the National Centre for Biotechnology (NCBI) Gene Expression Omnibus (GEO) under the accession number GSE17867.

Analysis of urea uptake using [^{14}C]urea

To assess the effect of constitutive *DUR3* expression on urea uptake activity, a [^{14}C]urea uptake assay was performed as previously described (Cooper and Sumrada 1975). Briefly, the strains K7, K7^{DUR1,2}, K7^{DUR3} and K7$^{DUR1,2/DUR3}$ were grown in uptake media (UM – 1·7 g l^{-1} yeast nitrogen base without amino acids or ammonium sulfate, 20 g l^{-1} glucose and 1 g l^{-1} ammonium sulfate) at 30°C to approx. 1×10^7 cells per ml. An 11-ml sample of cell culture was transferred to a 250-ml Erlenmeyer flask containing 80 μl of 36·6 mmol l^{-1} [^{14}C]urea (6·8 mCi mmol^{-1}; Sigma, St Louis, MO). Cells were then cultured at 30°C in a shaker water bath (180 rev min^{-1}) for 20 min, and 1-ml samples were taken every 2 min. Samples were applied to 0·22-μm nylon filters (Millipore) and washed twice with 25-ml aliquots of UM media to which 10 mmol l^{-1} urea had been added. Filters were placed in scintillation vials, filled with scintillation fluid (Fisher, Hampton, NH) and left overnight to equilibrate. Samples were counted in a Beckman LS6000IC liquid scintillation counter using the counter's factory '^{14}C quench' mode. Disintegrations were converted into nanomole urea transported per OD_{600} and plotted against time.

Production of 'Doburoku' (Homebrew) style Sake wine

Koji seeds (*Aspergillus oryzae*) were purchased from Vision Brewing (Nedlands, WA) and Koji rice was prepared in 400-g batches from short grain Japanese Kokoho Rose rice (Safeway, Vancouver, Canada) as per manufacturer's instructions. To make Sake wine, single colonies of the functionally improved and parental strains were inoculated into 5 ml YPD and grown overnight at 30°C on a rotary wheel. Cells were subcultured into 50 ml YPD (final OD_{600} = 0·05) and grown overnight at 30°C in a water shaker bath (180 rev min^{-1}). Cells were harvested by centrifugation and washed once with 50 ml sterile water. Cell pellets were resuspended in 5 ml sterile water and OD_{600} measured. The cell suspension was used to inoculate (final OD_{600} = 0·1) sterile 250-ml Schott bottles filled with 13 g 'koji' rice, 48 g freshly steamed rice and 100 ml of water containing 0·125 g l^{-1} citric acid. Bottles were aseptically sealed with sterilized (70% ethanol) vapour locks filled with sterile water. Sealed bottles were incubated at 18°C and weighed daily to monitor fermentation progress.

Analysis of Sake wine

Sake wine samples were analysed for ethanol content on an Agilent 1100 series liquid chromatograph (LC; Agilent, Santa Clara, CA) as previously described for Chardonnay wine samples (Dahabieh *et al.* 2009). The content of EC in Sake wine was quantified by solid-phase microextraction and gas chromatograph/mass spectrometry (GC/MS) as previously described (Coulon *et al.* 2006).

Results

Integration and characterization of the linear *DUR1,2* cassette in the genomes of the Sake yeast strains K7 and K9

To constitutively express *DUR1,2*, the Sake yeast strains K7 and K9 were transformed with the linear *DUR1,2* cassette (Fig. 1, panel a). After screening of approx. 1500 yeast transformants for the integration of the *DUR1,2* cassette, two metabolically improved strains, K7^{DUR1,2} and K9^{DUR1,2}, were obtained. The correct integration of the *DUR1,2* cassette into one of the *URA3* loci of K7^{DUR1,2} and K9^{DUR1,2} was confirmed by Southern blotting with *DUR1,2* and *URA3* probes (data not shown). To verify the sequence of the *DUR1,2* cassette integrated into the *URA3* locus, single-strand DNA sequencing of the cassette in K7^{DUR1,2} and K9^{DUR1,2} was completed. *In silico* sequence assembly and subsequent analysis revealed one nucleotide in the cassette sequence of K9^{DUR1,2} that did not match previously reported sequences. The C to T switch (theoretical to sequenced data) at nucleotide position 821 is located within the 5′*URA3* flanking region of the cassette and is likely because of a genetic polymorphism between the Sake strain K9 and the laboratory strain S288C upon which the Saccharomyces Genome Database (SGD) data are based.

After transformation, K7^{DUR1,2} and K9^{DUR1,2} were successively subcultured on nonselective media to eliminate pUT332, whose only purpose was to facilitate early screening for transformants. A Southern blot using probes

specific for the genes *bla* (Ampicillin resistance) and Tn5*ble* (Phleomycin resistance) (data not shown) revealed that these genes were indeed absent from $K7^{DUR1,2}$ and $K9^{DUR1,2,}$ as well as the parental strains K7 and K9, thus making the functionally improved yeast strains non-transgenic.

Integration of the *DUR1,2* cassette into the genomes of $K7^{DUR1,2}$ and $K9^{DUR1,2}$ results in constitutive expression of *DUR1,2* under conditions of NCR and has a minimal effect on the transcriptome of $K7^{DUR1,2}$

Total RNA from yeast cells in 24-h fermentations of Chardonnay must was used to confirm and quantify the constitutive expression of *DUR1,2* in $K7^{DUR1,2}$ and $K9^{DUR1,2}$. Integration of the *DUR1,2* cassette in the functionally improved strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$ up-regulated *DUR1,2* expression by 9·3-fold and 12·8-fold, respectively, when compared to the parental strains K7 and K9 (Fig. 2). Expression of *DUR1,2* in $K7^{DUR1,2}$ and $K9^{DUR1,2}$ was detected in noninducing conditions, indicating that the *PGK1* promoter and terminator signals are effective at overcoming repression by NCR during fermentation.

Total RNA from yeast cells in 24-h fermentations of Chardonnay must was also used assess the impact of the integrated *DUR1,2* cassette on global gene expression in $K7^{DUR1,2}$. Chardonnay must was used for microarray analysis of Sake yeast strains to minimize technical difficulties during RNA extraction, which would be complicated during Sake fermentation because of the presence of a competing micro-organism in the Sake fermentations (koji – *A. oryzae*). Additionally, isolating high-quality RNA from industrial yeast strains is hindered by particulate matter in Sake mash. As such, to be able to assess the global effect of the *DUR1,2* and *DUR3* cassettes, microarray analysis on Sake yeast strains in this study was performed on fermentations of filtered Chardonnay grape must.

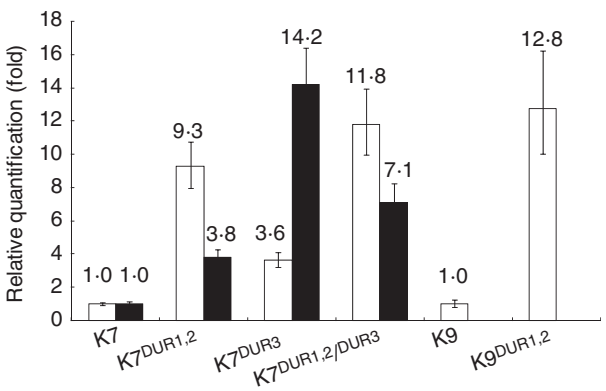

Figure 2 Integration of the *DUR1,2* and *DUR3* linear cassettes in the strains K7, $K7^{DUR1,2}$, $K7^{DUR3}$, $K7^{DUR1,2/DUR3}$, K9 and $K9^{DUR1,2}$ results in constitutive expression of *DUR1,2* and *DUR3*, respectively, under noninducing conditions. *DUR1,2* (white bars) and *DUR3* (black bars) gene expression was quantified by quantitative reverse transcriptase PCR, standardized to *ACT1* expression and calibrated to parental strains. Fermentations were conducted in triplicate, and the data were averaged; error bars represent 95% confidence intervals.

Besides *DUR1,2*, nine genes were affected ≥4-fold; thus, it is evident that integration of the *DUR1,2* cassette into the genome of *S. cerevisiae* K7 had a minimal effect (0·15% change) on the transcription of the 6607 ORFs (4852 verified and 944 uncharacterized, 811 dubious, SGD, February, 2009) in the yeast cell. One gene of interest that was up-regulated ≥4-fold in $K7^{DUR1,2}$ (Table 1) is *HAC1* (4·23-fold), a transcription factor involved in the unfolded protein response (Cox and Walter 1996; Mori 1996; Nikawa *et al.* 1996). This response is likely needed to support the increased translation and folding of DUR1,2p constitutively expressed from the strong *PGK1* promoter; indeed, *HAC1* was also up-regulated (5·67-fold) in the improved strain $K7^{DUR3}$ that contains the *DUR3* gene under the control of the *PGK1* promoter and terminator signals (Table 2). *URA3* was down-regulated by 2·35-fold but was not included in Table 1 as it fell below the fourfold cut-off. No metabolic pathways were affected by the presence of the integrated *DUR1,2* cassette; however, integration of the *DUR1,2* cassette down-regulated three unrelated genes involved in meiosis/sporulation (*RME1*, *SSP1* and *SDS3*), indicating a possible effect on sporulation efficiency (Table 1). As sporulation can be triggered by nutrient deficiency (Malone 1990), it is reasonable that the integration of the *DUR1,2* cassette, which results in constitutive utilization of urea as a nitrogen source, may cause yeast cells to alter or delay their response to nutrient limitation.

Integration and characterization of the linear *DUR3* cassette into the genomes of the Sake yeast strains K7 and $K7^{DUR1,2}$

To facilitate constitutive expression of *DUR3* in industrial Sake yeast strains, a linear *DUR3* expression cassette with a positive *kanMX* selection marker was constructed (Fig. 1, panel b). Single-strand sequencing revealed that this cassette contained the desired DNA fragments in the correct order and orientation. Furthermore, *in silico* assembly of the *DUR3* coding region revealed that the *DUR3* ORF was identical in amino acid sequence and length to that published on SGD.

To constitutively express *DUR3* in industrial *S. cerevisiae* strains, the linear *DUR3* cassette was transformed into Sake yeast strains K7 and $K7^{DUR1,2}$. We obtained two functionally improved Sake yeast strains ($K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$); each of the two recombinant strains was confirmed by Southern blot to contain a single copy of

Table 1 Effect of the integrated *DUR1,2* cassette in the genome of K7 on global gene expression patterns of *Saccharomyces cerevisiae* $K7^{DUR1,2}$ (≥4-fold change). Reported changes are relative to the parental strain K7. Total RNA from K7 and $K7^{DUR1,2}$, harvested at 24 h into fermentation of filter-sterilized Chardonnay must, was used for hybridization to microarrays. Fermentations were conducted in duplicate, and the data were averaged ($P \leq 0{\cdot}005$)

Fold change	Gene symbol	Biological process
Genes expressed at higher levels in $K7^{DUR1,2}$		
6·60	*RTG1*	Transcription factor (bHLH) involved in interorganelle communication
6·35	*DUR1,2*	Urea amidolyase
4·23	*HAC1*	bZIP (basic-leucine zipper) protein involved in unfolded protein response
Genes expressed at lower levels in $K7^{DUR1,2}$		
−5·64	*RME1*	Zinc finger protein involved in control of meiosis
−4·97	*SEO1*	Permease involved in methionine transport
−4·67	*MF(Alpha)1*	Mating factor alpha
−4·64	*SSP1*	Protein involved in the control of meiotic nuclear divisions and spore formation
−4·21	*SDS3*	Protein involved in deacetylase complex and transcriptional silencing during sporulation
−4·16	*CBP1*	Protein required for Cytochrome B mRNA stability or 5′ processing
−4·12	*RUD3*	Protein involved in organization of Golgi

Table 2 Effect of the integrated *DUR3* cassette in the genome of K7 on global gene expression patterns of *Saccharomyces cerevisiae* $K7^{DUR3}$ (≥4-fold change). Reported changes are relative to the parental strain K7. Total RNA from K7 and $K7^{DUR3}$, harvested at 24 h into fermentation of filter sterilized Chardonnay must, was used for hybridization to microarrays. Fermentations were conducted in duplicate, and the data were averaged ($P \leq 0{\cdot}005$)

Fold change	Gene symbol	Biological process
Genes expressed at higher levels in $K7^{DUR3}$		
36·95	*DUR3*	Urea permease
5·67	*HAC1*	Basic-leucine zipper (bZIP) protein involved in unfolded protein response
4·93	*BRN1*	Protein required for chromosome condensation
4·24	*FIG1*	Integral membrane protein required for efficient mating and low affinity Ca^{2+} transport
Genes expressed at lower levels in $K7^{DUR3}$		
−8·18	*TID3*	Meiotic protein required for synapsis and meiotic recombination; interaction partner with DMC1p
−7·31	*SNT309*	Splicing factor protein
−5·98	*TOA1*	Transcription factor IIA, large chain

the linear *DUR3* cassette integrated into one of their *TRP1* loci (data not shown). The strain $K7^{DUR1,2/DUR3}$ also contains a single copy of the *DUR1,2* cassette integrated into one of its *URA3* loci.

Integration of the *DUR3* cassette into the genomes of $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ results in constitutive expression of *DUR3* under conditions of NCR and has a minimal effect on the transcriptome of $K7^{DUR3}$

Expression of *DUR1,2* and *DUR3* was quantified by qRT-PCR analysis of cDNA reverse-transcribed from the total RNA of K7, $K7^{DUR1,2}$, $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ (Fig. 2). *DUR1,2* was up-regulated 11·8-fold in $K7^{DUR1,2}$, while *DUR3* was up-regulated 14·2-fold in $K7^{DUR3}$. High-level expression of both *DUR1,2* and *DUR3* (11·8-fold and 7·1-fold, respectively) was sustained in $K7^{DUR1,2/DUR3}$, when both the *DUR1,2* and *DUR3* cassettes were integrated into the genome. In strains in which only one cassette (*DUR1,2* or *DUR3*) was integrated, constitutive expression of that gene induced expression of the other. Constitutive expression of *DUR1,2* in $K7^{DUR1,2}$ induced expression of *DUR3* by 3·8-fold; likewise, constitutive expression of *DUR3* in $K7^{DUR3}$ induced expression of *DUR1,2* by 3·6-fold (Fig. 2).

Total RNA from yeast cells isolated from 24-h fermentations in Chardonnay must was used analyse the impact of the integrated *DUR3* cassette on global gene expression in $K7^{DUR3}$. *DUR3* was up-regulated by 36·95-fold in $K7^{DUR3}$. Besides *DUR3*, three genes were up-regulated >4-fold in $K7^{DUR3}$ (Table 2); one gene (*HAC1*), which is crucial to the unfolded protein response, was common to both of the engineered strains $K7^{DUR1,2}$ and $K7^{DUR3}$ (Tables 1 and 2). Four genes were down-regulated more than fourfold in $K7^{DUR3}$. *TRP1* was down-regulated by 1·25-fold but was not included in Table 2 as it fell below the fourfold cut-off. As so few genes were affected, it is evident that integration of the *DUR3* cassette into the genome of *S. cerevisiae* K7 had a minimal effect (0·1% change) on the transcription of the 6607 ORFs in the yeast cell. No metabolic pathways were affected by the presence of the integrated *DUR3* cassette; however,

integration of the *DUR3* cassette up-regulated one gene (*FIG1*) and down-regulated one gene (*TID3*) involved in meiosis/sporulation, indicating a possible effect on nutritionally triggered meiosis/sporulation (Malone 1990) (Table 2). The possible effect on meiosis/sporulation, which is likely the result of altered urea utilization in urea-importing strains, was also observed in the global gene expression analysis of $K7^{DUR1,2}$ (Table 1).

Recombinant strains $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ exhibit highly enhanced urea uptake ability under conditions of strong NCR

Radiolabelled [^{14}C]urea uptake assays were performed to confirm constitutive urea uptake as a result of integration of the *DUR3* cassette (Fig. 3). The parental Sake strain K7 and the urea-degrading Sake strain $K7^{DUR1,2}$ containing the *DUR1,2* cassette integrated into the *URA3* locus failed to incorporate any significant amount of radiolabelled urea in a minimal medium containing 1% (w/v) ammonium sulfate (Fig. 3). This is likely because of the NCR of the *DUR* genes in the presence of the preferred nitrogen source (ammonium sulfate). In contrast, $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ were highly efficient at urea uptake (Fig. 3), indicating that integration of the *DUR3* cassette results in the production of a functional protein that, presumably, localizes to the yeast plasma membrane and that control of *DUR3* by the *PGK1* promoter and terminator signals is capable of overcoming native repression by NCR. A marked difference in the urea uptake rates of $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ was observed (Fig. 3), despite integration of identical *DUR3* cassettes in both strains; $K7^{DUR3}$ incorporated approx. threefold more [^{14}C]urea than $K7^{DUR1,2/DUR3}$. The observed difference in urea uptake between $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ is likely explained by the need for urea degradation after its import by the cell. While *DUR1,2* must be induced and then synthesized in $K7^{DUR3}$, DUR1,2p is constitutively expressed in $K7^{DUR1,2/DUR3}$ leading to rapid degradation of urea; degradation may mask an otherwise increased uptake because the radiolabel is quickly lost as $^{14}CO_2$. Indeed, the identical phenomenon was also observed when looking at the difference in urea uptake by K7 and $K7^{DUR1,2}$ (Fig. 3, insert). Although both strains failed to uptake any appreciable amount of urea when compared to $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$, K7 did uptake approx. threefold more urea than its urea-degrading counterpart $K7^{DUR1,2}$.

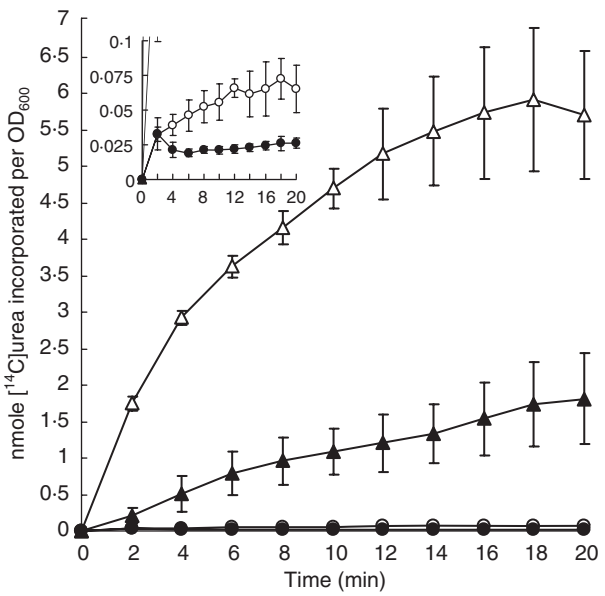

Figure 3 Uptake of [^{14}C]urea by K7 (○), $K7^{DUR1,2}$ (●), $K7^{DUR3}$ (△) and $K7^{DUR1,2/DUR3}$ (▲) under conditions of nitrogen catabolite repression. Strains were cultured to a final OD_{600} = 1 in a 1% (w/v) ammonium sulfate minimal medium prior to exposure to 0·27 mmol l^{-1} [^{14}C]urea (6·8 mCi $mmol^{-1}$). Assays were conducted in triplicate, and the data were averaged; error bars represent one standard deviation. Insert: Plot redrawn with *Y*-axis scale reduced from 7 to 0·1 nmole so as to highlight the difference between K7 and $K7^{DUR1,2}$.

Constitutive expression of *DUR1,2* and/or *DUR3* reduced EC in Sake wine

To assay the EC reduction of functionally enhanced yeast strains during Sake making, Sake wine was brewed with the parental strains K7 and K9 and the enhanced strains $K7^{DUR1,2}$, $K7^{DUR3}$, $K7^{DUR1,2/DUR3}$, $K9^{DUR1,2}$ and the EC content quantified by GC/MS at the end of fermentation. Fermentation profiles (Fig. 4) and ethanol production (data not shown) indicate a high degree of similarity between the parental strains and their functionally improved counterparts. The functionally enhanced Sake yeasts $K7^{DUR1,2}$ and $K7^{DUR1,2/DUR3}$ reduced EC content by 87 and 83%, respectively (Table 3), while the strain $K9^{DUR1,2}$ reduced EC by 68%. Under the conditions tested, constitutive expression of *DUR3* alone did not reduce EC as effectively as constitutive expression of *DUR1,2*; $K7^{DUR3}$ reduced EC by 15%, while $K7^{DUR1,2}$ reduced EC by 87% (Table 3). Constitutive co-expression of *DUR1,2* and *DUR3* had no synergistic effect on EC reduction during Sake production (Table 3).

Discussion

Constitutive expression of *DUR1,2* reduces EC production in Sake wine

Despite the limits imposed on the EC content in food and beverages by the Canadian and US governments, the

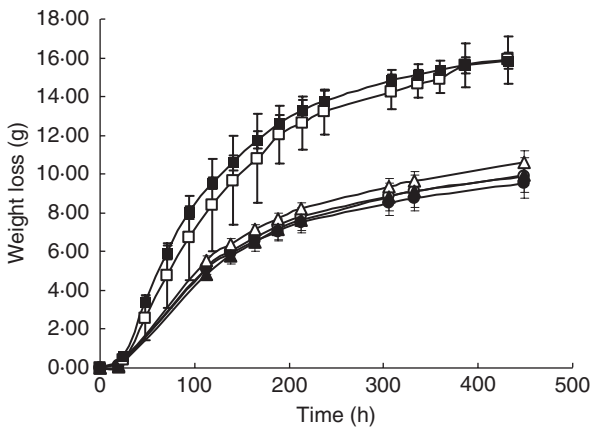

Figure 4 Fermentation profiles (weight loss) of Sake yeast strains K7 (○), $K7^{DUR1,2}$ (●), $K7^{DUR3}$ (Δ), $K7^{DUR1,2/DUR3}$ (▲), K9 (□) and $K9^{DUR1,2}$ (■) in Sake. Sake wine was produced from white rice and koji mash inoculated to a final OD_{600} = 0·1 and incubated to completion (*c.* 450 h) at 18°C. Fermentations were conducted in triplicate, and data were averaged; error bars indicate one standard deviation.

Table 3 Reduction of ethyl carbamate (EC) by functionally enhanced yeast strains during Sake making. The concentration of EC ($\mu g\ l^{-1}$) in Sake wine produced by Sake yeast strains K7, $K7^{DUR1,2}$, $K7^{DUR3}$, $K7^{DUR1,2/DUR3}$, K9 and $K9^{DUR1,2}$ from white rice and koji mash was quantified by gas chromatograph/mass spectrometry (GC/MS). Triplicate fermentations were incubated to completion (*c.* 450 h) at 18°C. Sake wine was heated at 70°C for 48 h prior to GC/MS to promote the evolution of EC. Percentage reduction is reported as compared to the respective parental strain

Yeast strain	K7	$K7^{DUR1,2}$	$K7^{DUR3}$	$K7^{DUR1,2/DUR3}$	K9	$K9^{DUR1,2}$
Replicate 1	109·8	13·4	93·4	15·7	241·7	108·9
Replicate 2	80·7	12·3	79·9	18·6	265·0	106·0
Replicate 3	108·3	13·0	80·8	17·8	525·7	112·2
Average (n = 3)	99·6	12·9	84·7	17·4	344·2	109·0
STDEV	16·4	0·5	7·6	1·5	157·7	3·1
% Reduction		– 87·1	15·0	82·6	–	68·3

presence of EC continues to be a pervasive problem. A recent survey by our group of the potential EC levels in 20 randomly chosen, commercially available wines from six wine-producing countries found that 14 and 17 exceeded the Canadian and US limits, respectively (Coulon *et al.* 2006). It is therefore obvious that current methods of EC reduction in wine are largely ineffective. Methods currently suggested to reduce EC include agricultural practices to control grape must arginine content (Butzke and Bisson 1998), the addition of lyophilized acid urease preparations to wine (Ough and Trioli 1988; Kodama *et al.* 1994), the expression of a bacterial acid urease in fermenting yeast strains (Ough and Trioli 1988), and the metabolic engineering of *CAR1*-deficient yeast strains to limit EC in Sake and stone fruit spirits (Kitamoto *et al.* 1991; Yoshiuchi *et al.* 2000; Park *et al.* 2001). Each of the proposed solutions, however, is not without serious limitation. Lyophilized urease additions incur additional cost and time for winemakers as well as exhibit limited functionality at natural wine pH (Kodama *et al.* 1994). Expression of bacterial ureases has proven to be ineffective because of the lack of accessory genes and cofactors necessary for proper protein folding and secretion (Visser *et al.* 1999). Finally, while knocking out *CAR1* in Sake yeast strains yields Sake with almost no detectable EC (Kitamoto *et al.* 1991), the approach is industrially impractical because of the loss of ability to metabolize arginine, a major amino acid in grape must and Sake mash, thus leading to growth and fermentation defects. Furthermore contamination from wild-type yeasts hinders the use of *CAR1* null mutants, because of the competitive advantage of wild-type yeasts. Until recently, the previously mentioned methods were the only methods of EC reduction available to winemakers. However, in 2006, our group developed a functionally enhanced strain of the popular industrial wine yeast 522 that is capable of reducing EC levels in Chardonnay wine by 89% (Coulon *et al.* 2006). This result, which is a consequence of the constitutive expression of an otherwise inactive *DUR1,2* gene, far exceeds any other method of EC reduction, is nontransgenic and does not lengthen production time or incur any additional costs to winemakers and/or consumers.

To address the EC problem in Sake wine, we integrated the *DUR1,2* cassette previously developed by our group (Coulon *et al.* 2006) into the *URA3* locus of the popular Sake yeast strains K7 and K9, yielding the urea-degrading strains $K7^{DUR1,2}$ and $K9^{DUR1,2}$, which are the first nontransgenic, phenotypically equivalent, EC-reducing Sake yeast strains to be developed. During Sake-brewing trials, the functionally enhanced yeasts exhibited a high degree of similarity to their parental strains in terms of fermentation rate (Fig. 4) and ethanol production (data not shown) and were highly efficacious EC reducers (Table 3), thus making them an important tool for modern Sake makers.

Constitutive expression of the urea permease, *DUR3*, in K7 yeast cells results in a urea-importing phenotype

Like *DUR1,2*, *DUR3* is subject to transcriptional repression by NCR during fermentation, resulting in the inability of *S. cerevisiae* to re-absorb excreted urea in the presence of good nitrogen sources (ElBerry *et al.* 1993; Hofman-Bang 1999). This inability to absorb excreted urea is a contributing factor in the production of wines with high residual urea, which in turn leads to high EC. To constitutively express *DUR3* throughout fermentation, the *DUR3* cassette, containing the *DUR3* ORF under the control of the constitutive *PGK1* promoter, was integrated

into the *TRP1* locus of K7 and $K7^{DUR1,2}$, which yielded the functionally enhanced, substantially equivalent strains $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$.

Gene expression analysis of the modified strains revealed that the *PGK* promoter is capable of driving constitutive *DUR3* expression despite noninducing conditions early in fermentation (Fig. 2). Furthermore, high-level expression of both *DUR1,2* and *DUR3* was maintained in $K7^{DUR1,2/DUR3}$, which had both the *DUR1,2* and *DUR3* cassettes integrated into its genome. In strains in which only one cassette (*DUR1,2* or *DUR3*) was integrated, constitutive expression of that gene induced expression of the other gene, indicating a certain amount of cross-talk between the regulatory mechanisms for *DUR1,2* and *DUR3*. Presumably, when cells are actively degrading urea instead of exporting it, the urea degradation intermediate, allophanate, causes induction of *DUR3*. Allophanate is a known inducer of many of the *DUR* genes (Hofman-Bang 1999; Cooper 2002). Similarly, when cells are actively importing urea, the increased intracellular urea concentration induces *DUR1,2* expression such that the intracellular concentration of urea can be lowered before it becomes toxic.

To confirm the production of a functional urea permease encoded by the integrated *DUR3* cassette and to correlate *DUR3* constitutive expression with increased urea uptake, the uptake of radiolabelled urea by K7, $K7^{DUR1,2}$, $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ was studied. Under the conditions tested, the strains $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$ were both highly capable of importing [^{14}C]urea, while K7 and $K7^{DUR1,2}$ were unable to incorporate any appreciable amounts of [^{14}C]urea (Fig. 3). These data indicate that integration of the *DUR3* cassette is responsible for the constitutive expression of an active urea permease (DUR3p) under NCR conditions that are common to Sake and wine making. Interestingly, strains constitutively expressing DUR3p did not require over-expression of the kinase *PTK2* to be highly active urea importers. *PTK2* was previously shown to be important in positive regulating DUR3p-mediated polyamine uptake by phosphorylation of three cytoplasmic residues (Uemura *et al.* 2007). Our results suggest that either wild-type levels of PTK2p are sufficient to activate constitutively expressed DUR3p or, more interestingly, PTK2p-mediated phosphorylation is not required for DUR3p to import urea.

To assess the EC reduction potential of the engineered strains $K7^{DUR3}$ and $K7^{DUR1,2/DUR3}$, small-scale fermentations of 'Doburoku' style Sake were conducted after which the EC content of the resultant wines was quantified. Despite the constitutively expressed and functional urea permease and despite the induction of *DUR1,2* in $K7^{DUR3}$ cells, $K7^{DUR3}$ was much less effective in reducing EC than $K7^{DUR1,2}$ (Table 3). These data suggest that for EC reduction in Sake wine, under the conditions tested, constitutive expression of *DUR1,2* is superior to that of *DUR3*. However, given that the *DUR3* cassette constitutively produces functional DUR3p (as determined by urea uptake assay), $K7^{DUR3}$ would likely be the strain of choice for reduction of EC in Sake made from mash with high endogenous urea. Indeed, constitutive expression of *DUR3* should not be ignored as it has been shown to be highly effective at reducing EC in Chardonnay wine made from high urea must (Dahabieh *et al.* 2009). Specifically, in wine made from Chardonnay must with high endogenous urea, a yeast strain expressing *DUR3* was approx. four times as effective at reducing EC as the same yeast strain expressing *DUR1,2*.

Despite the high-level expression of both *DUR1,2* and *DUR3* and despite the functionality of both proteins, $K7^{DUR1,2/DUR3}$ was not capable of producing Sake wine with less EC than $K7^{DUR1,2}$ (Tables 3). The lack of synergistic EC reduction in strains constitutively co-expressing *DUR1,2* and *DUR3* and lack of efficacious EC reduction in strains constitutively expressing only *DUR3* suggest the presence of confounding factors that must still be investigated. One such factor that may explain the lack of effective EC reduction by urea-importing yeasts during Sake brewing is a change in the primary physiological role of DUR3p. As noted previously, DUR3p is primarily an active transporter of urea; however, it has also been shown to be involved in boron transport (Nozawa *et al.* 2006) and polyamine uptake (Uemura *et al.* 2007). While little is known about boron's role in fermentation, polyamines have been shown to be crucial for cell growth (Tabor and Tabor 1999); polyamine levels are generally elevated in actively growing cells (Monteiro and Bisson 1992). Polyamines may be especially important for growth in the substantially different nutrient environment of rice mash; thus, during Sake brewing, an increased need for polyamines, and perhaps boron, may decrease the amount of over-expressed DUR3p available for urea uptake, thereby lowering EC reduction ability. Indeed, fermented foods and beverages have been shown to be especially high in polyamines; specifically, Sake wine is high in agmatine (Okamoto *et al.* 1997). Given that Sake mash is generally low in free amino acids and ammonia (Kliewer 2006; Kizaki *et al.* 1991), polyamines may make up a larger proportion of available nitrogen in Sake fermentation when compared to grape must fermentation, thereby switching DUR3p from a urea import role to a polyamine uptake role.

Acknowledgements

This work was funded by grants from NSERC (217271-04) and Functional Technologies Corporation to

H.J.J. van Vuuren. The authors also thank Lina Madilao of the UBC Wine Research Centre for GC/MS analysis of wines.

References

An, D. and Ough, C.S. (1993) Urea excretion and uptake by wine yeasts as affected by various factors. *Am J Enol Vitic* **44**, 35–40.

Beland, F.A., Benson, R.W., Mellick, P.W., Kovatch, R.M., Roberts, D.W., Fang, J.L. and Doerge, D.R. (2005) Effect of ethanol on the tumorigenicity of urethane (ethyl carbamate) in B6C3F1 mice. *Food Chem Toxicol* **43**, 1–19.

Butzke, C.E. and Bisson, L.F. (1998) Ethyl carbamate preventative action manual. University of California, Davis. http://www.fda.gov/Food/FoodSafety/FoodContaminants Adulteration/ChemicalContaminants/EthylCarbamate Urethane/ucm078546.htm.

Canas, B.J., Havery, D.C., Robinson, L.R., Sullivan, M.P., Joe, F.L. Jr and Diachenko, G.W. (1989) Ethyl carbamate levels in selected fermented foods and beverages. *J Assoc Off Anal Chem* **72**, 873–876.

Cooper, T.G. (1982) Nitrogen metabolism in *S. cerevisiae*. In *The Molecular Biology of the Yeast: Metabolism and Gene Expression* ed. Strathern, J.N., Jones, E.W. and Broach, J.R. pp. 39–99. New York: Cold Spring Harbor Press.

Cooper, T.G. (2002) Transmitting the signal of excess nitrogen in *Saccharomyces cerevisiae* from the Tor proteins to the GATA factors: connecting the dots. *FEMS Microbiol Rev* **26**, 223–238.

Cooper, T.G. and Sumrada, R. (1975) Urea transport in *Saccharomyces cerevisiae*. *J Bacteriol* **121**, 571–576.

Coulon, J., Husnik, J.I., Inglis, D.L., van der Merwe, G.K., Lonvaud, A., Erasmus, D.J. and van Vuuren, H.J.J. (2006) Metabolic engineering of *Saccharomyces cerevisiae* to minimize the production of ethyl carbamate in wine. *Am J Enol Vitic* **57**, 113–124.

Cox, J.S. and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell*, **87**, 391–404.

Dahabieh, M.S., Husnik, J.I. and van Vuuren, H.J.J. (2009) Functional expression of the DUR3 gene in a wine yeast strain to minimize ethyl carbamate in Chardonnay wine. *Am J Enol Vitic* **60**, 537–541.

ElBerry, H.M., Majumdar, M.L., Cunningham, T.S., Sumrada, R.A. and Cooper, T.G. (1993) Regulation of the urea active transporter gene (DUR3) in *Saccharomyces cerevisiae*. *J Bacteriol* **175**, 4688–4698.

Genbauffe, F.S. and Cooper, T.G. (1986) Induction and repression of the urea amidolyase gene in *Saccharomyces cerevisiae*. *Mol Cell Biol* **6**, 3954–3964.

Genbauffe, F.S. and Cooper, T.G. (1991) The urea amidolyase (DUR1,2) gene of *Saccharomyces cerevisiae*. *DNA Seq* **2**, 19–32.

Hofman-Bang, J. (1999) Nitrogen catabolite repression in *Saccharomyces cerevisiae*. *Mol Biotechnol* **12**, 35–73.

Hubner, P., Groux, P.M., Weibel, B., Sengstag, C., Horlbeck, J., Leong-Morgenthaler, P.M. and Luthy, J. (1997) Genotoxicity of ethyl carbamate (urethane) in Salmonella, yeast and human lymphoblastoid cells. *Mutat Res* **390**, 11–19.

Ingledew, W.M., Magnus, C.A. and Patterson, J.R. (1987) Yeast foods and ethyl carbamate formation in wine. *Am J Enol Vitic* **38**, 332–335.

Kitamoto, K., Oda, K., Gomi, K. and Takahashi, K. (1991) Genetic engineering of a sake yeast producing no urea by successive disruption of arginase gene. *Appl Environ Microbiol* **57**, 301–306.

Kizaki, Y., Inoue, Y., Okazaki, N. and Kobayashi, S. (1991) Isolation and determination of protein bodies (PB-I, PB-II) in polished rice endosperm. *J Brew Soc Jpn* **86**, 293–298.

Kliewer, W.M. (2006) Free amino acids and other nitrogenous fractions in wine grapes. *J Food Sci* **35**, 17–21.

Kodama, S., Suzuki, T., Fujinawa, S., de la Teja, P. and Yotsuzuka, F. (1994) Urea contribution to ethyl carbamate formation in commercial wines during storage. *Am J Enol Vitic* **45**, 17–24.

Leithauser, M.T., Liem, A., Stewart, B.C., Miller, E.C. and Miller, J.A. (1990) 1,N6-ethenoadenosine formation, mutagenicity and murine tumor induction as indicators of the generation of an electrophilic epoxide metabolite of the closely related carcinogens ethyl carbamate (urethane) and vinyl carbamate. *Carcinogenesis* **11**, 463–473.

Malone, R.E. (1990) Dual regulation of meiosis in yeast. *Cell*, **61**, 375–378.

Marks, V.D., Sui, S.J.H., Erasmus, D., van der Merwe, G.K., Brumm, J., Wasserman, W.W., Bryan, J. and van Vuuren, H.J.J. (2008) Dynamics of the yeast transcriptome during wine fermentation reveals a novel fermentation stress response. *FEMS Yeast Res* **8**, 35–52.

Monteiro, F.F. and Bisson, L.F. (1991) Amino acid utilization and urea formation during vinification fermentations. *Am J Enol Vitic* **42**, 199–208.

Monteiro, F.F. and Bisson, L.F. (1992) Nitrogen supplementation of grape juice. I. Effect on amino acid utilization during fermentation. *Am J Enol Vitic* **43**, 1–10.

Monteiro, F.F., Trousdale, E.K. and Bisson, L.F. (1989) Ethyl carbamate formation in wine: use of radioactively labeled precursors to demonstrate the involvement of urea. *Am J Enol Vitic* **40**, 1–8.

Mori, K. (1996) Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. *Genes Cells* **1**, 803–817.

Nikawa, J., Akiyoshi, M., Hirata, S. and Fukuda, T. (1996) *Saccharomyces cerevisiae* IRE2/HAC1 is involved in IRE1-mediated KAR2 expression. *Nucleic Acids Res* **24**, 4222–4226.

Nozawa, A., Takano, J., Kobayashi, M., von Wiren, N. and Fujiwara, T. (2006) Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in *Saccharomyces cerevisiae*. *FEMS Microbiol Lett* **262**, 216–222.

Okamoto, A., Sugi, E., Koizumi, Y., Yanagida, F. and Udaka, S. (1997) Polyamine content of ordinary foodstuffs and various fermented foods. *Biosci Biotechnol Biochem* **61**, 1582–1584.

Ough, C.S. (1976a) Ethylcarbamate in fermented beverages and foods. I. Naturally occurring ethylcarbamate. *J Agric Food Chem* **24**, 323–328.

Ough, C.S. (1976b) Ethylcarbamate in fermented beverages and foods. II. Possible formation of ethylcarbamate from diethyl dicarbonate addition to wine. *J Agric Food Chem* **24**, 328–331.

Ough, C.S. and Trioli, G. (1988) Urea removal from wine by an acid urease. *Am J Enol Vitic* **39**, 303–307.

Ough, C.S., Crowell, E.A. and Gutlove, B.R. (1988a) Carbamyl compound reactions with ethanol. *Am J Enol Vitic* **39**, 239–242.

Ough, C.S., Crowell, E.A. and Mooney, L.A. (1988b) Formation of ethyl carbamate precursors during grape juice (chardonnay) fermentation. I. Addition of amino acids, urea, and ammonia: effects of fortification on intracellular and extracellular precursors. *Am J Enol Vitic* **39**, 243–249.

Ough, C.S., Stevens, D., Sendovski, T., Huang, Z. and An, D. (1990) Factors contributing to urea formation in commercially fermented wines. *Am J Enol Vitic* **41**, 68–73.

Park, H., Shin, M. and Woo, I. (2001) Antisense-mediated inhibition of arginase (CAR1) gene expression in *Saccharomyces cerevisiae*. *J Biosci Bioeng* **92**, 481–484.

Park, S., Ha, S., Yoon, J., Lee, S., Hong, K., Lee, E., Yeom, H., Yoon, N. *et al.* (2009) Exposure to ethyl carbamate in alcohol-drinking and nondrinking adults and its reduction by simple charcoal filtration. *Food Control* **20**, 946–952.

Schehl, B., Senn, T., Lachenmeier, D.W., Rodicio, R. and Heinisch, J.J. (2007) Contribution of the fermenting yeast strain to ethyl carbamate generation in stone fruit spirits. *Appl Microbiol Biotechnol* **74**, 843–850.

Schlatter, J. and Lutz, W.K. (1990) The carcinogenic potential of ethyl carbamate (urethane): risk assessment at human dietary exposure levels. *Food Chem Toxicol* **28**, 205–211.

Sumrada, R., Gorski, M. and Cooper, T. (1976) Urea transport-defective strains of *Saccharomyces cerevisiae*. *J Bacteriol* **125**, 1048–1056.

Tabor, H. and Tabor, C.W. (1999) A guide to the polyamines. Seymour S. Cohen. *Anal Biochem*, **274**, 150.

Uemura, T., Kashiwagi, K. and Igarashi, K. (2007) Polyamine uptake by DUR3 and SAM3 in *Saccharomyces cerevisiae*. *J Biol Chem* **282**, 7733–7741.

Visser, J.J., Coton, E.P.N., Bauer, F., Viljoen, M. and van Vuuren, H.J.J. (1999) Engineering an acid urease for heterologous expression in *Saccharomyces cerevisiae*. *Curr Genet* **35**, 321.

Weber, J. and Sharypov, V. (2009) Ethyl carbamate in foods and beverages: a review. *Environ Chem Lett* **7**, 233–247.

Whitney, P.A. and Cooper, T.G. (1972) Urea carboxylase and allophanate hydrolase. Two components of adenosine triphosphate:urea amido-lyase in *Saccharomyces cerevisiae*. *J Biol Chem* **247**, 1349–1353.

Whitney, P.A. and Cooper, T. (1973) Urea carboxylase from *Saccharomyces cerevisiae.* Evidence for a minimal two-step reaction sequence. *J Biol Chem* **248**, 325–330.

Whitney, P.A., Cooper, T.G. and Magasanik, B. (1973) The induction of urea carboxylase and allophanate hydrolase in *Saccharomyces cerevisiae*. *J Biol Chem* **248**, 6203–6209.

Yoshiuchi, K., Watanabe, M. and Nishimura, A. (2000) Breeding of a non-urea producing sake yeast with killer character using a kar1-1 mutant as a killer donor. *J Ind Microbiol Biotechnol* **24**, 203–209.

Zimmerli, B. and Schlatter, J. (1991) Ethyl carbamate: analytical methodology, occurrence, formation, biological activity and risk assessment. *Mutat Res* **259**, 325–350.