METABOLIC ENGINEERING OF INDUSTRIAL YEAST STRAINS TO MINIMIZE THE PRODUCTION OF ETHYL CARBAMATE IN GRAPE AND SAKE WINE

by

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ABSTRACT

During alcoholic fermentation *Saccharomyces cerevisiae* metabolizes L-arginine to ornithine and urea. *S. cerevisiae* can metabolize urea through the action of urea amidolyase, encoded by the *DUR1,2* gene; however, *DUR1,2* is subject to nitrogen catabolite repression (NCR) in the presence of high quality nitrogen sources during fermentation. Being cytotoxic at high concentrations, urea is exported into wine where it spontaneously reacts with ethanol, and forms the carcinogen ethyl carbamate (EC).

Urea degrading yeast strains were created by integrating a linear cassette containing the *DUR1,2* gene under the control of the *S. cerevisiae PGK1* promoter and terminator signals into the *URA3* locus of the Sake yeast strains K7 and K9. The 'self-cloned' strains $K7^{EC-}$ and $K9^{EC-}$ produced Sake wine with 68% less EC. The Sake strains $K7^{EC-}$ and $K9^{EC-}$ did not efficiently reduce EC in Chardonnay wine due to the evolutionary adaptation of said strains to the unique nutrients of rice mash; therefore, the functionality of engineered yeasts must be tested in their niche environments as to correctly characterize new strains.

S. cerevisiae possesses an NCR controlled high affinity urea permease (*DUR3*). Urea importing yeast strains were created by integrating a linear cassette containing the *DUR3* gene under the control of the *PGK1* promoter and terminator signals into the *TRP1* locus of the yeast strains K7 (Sake) and 522 (wine). In Chardonnay wine, the urea importing strains $K7^{D3}$ and 522^{D3} reduced EC by 7% and 81%, respectively; reduction by these strains was equal to reduction by the urea degrading strains $K7^{EC-}$ and 522^{EC-}. In Sake wine, the urea degrading strains $K7^{EC-}$ and 522^{EC-} reduced EC by 87% and 84% respectively, while the urea importing strains $K7^{D3}$ and 522^{D3} were significantly less capable of reducing EC (15% and 12% respectively). In Chardonnay and Sake wine, engineered strains that constitutively co-expressed *DUR1,2* and *DUR3* did not reduce EC more effectively than strains in which either gene was expressed solely. Uptake of ^{14}C-urea under non-inducing conditions was enhanced in urea importing strains; parental strains failed to incorporate any ^{14}C-urea thus confirming the functionality of the urea permease derived from the integrated *DUR3* cassette.

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LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
aa	Amino acid
Abs	Absorbance
ANOVA	Analysis of variance
BC	British Columbia
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Ci	Curie
cm	Centimetre
cM	Centimorgan
Corp.	Corporation
cRNA	Ribonucleic acid derived from cDNA
DAP	Diammonium phosphate
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC	Ethyl carbamate
EDTA	Ethylenediamine tetraacetic acid
ER	Endoplasmic reticulum
EtOH	Ethanol
FDA	Food and Drug Administration
g	Gram
GC	Gas chromatograph
GC/MS	Gas chromatograph coupled mass spectrometry
GM	Genetically modified
GMO	Genetically modified organism
GO	Gene ontology
GRAS	Generally regarded as safe
HPLC	High pressure liquid chromatography
kb	Kilo base pair

kg	Kilogram
L	Litre
LB	Luria-Bertani medium
LC	Liquid chromatograph
log	Logarithm
LSD	Least significant difference
m	Metre
M	Molarity
m/v	Mass per volume
mCi	Millicurie
mg	Milligram
min	Minute
mL	Millilitre
MLF	Malolactic fermentation
mM	Millimolar
mRNA	Messenger RNA
MS	Mass spectrometry
NAPS	Nucleic Acid Protein Service Unit at The University of British Columbia
NCR	Nitrogen catabolite repression system of *S. cerevisiae*
ng	Nanogram
nM	Nanomolar
nt	Nucleotide
°C	Degree Celsius
OD	Optical density
O/N	Overnight
ORF	Open reading frame
PB	Protein body
PCR	Polymerase Chain Reaction
PDM	Prise de Mousse
pH	Potential of Hydrogen
ppb	Parts per billion

ppm	Parts per million
qPCR	Quantitative PCR
qRT-PCR	Quantitative Reverse Transcriptase PCR
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
ROX	Passive reference dye used in qPCR
rpm	Revolutions per minute
RQ	Relative quantification
rRNA	Ribosomal RNA
RSD	Relative standard deviation
RT-PCR	Reverse Transcriptase PCR
s	Second
SAP	Shrimp Alkaline Phosphatase
STDEV	Standard deviation
SGD	*Saccharomyces* Genome Database (www.yeastgenome.org)
SLR	Signal log ratio (log base = 2)
TBE	Buffer consisting of Tris base, boric acid, EDTA, and water
TE	Buffer consisting of Tris base, EDTA, and water
tRNA	Transfer RNA
TRP	Tryptophan
UAS	Upstream activation sequence
UAS_{NTR}	Upstream activation sequence – nitrogen regulated
UIS	Upstream induction sequence
UK	United Kingdom
URA	Uracil
URS	Upstream repression sequence
USA	United States of America
v	Volt
v/v	Volume per volume
w/o	Without
YAN	Yeast assimilable nitrogen

YEG	Medium consisting of yeast extract and dextrose
YNB	Yeast Nitrogen Base
YPD	Medium consisting of yeast extract, peptone, and dextrose

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1 INTRODUCTION

1.1 *Saccharomyces cerevisiae*

In the early 20th century intensive genetic research began on the budding yeast *Saccharomyces cerevisiae* and, ever since, scientists have championed yeast as the "*Escherichia coli* of eukaryotes" (Miklos and Rubin 1996). *S. cerevisiae* combines the ease of use and brute force genetics of bacteria with the sophistication and elegance of higher eukaryotes, thus making it an ideal organism to study processes fundamental to all eukaryotic cells. Such processes, many of which are conserved from yeast to humans, include complex cell cycle control, eukaryotic meiotic recombination, mitochondrial respiration, and cell fusion events (Griffiths, et al. 2005).

S. cerevisiae is a unicellular eukaryotic fungus which is ubiquitous to wineries worldwide (Perez-Ortin, Garcia-Martinez and Alberola 2002). While it is assumed that its natural environment is the winery itself, *S. cerevisiae* can also be found naturally in rotting grapes and fruits, and in addition, there may be some yet undiscovered natural habitat of budding yeast (Perez-Ortin, Garcia-Martinez and Alberola 2002). *S. cerevisiae* feeds on the sugars and nutrients of crushed and rotting fruit and, when conditions are optimal, reproduce approximately every 90 minutes (Herskowitz 1988). Approximately 10 microns in diameter, yeast reproduce asexually by mitotic budding; however, they can also undergo sexual reproduction when haploid cells (created by meiotic division) of opposite mating type fuse, thus yielding a stable diploid cell (Herskowitz 1988). The yeast mating types (MATa and MATalpha) can be likened to the male and female genders.

Wild isolates of *S. cerevisiae* also possess the ability to switch mating types such that a haploid population of one mating type can mate with itself and achieve diploidy (Herskowitz 1988). The mechanism of mating type switching involves the HO endonuclease and has been well characterized (Nasmyth 1993). Yeasts with this ability are known as homothallic and, from a Darwinian point of view, achieving diploidy is desirable. Having two copies of every gene makes an organism genetically more stable, being able to tolerate loss of function "recessive" mutations more readily than a haploid equivalent (Greig and Travisano 2003). However, the haploid state can confer certain advantages on the yeast cell; a second copy of every gene buffers deleterious mutations, but it also buffers advantageous

mutations. Thus, a diploid organism may, under certain circumstances, 'evolve' or 'adapt' to changes in the environment more slowly than its haploid counterpart (Greig and Travisano 2003). Nevertheless, as of present, *S. cerevisiae* has evolved into an organism which seems to function and prosper in a life cycle stuck between its haploid prokaryotic predecessors and its diploid eukaryotic successors.

Like most bacteria and other microorganisms, yeast replicate rapidly and can be easily cultivated in the laboratory. They grown well in liquid culture and on solid media, and can be manipulated via standard microbiological techniques (Ausubel, et al. 2005). In addition to other common techniques, yeast cells are remarkably amenable to chemical or UV mutagenesis, genetic selection, recombinant DNA methods, rescue cloning, complementation, and high efficiency transformation (Griffiths, et al. 2005). However, the real value of *S. cerevisiae* lies in the fact that yeasts incorporate all of these advantages into a eukaryotic background. Thus, fundamental eukaryotic processes can be dissected on a molecular level when such analysis would be exceedingly difficult or impossible in higher eukaryotes.

The full sequence of the *S. cerevisiae* genome was published in 1996 (Goffeau, et al. 1996) and this has made *S. cerevisiae* even more powerful as a model organism. *S. cerevisiae* was in fact the first eukaryotic organism to be completely sequenced and subsequent analysis has revealed that the genome of the common laboratory strain S288C is approximately 12 Mb in size and consists of 16 independently assorting chromosomes (Goffeau, et al. 1996). It contains approximately 6000 genes, ~1000 of which are essential for growth on rich media (Maftahi, Gaillardin and Nicaud 1998), and ~25% of which have human homologues (Griffiths, et al. 2005). The remaining 5000 non-essential genes have been systemically knocked out in the '*Saccharomyces* Genome Deletion project' resulting in a knock-out collection (Maftahi, Gaillardin and Nicaud 1998) that allows for streamlined reverse genetic analysis. In addition to the set of 5000 knockouts available, the remaining 1000 essential genes can be investigated through the use of readily available temperature sensitive (TS) conditional alleles (Dohmen and Varshavsky 2005). Finally, both expression and tiling DNA microarrays exist for various yeast strains allowing complex global analysis of the yeast genome, transcriptome and proteome (Dunn, Levine and Sherlock 2005; Hauser, et al. 2001; Perez-Ortin, Garcia-Martinez and Alberola 2002; Rossignol, et al. 2003; Shobayashi, et al. 2007; Wu, et al. 2006).

Of the 6000 yeast genes, the *Saccharomyces* Genome Database (SGD – http://www.yeastgenome.org) lists known functions or GO (Gene ontology) annotations for about 5000 genes. The remaining 1000 yeast genes remain uncharacterized despite 70 years of yeast genetics. While some speculate that many of these 1000 genes (uncharacterized ORFs) may not actually code for functional protein, the general consensus is that they are indeed functional (Pena-Castillo and Hughes 2007). Upon *in silico* analysis, many of the 1000 uncharacterized genes contain putative protein domains which suggest some sort of metabolic function (Pena-Castillo and Hughes 2007). Furthermore, a large proportion of the 1000 uncharacterized genes are homologues to genes found solely in other species of fungi (Pena-Castillo and Hughes 2007). Thus, these genes may be important in aspects of fungal metabolism/physiology, and thus do not assay well under standard laboratory conditions. In fact, a growing consensus amongst yeast biologists is that these 1000 dubious genes will never become characterized until more focus is placed on *S. cerevisiae* outside the laboratory (Pena-Castillo and Hughes 2007). This requires doing away with traditional laboratory screens and looking at culturing *S. cerevisiae* under natural conditions, most notably fermentative conditions. Under conditions of fermentation, yeast cells are subjected to profoundly different stresses than under laboratory conditions. These stresses include, but are not limited to, osmotic stress, ethanol stress, nutrient limitation, oxidative stress, and temperature stress. If some of the 1000 uncharacterized genes are vital to these types of stress responses, their functions will only be revealed when yeast cells are cultured under conditions that create such stresses. For example, a recent study of the yeast transcriptome during wine fermentation identified a previously uncharacterized fermentation stress response containing approximately 223 genes, many of which are part of the 1000 remaining uncharacterized yeast genes (Marks, et al. 2008).

1.2 Yeast strains from nature *vs.* laboratory yeasts

The majority of laboratory strains are descendents of isolates from nature, such as the common laboratory strain S288C, which is a descendent of a yeast strain isolated from a rotten fig in California in 1938 (Perez-Ortin, Garcia-Martinez and Alberola 2002). However, today's laboratory strains are significantly different, both genetically and physiologically, from their wild type parents. Given the 90 minute generation time of *S. cerevisiae*, it is not difficult to imagine that over 70 years of growth on rich laboratory media, a wild strain could evolve such that it no longer requires much of the genetic diversity

and robustness needed to deal with constantly changing environmental conditions (Dunn, Levine and Sherlock 2005). Without environmental pressures to maintain robust pathways needed for growth in nature (e.g. sporulation, pseudohyphal growth), natural isolates could quickly become homogenized into the less vigorous laboratory strains we see today. As such, many laboratory strains exhibit dramatically different transcriptional profiles from wild yeasts and also differ in their ability to conduct robust and efficient alcoholic fermentations of high sugar grape musts (Hauser, et al. 2001). Additionally, in order to maintain stable haploid populations, the HO locus of various *S. cerevisiae* laboratory strains has been purposely disrupted (Nasmyth 1993). Other important differences in laboratory yeast strains include the addition of various auxotrophic markers which can be used to select transformants. These auxotrophic markers often map to defects in amino acid or nucleotide biosynthetic pathways; common markers include *URA3*, *TRP1*, *LEU2*, *ADE2*, etc. (Ausubel, et al. 1995; Dohmen and Varshavsky 2005).

In nature, many distinct strains of *S. cerevisiae* have been isolated from various environments worldwide (Dunn, Levine and Sherlock 2005). Environments can vary widely in terms of nutrient (carbon, nitrogen, and minerals) availability, temperature, osmolarity, etc. Thus, while fundamentally similar, each of these strains has adapted, but not yet undergone speciation, in response to various niches in the environment. These adaptations manifest themselves as differences in growth rate, fermentation rate, ethanol production, and various resistances when different strains are grown in identical media (Dunn, Levine and Sherlock 2005; Hauser, et al. 2001).

Despite differences between wild type strains from nature, they tend to share some common distinguishing genetic characteristics when comparing them to laboratory strains. As stated previously, most laboratory strains exist as stable populations of haploids; however, most wild strains are homothallic and diploid, polyploid, or aneuploid (Bond, et al. 2004; Dunn, Levine and Sherlock 2005; Hauser, et al. 2001; Hughes, et al. 2000; Perez-Ortin, Garcia-Martinez and Alberola 2002). Compared to laboratory strains, wild type strains also differ in chromosome length, contain large scale (~50 kb) deletions or insertions, contain many more transposons (*Ty* elements), differ in sporulation rate (0-75%) and spore viability (0-98%), exhibit variable pseudohyphal growth, and are largely heterozygous (Perez-Ortin, Garcia-Martinez and Alberola 2002).

1.3 *S. cerevisiae* and industry: Wine yeasts

The interaction between *Homo sapiens* and *S. cerevisiae* dates back almost 8000 years (Perez-Ortin, Garcia-Martinez and Alberola 2002; Vine, Harkness and Linton 2002). It was first reported by ancient Egyptian civilization that crushed grapes would 'ferment spontaneously' and that the resultant wine contained 'magical and anesthetic properties' (Vine, Harkness and Linton 2002). Since that time, humans have been selectively breeding the then unknown microorganism, *S. cerevisiae*, for desirable characteristics such as tolerance to high sugar stress, robust fermentation, ethanol tolerance, and good flavour production (Hauser, et al. 2001).

As an art form, winemaking flourished in the ancient Mediterranean and was quickly adopted anywhere where the climate was suitable for viticulture (Goode 2005; Vine, Harkness and Linton 2002). As a science, however, winemaking was not understood until 1863; Louis Pasteur was the first to isolate *S. cerevisiae* and show that it was responsible for the production of ethyl alcohol (ethanol) and carbon dioxide from simple sugars (glucose) (Figures 1 and 2) (Perez-Ortin, Garcia-Martinez and Alberola 2002; Vine, Harkness and Linton 2002).

$$\text{Sugar (glucose or fructose)} \rightarrow \text{Ethanol} + \text{Carbon dioxide} + \text{ATP}$$

$$C_6H_{12}O_6 + 2P_i + 2ADP \rightarrow 2CH_3CH_2OH + 2CO_2 + 2\ ATP\ (\text{energy released: } 118\ kJ/mol)$$

Figure 1. Chemical basis of anaerobic fermentation. Under anaerobic conditions *S. cerevisiae* creates energy (ATP) for biomass by converting sugar into ethanol and carbon dioxide.

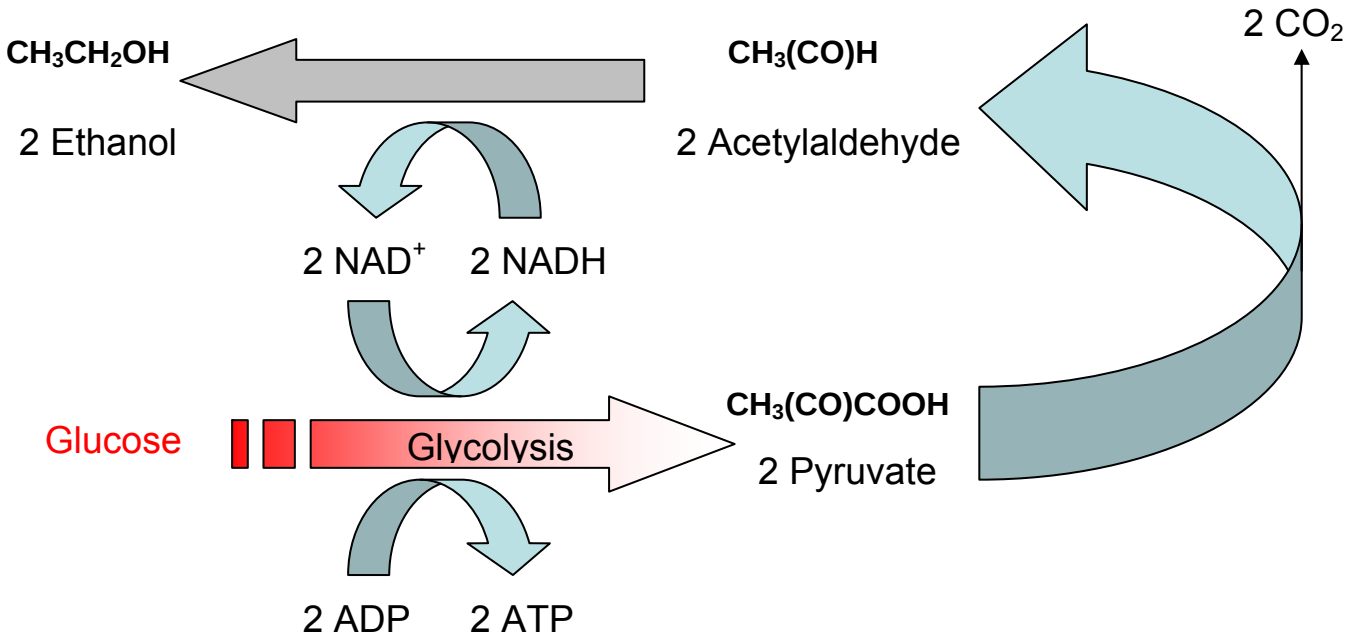


Figure 2. Simplified diagram of the Embden-Meyerhof pathway. In the presence of oxygen, pyruvate enters the mitochondrial Krebs cycle and then undergoes oxidative phosphorylation to create maximal ATP. Under anaerobic conditions, yeast shunt pyruvate through alcoholic fermentation in order to create energy from sugar and restore the intracellular pool of NAD^+ that is depleted through glycolysis.

Today, no other microorganism is as important to human diet and the global economy as *S. cerevisiae* (Goode 2005; Vine, Harkness and Linton 2002). Fermentation by yeast is vital to winemaking, brewing, baking, and the distillation of spirits. Furthermore, in the face of global warming, *S. cerevisiae* may soon play a vital role in the paradigm shift from fossil fuel dependency to the use of bio-ethanol as a fuel source (Farrell, et al. 2006).

1.4 Winemaking and Sake brewing

Although it may be a seemingly simple process, winemaking (enology) and thus wine itself is incredibly complex. Recent estimates suggest that wine contains approximately 1000 volatile flavor and aroma compounds (Goode 2005). Furthermore, of the 1000 compounds, an estimated 400 are produced by *S. cerevisiae* itself (Goode 2005). In its most fundamental form winemaking can be described as, "the product of fermenting [with *S. cerevisiae*] and processing grape juice or must" (Vine, Harkness and Linton 2002).

While human beings have been actively fermenting grapes and other fruits for thousands of years, the fundamental process has changed very little. The steps involved in modern grape winemaking are outlined in Figure 3 (Vine, Harkness and Linton 2002).

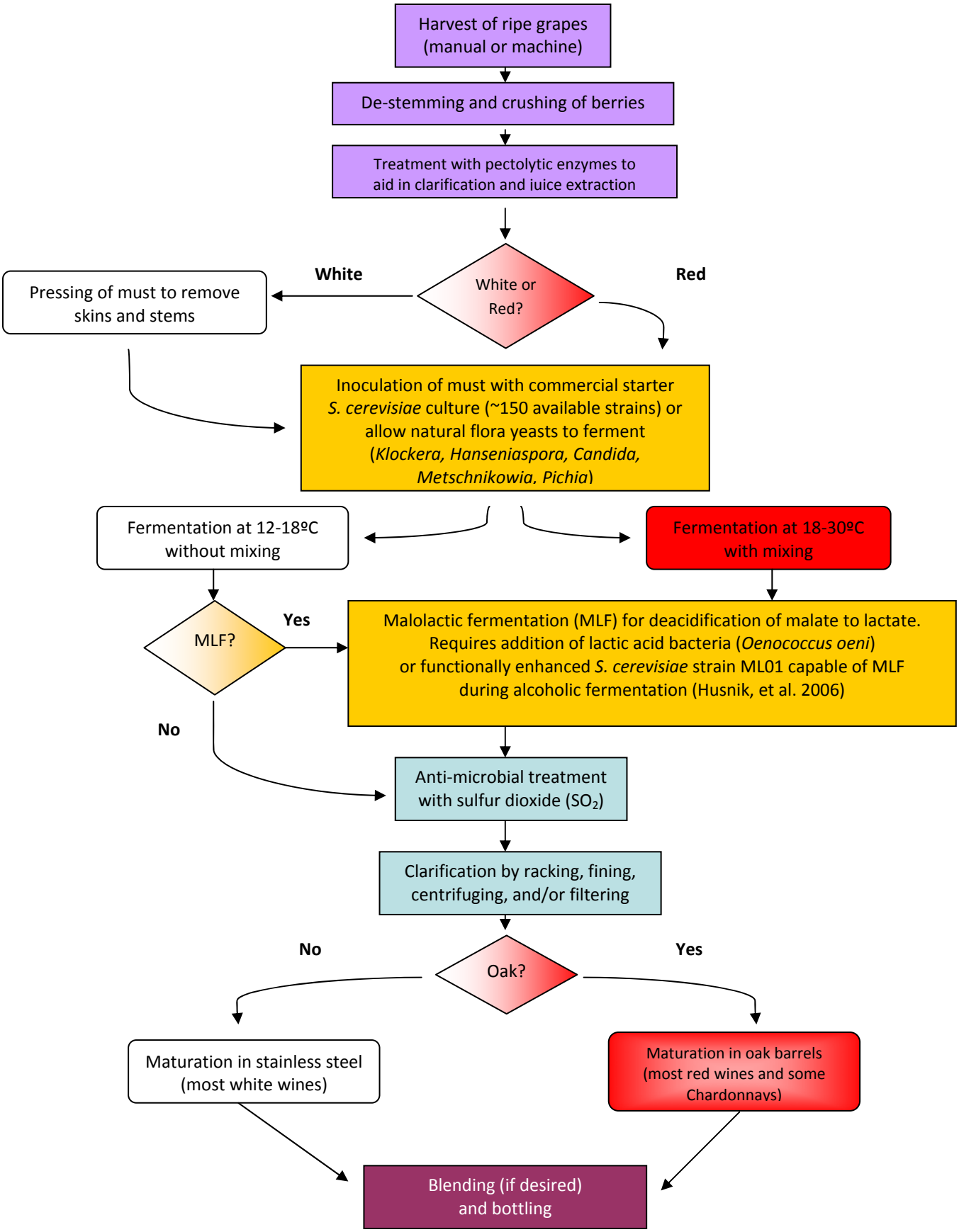


Figure 3. Processes involved in red and white winemaking.

In contrast to grape wine, Sake wine is produced from the fermentation of milled rice grains by *S. cerevisiae*. Sake is native to Japan but has steadily been gaining popularity around the world. Sake is characterized by a translucent hue, mild fruity bouquet, relatively high natural alcohol content (15-20% v/v), and is served either hot or cold (Shobayashi, et al. 2007). As is the case with wine yeasts, many different Sake yeast strains exist and certain strains have become popular for particular sensory characteristics that they impart to the final product. Some strains produce Sake wine which is rich in fruitiness and has high acidity, while others produce wine which is milder and more aromatic in bouquet. Sake strains K7 and K9 are amongst the most popular and widely used yeasts in the industry (Kodama 1993; Wu, et al. 2006). The process of alcoholic fermentation is fundamental to both winemaking and Sake brewing and yeast cells are subjected to substantial temperature, acid, hypoxic and ethanol stresses during both wine making and Sake brewing (Kodama 1993; Rossignol, et al. 2003; Wu, et al. 2006). However, one of the most profound differences is the level of osmotic stress experienced by yeast cells during wine making. High ethanol levels produced during Sake fermentations also exert significant ethanol stress on yeast cells.

Typical grape juice is a complex mixture high in carbohydrates, rich in assimilable nitrogen, and high in vitamin/mineral content (Ingledew, Magnus and Patterson 1987; Rossignol, et al. 2003). On average, grape must contains approximately 20% w/v sugar (200 g/L) in the form of a mixture of sucrose, glucose and fructose (Rossignol, et al. 2003). Consequently, at the start of grape must fermentation yeast cells are subject to substantial osmotic stress. Although yeast possess a cell wall composed of crosslinked 1,3- and 1,6-glucans that protects them against osmotic forces, growth in grape must induces several other metabolic methods of coping with said stress (Westfall, Ballon and Thorner 2004). The primary method of dealing with osmotic stress is induction of the high osmolarity growth (HOG) pathway (Reviewed in Westfall, Ballon and Thorner 2004; Han, et al. 1994). This pathway, which is highly conserved between most eukaryotic cells, is responsible for the production of intracellular small molecules which help offset the osmotic pressure difference. The principle molecules produced in *S. cerevisiae* are glycerol (1,2,3-propanetriol) and trehalose (α1,1 glucose disaccharide) (Westfall, Ballon and Thorner 2004). Both molecules are produced after the induction of biosynthetic genes by the MAP kinase mediated HOG pathway, which occurs within minutes of osmotic stress (Westfall, Ballon and Thorner 2004). In contrast to the high osmolarity of typical grape must, Sake rice

mash contains much less initial free sugar as the majority of carbon is tied up in the form of insoluble starch (Kodama 1993; Shobayashi, et al. 2007; Wu, et al. 2006).

1.5 *Aspergillus oryzae* and its role in Sake brewing

Since *S. cerevisiae* does not possess the necessary amylases needed to convert starch (β1,4 glucose polymer) to glucose, yeast cells are not able to consume starch as a sole carbon source (Kodama 1993; Shobayashi, et al. 2007; Wu, et al. 2006). As a result, steamed rice must be pre-treated in preparation for alcoholic fermentation (Figure 4). Sake brewers have long used the fungus *Aspergillus oryzae* as a source of amylases (Kodama 1993; Shobayashi, et al. 2007; Wu, et al. 2006).

At the start of fermentation steamed rice is mixed with rice that has been inoculated with *A. oryzae,* traditionally referred to as 'koji'. Koji rice is rich in free glucose as well as amylases that are free to act on the starch of freshly steamed rice. As a result of the presence of koji, glucose is fed into the fermentation mixture at a controlled rate (amylase limited) which substantially lowers the osmotic stress experienced by yeast cells (Kodama 1993; Shobayashi, et al. 2007; Wu, et al. 2006). This decrease in osmotic stress allows more energy to be devoted to biomass and thus enables Sake fermentations to reach higher titres and higher alcohol concentrations (Shobayashi, et al. 2007; Takagi, et al. 2005; Wu, et al. 2006). Consequently, Sake wine contains the highest concentration of ethanol of any non-distilled alcoholic beverage (Kodama 1993).

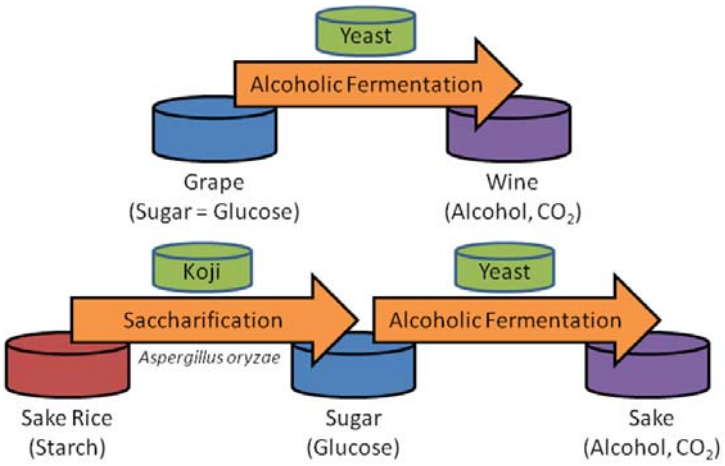


Figure 4. Contrasting processes in wine and Sake making. During winemaking, a saccharification step prior to alcoholic fermentation is unnecessary as the carbon source in grape must is mostly glucose and fructose. During Sake brewing, saccharification must precede alcoholic fermentation as *S. cerevisiae* cannot consume starch as a carbon source.

1.6 Yeast nitrogen metabolism during alcoholic fermentation

In order to build significant biomass and then conduct an efficient alcoholic fermentation, yeast cells require significant amounts of nitrogen. Free nitrogen, in the form of ammonia, is used in many anabolic pathways, while peptides and free amino acids are either used in cellular processes directly, or are broken down to ammonia, glutamate, and glutamine via catabolic pathways (Cooper 2002; Hofman-Bang 1999). The principle nitrogen source present in grape must is arginine, of which the metabolism leads directly to the formation of intracellular urea (Figure 5) (Monteiro and Bisson 1991). Urea forms from the arginase (*CAR1*- EC 3.5.3.1) dependent breakdown of arginine to ornithine and urea (Cooper 1982). At high concentrations, urea is a toxic and poor nitrogen source for *S. cerevisiae*, and is therefore exported to the surrounding medium (Hofman-Bang 1999). *S. cerevisiae* possesses the ability to degrade urea via urea amidolyase (*DUR1,2* - EC 3.5.1.54) (Genbauffe and Cooper 1991); however, in the presence of higher quality nitrogen sources, *DUR1,2* expression is repressed while expression of the urea exporter (*DUR4*) is not (Whitney, Cooper and Magasanik 1973). Consequently, as long as yeast cells are not starved for nitrogen, which forces them to degrade urea, they will preferentially export urea to the

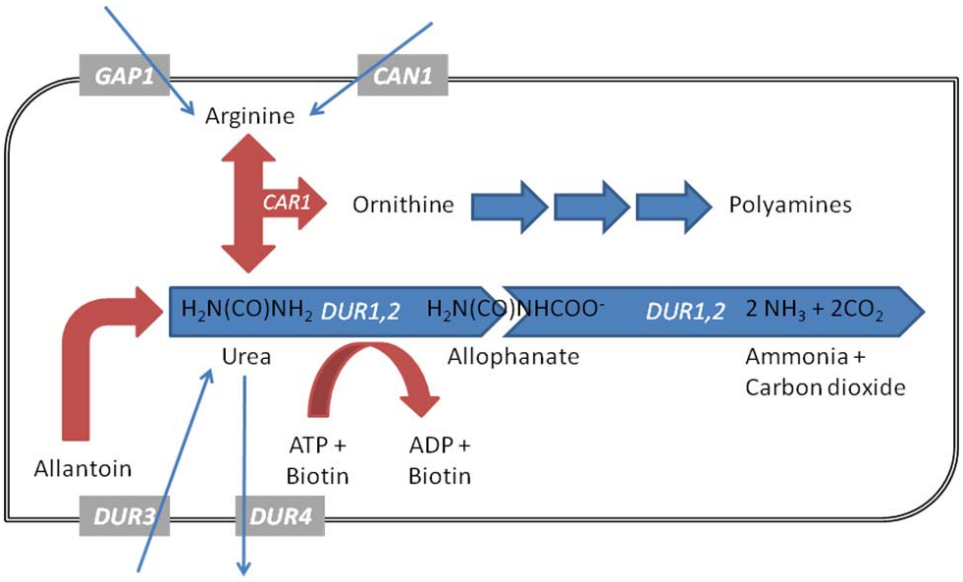


Figure 5 Overview of urea metabolism in *S. cerevisiae*. Arginine is imported into the cell either by the Arginine specific transporter *CAN1* or by the general amino acid permease *GAP1*. Following import arginine is degraded to ornithine and urea by arginase, the product of the *CAR1* gene. Urea can either be exported by *DUR4* or degraded to ammonia and carbon dioxide by *DUR1,2*. Cells can also reabsorb urea through the importer *DUR3*. Adapted from Coulon, et al. (2006).

extracellular environment. If, in the later stages of fermentation, yeast become starved for nitrogen, urea can be reabsorbed (*DUR3* - TC 2.A.21.6) (Cooper and Sumrada 1975; ElBerry, et al. 1993) and metabolized; however, finished wines made from grape varietals with high assimilable nitrogen tend to possess significant residual urea (Ough, Crowell and Gutlove 1988; Ough, Crowell and Mooney 1988; Ough, et al. 1990; Ough, et al. 1991).

1.7 Nitrogen metabolism and Nitrogen Catabolite Repression (NCR) in *S. cerevisiae*

The ability to discriminate between various nutrient sources is an important and evolutionarily conserved theme in biology. Prokaryotes and eukaryotes alike exhibit exquisite regulatory control over their metabolic pathways in order to exist in the most energetically efficient way possible. Such regulatory systems are often referred to as 'catabolite repression' systems because they repress genes necessary to metabolize a particular nutrient source in the presence of a more favored one (Griffiths, et al. 2005). Catabolite repression systems occur for both various carbon (Bruckner and Titgemeyer 2002; Gancedo 1992) and nitrogen sources (Cooper 2002; Hofman-Bang 1999; Salmon and Barre 1998). Particularly well characterized examples of carbon catabolite repression systems include the lactose (*lac*) operon in *E. coli* (Griffiths, et al. 2005) as well as the galactose (*GAL*) genes in *S. cerevisiae* (Lohr, Venkov and Zlatanova 1995). In terms of nitrogen catabolite repression both prokaryotes and eukaryotes utilize a more global repression system that encompasses many different catabolic pathways.

Yeast nitrogen utilization is centered on the usage of both glutamate and glutamine (Hofman-Bang 1999). From glutamate and glutamine, wild type *S. cerevisiae* can synthesize any other amino acid necessary and thus, glutamate and glutamine, along with ammonia, are the nitrogen sources most preferred by yeast (Hofman-Bang 1999).

Due to its diverse repertoire of nitrogen catabolic pathways, *S. cerevisiae* can grow solely on a wide range of nitrogenous compounds (e.g. common and uncommon amino acids, urea, GABA, allophanate, allantoin), as each of these may be converted in to glutamate, glutamine, and ammonia (Hofman-Bang 1999). However, because each non-optimal nitrogenous compound varies in terms of ease of import and degradation, the various nitrogen sources can be ranked in terms of quality or

preference (Hofman-Bang 1999). Furthermore, since it is advantageous to utilize higher quality sources preferentially, and because yeast posses a catabolite repression system for nitrogen sources, the various compounds can also be ranked in terms of NCR strength (Hofman-Bang 1999). A few common yeast nitrogen sources are shown in Table 1.

Table 1. Ranking of various yeast nitrogen sources according to NCR repression strength. Low repression strength indicates a poor nitrogen source. Adapted from Hofman-Bang (1999).

NCR repression under growth in listed media (Low to High)	Nitrogen Source
1	Proline
2	GABA
3	Urea
4	Glutamate
5	Ammonium
6	Asparagine/Glutamine

The global nitrogen catabolite repression system of *S. cerevisiae* has been well studied but is far from being understood in absolute detail (Reviewed in Cooper 2002; Hofman-Bang 1999). At its core, the NCR system of *S. cerevisiae* makes use of four known regulatory transcription factors (*GLN3*, *GAT1*, *DAL80*, and *DEH1*) to control the expression of all NCR sensitive genes (Cooper 2002; Hofman-Bang 1999). Two of the factors, *GLN3* and *GAT1*, are positive regulators (activators), while the other two factors, *DAL80* and *DEH1*, are negative regulators (repressors) (Cooper 2002; Hofman-Bang 1999). The complex interaction of all four transcription factors, as well as various inducers and repressors, at NCR sensitive promoters allows for highly regulated nitrogen catabolite gene expression (Figures 6 and 7).

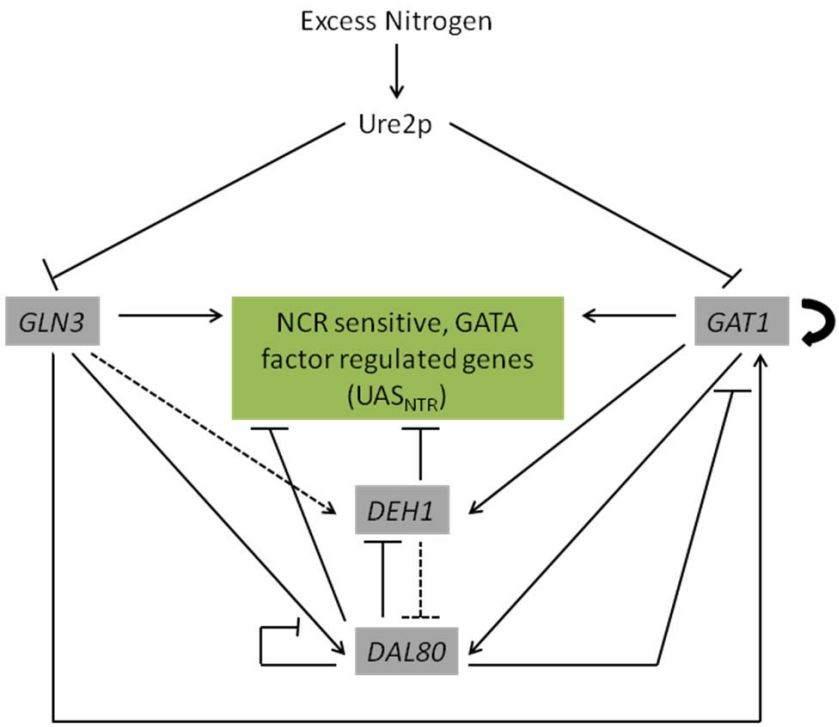


Figure 6. Model of reciprocal regulation of GATA factor gene expression and GATA factor regulation of NCR-sensitive gene expression. Dashed lines indicate weak association/regulation. Adapted from Cooper (2002).

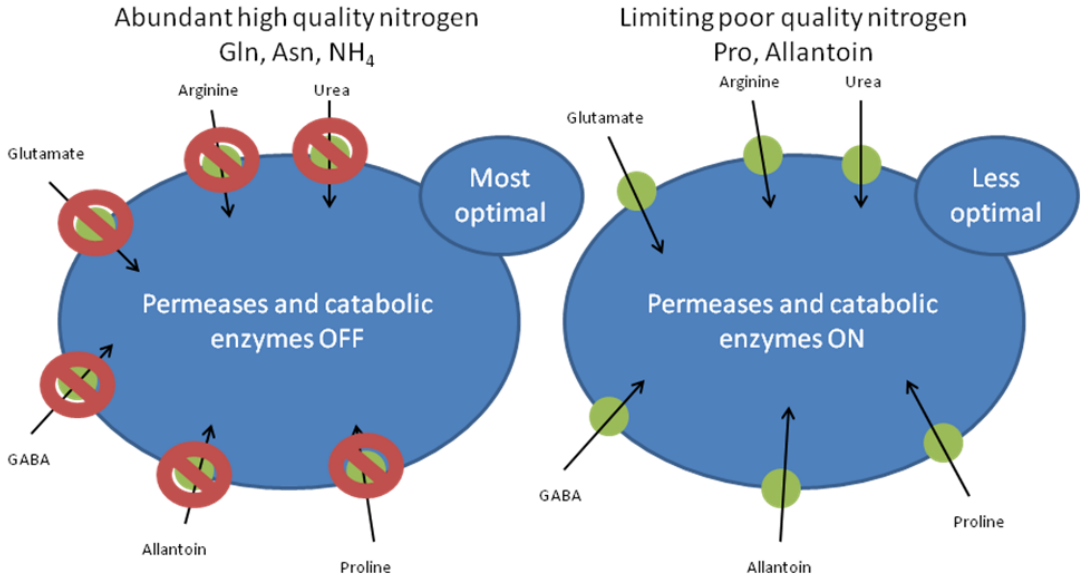


Figure 7. Permeases and degradative enzymes needed to utilize poor nitrogen sources are transcriptionally silenced during growth in abundant high quality nitrogen sources. Adapted from Cooper (2002).

The four NCR transcription factors are referred to as GATA factors because they all exert their effect though the DNA consensus sequence 5'-GATAA-3'. Each factor binds DNA at the GATA site through a conserved zinc finger binding domain and each factor is homologous to many other zinc finger proteins found in higher eukaryotes, including mammals (Bysani, Daugherty and Cooper 1991; Cooper 2002; Cox, et al. 2000; Cox, et al. 2004; Hofman-Bang 1999; van Vuuren, et al. 1991).

The four NCR GATA factors are controlled by the upstream negative regulator *URE2*, which is in turn regulated by the TOR pathway (Target of Rapamycin) (Cooper 2002; Hofman-Bang 1999). The TOR pathway, which is conserved throughout most eukaryotic organisms, acts a master regulatory sensor and signal transduction cascade that assesses and responds, with highly pleiotropic effects, to general cell health, nutrient availability, and growth (Cooper 2002; Dann and Thomas 2006; De Virgilio and Loewith 2006). The TOR pathway acts primarily through two kinases, *TOR1* and *TOR2,* and regulates fundamental cell functions such as the cell cycle, cell division, protein synthesis, etc. (Cooper 2002; Dann and Thomas 2006; De Virgilio and Loewith 2006).

In terms of controlling NCR, the exact mechanism linking the GATA transcription factors, *URE2* and *TOR1/2* has yet to be fully worked out. Despite confusion and conflicting data, researchers do agree on the following relationships (Cooper 2002; Hofman-Bang 1999):

a. GLN3p localization correlates highly with active transcription of NCR regulated genes
b. NCR regulated genes are largely activated when GLN3p is nuclear
c. URE2p complexes with GLN3p and the complex localizes to the cytoplasm
d. Inhibition of TOR1/2p correlates highly with decreased GLN3p phosphorylation, which in turn correlates with GLN3p being nuclear and NCR regulated genes being expressed

The most commonly accepted model of *TOR1/2* control on NCR is depicted in Figure 8. In this model *TOR1/2* keep NCR genes repressed by inhibiting a phosphatase which is necessary for GLN3p dephosphorylation and nuclear import.

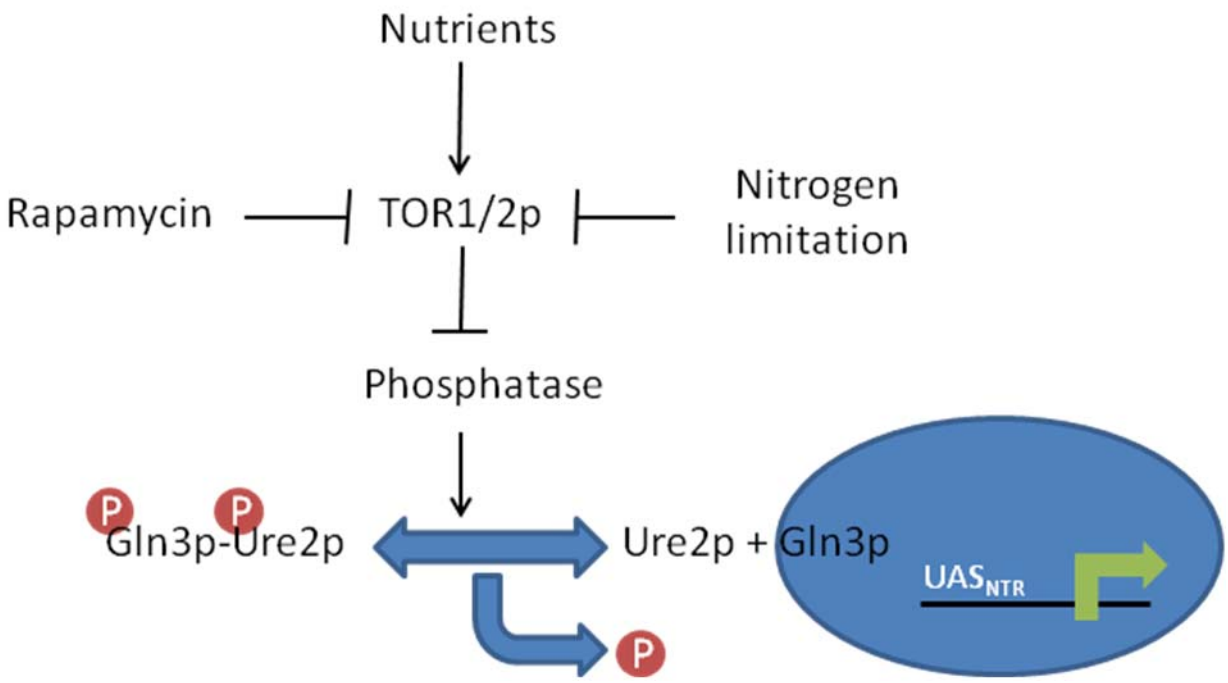


Figure 8. Model of the regulatory pathway by which rapamycin and nitrogen starvation induce NCR regulated gene expression. Adapted from Cooper (2002).

1.8 Urea and ethyl carbamate (EC)

The excretion of non-metabolized urea into wine is the major factor involved in formation of the compound ethyl carbamate (EC) (Monteiro and Bisson 1991). Under ambient conditions (wine storage), ethanol reacts with carbamyl compounds present in fermented beverages to form EC (Ingledew, Magnus and Patterson 1987; Kodama, et al. 1994; Ough, Crowell and Gutlove 1988) in a time and temperature dependent manner (Figure 9). Potentially reactive carbamyl compounds include citrulline and carbamyl phosphate, which result from arginine and nucleotide metabolism, as well as urea (Ough, Crowell and Gutlove 1988).

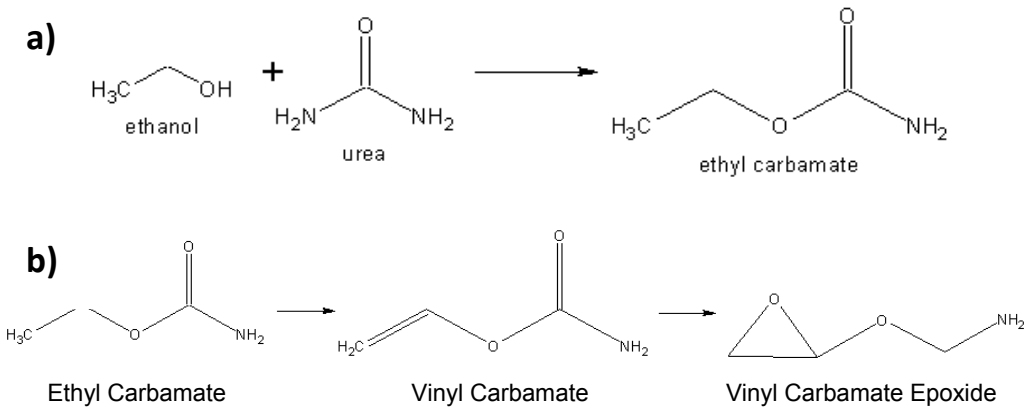


Figure 9. Synthesis reaction and bioactivation pathway of ethyl carbamate**.** a) The synthesis of EC in wines results from the spontaneous reaction of ethanol and urea. b) The epoxide degradation product of EC binds DNA, causing damage and resulting in increased rates of cancers in test animals.

1.9 The EC problem

1.9.1 The EC problem: History

During the early part of the 20th century, ethyl carbamate was often administered to humans as an anesthetic during surgeries. High incidence of lung cancer in surgical patients was the first evidence supporting EC's role as a toxic compound (Nettleship, Henshaw and Meyer 1943). Elucidation of the compound's bioactivation pathway and further studies into its carcinogenicity (Ashby 1991; Guengerich and Kim 1991; Leithauser, et al. 1990) fueled interest of both EC's prevalence and mechanism of action. Studies would soon show that vinyl carbamate epoxides, which are highly reactive oxidative degradation products of EC, interact with free nucleotides as well as RNA and DNA and induce mutations in both through mismatch pairing and direct damage (Dahl, Miller and Miller 1978; Leithauser, et al. 1990; Park, et al. 1993; Schlatter and Lutz 1990; Zimmerli and Schlatter 1991). Further supporting EC's role in cancer is the evidence that EC significantly increases the rates of liver, lung, and harderian gland cancers in male and female mice. Moreover, incidence of mammary and ovarian as well as skin and forestomach cancers was substantially increased in female and male mice, respectively (National Institutes of Health National Toxicology Program 2004).

Known to be a naturally occurring byproduct of fermentation, EC was soon shown to be ubiquitous to nearly all wine and spirits albeit in vastly different quantities (Canas, et al. 1989; Ingledew, Magnus and Patterson 1987; Ough 1976a; Ough 1976b; Schlatter and Lutz 1990; Zimmerli and Schlatter 1991). As a result of studies showing that most wines and spirits contain high levels of EC, during the mid 1980's Canada set a legal limit of 30 µg/L on the allowable EC content in wines; the US set a voluntary limit of 15 µg/L. Long term toxicology studies eventually gave rise to the 1997 action manual on the prevention of EC in wine published by the FDA in conjunction with the Department of Viticulture and Enology at the University of California Davis (http://vm.cfsan.fda.gov/~frf/ecaction.html) (Butzke and Bisson 1998).

Exposure to EC, which may be significantly increased by the regular consumption of alcoholic beverages (Zimmerli and Schlatter 1991), may be a significant factor involved in human cellular mutagenesis and resultant tumorigenesis. As a result, winemakers have been actively reducing EC levels in wines both by agricultural practices and, more recently, molecular biological means (Butzke and Bisson 1998).

1.9.2 The EC problem: Surveys of EC in alcoholic beverages

Despite the government imposed limits on EC levels, table wines, Sake and other alcoholic beverages currently available to consumers contain far more EC than was previously suggested.

In order to assess the scope of the EC problem, numerous stuides have assayed EC levels in various foods and beverages. The EC content of 20 randomly chosen wines from six wine producing countries (five whites – Riesling, Pinot Gris, and Chenin Blanc; 15 reds – Pinot Noir, Cabernet Sauvignon, Shiraz, Zinfandel, and Nebbiolo) is summarized in Table 2 (Reproduced from Coulon, et al. (2006)). As the data indicates, of the 20 wines, 14 exceeded the Canadian EC legal limit (30 µg/L) and 17 exceeded the US voluntary limit (15 µg/L) (Coulon, et al. 2006).

Table 2. Maximum potential ethyl carbamate detected by GC/MS in 20 wines from six countries. Wines were heated at 70°C for 48 hours prior to analysis. Adapted from Coulon et al. (2006).

Wine	Concentration EC (µg/L)	Area 1	Area 2	Area 3	Mean peak area	SD	RSD[a] (%)
1	**11.83**	4274	4542	4185	4334	186	4.29
2	**9.12**	3649	3586	3887	3707	159	4.28
3	**14.63**	5043	4959	4948	4983	52	1.04
4	**38.64**	10262	10112	11269	10548	629	5.97
5	**57.57**	14342	15833	14632	14936	791	5.29
6	**66.15**	16610	16734	17430	16925	442	2.61
7	**59.52**	15890	15015	15260	15388	451	2.93
8	**46.39**	11208	13611	12213	12344	1207	9.78
9	**55.41**	13865	14820	14618	14434	503	3.49
10	**24.36**	7012	6808	7895	7238	578	7.98
11	**44.76**	11650	12576	11677	11968	527	4.40
12	**41.32**	11186	10952	11368	11169	209	1.87
13	**27.5**	7664	8366	7868	7966	361	4.53
14	**38.22**	10375	10471	10508	10451	69	0.66
15	**57.68**	14069	14839	15974	14961	958	6.41
16	**33.82**	9034	9428	9832	9431	399	4.23
17	**51.99**	14146	12880	13899	13642	671	4.92
18	**18.61**	5734	5935	6047	5905	159	2.69
19	**34.99**	9396	9295	10417	9703	621	6.40
20	**36.57**	9635	9756	10811	10067	647	6.43

[a] – Relative standard deviation

In addition to the goal of reducing EC in table wines, Sake wine has some of the highest EC contents amongst fermented beverages, thus making the reduction of EC in Sake an intensely relevant and important pursuit. Typical Sake wines have anywhere from 100-250 µg/L EC (Canas, et al. 1989) due to a pasteurization process that all Sakes undergo prior to bottling.

1.9.3 The EC problem: Current methods of lowering EC

Current strategies for EC reduction generally fall into three categories: agricultural practices, the use of wine additives, and genetic engineering of yeasts.

1.9.3.1 Agricultural methods. The yeast assimilable nitrogen (YAN) and, more specifically, arginine content of the fermentation substrate directly influences the amount of EC present in the final product (Butzke and Bisson 1998). Thus, a reasonable approach to controlling EC levels is to limit arginine levels

in grape must and rice mash. It is possible to regulate the type and amount of nitrogen in fertilizers in order to minimize urea production. Furthermore, management of legume ground cover foliage and nitrogen fixing bacteria can keep juice arginine content below 1000 mg/L, however additional methods are needed to further reduce EC levels in wines (Butzke and Bisson 1998).

1.9.3.2 Additives (acid urease). Alternative methodologies for EC reduction include the use of post- or peri-fermentation additives (Ough and Trioli 1988). These additives, which are most often lyophilized preparations of urease from *Lactobacillus fermentum*, degrade urea in the wine before it has a chance to form EC; however, urease additives yield variable results due to pH and ethanol sensitivity (Kodama, et al. 1994) which, can significantly lengthen wine processing time due to necessary enzyme incubation. The enzyme is also expensive for winemakers to use.

1.9.3.3 Additives (DAP). Supplementation of grape musts with nitrogen is a common winemaking practice. In musts with low assimilable nitrogen, supplementation is crucial for preventing stuck or sluggish fermentations and subsequent spoilage. Given the obvious inappropriateness of supplementation with urea or arginine, the industry makes use of the cheap and efficient supplement diammonium phosphate (DAP); DAP is a source of free ammonia and, as a highly favorable nitrogen source, has been shown to downregulate *CAR1* during alcoholic fermentation (Marks, et al. 2003). Lessening the cell's dependence on arginine as a nitrogen source could prove to be a useful method for lowering EC content in wines.

1.9.3.4 Genetic engineering (urease expression). Another possible method for EC control in wine is the engineering of yeast strains which express bacterial or fungal ureases (Ough and Trioli 1988). This approach, whether integrated or plasmid borne, would allow yeast to directly degrade urea during fermentation, and eliminates the need for urease additives post-fermentation. However, because *S. cerevisiae* does not possess such an enzyme, a urease expressing strain would be classified as transgenic thus resulting in difficulty obtaining regulatory approval for use. Furthermore, transgenic organisms carry a strong negative connotation in today's society thus making their universal acceptance unlikely.

1.9.3.5 Genetic engineering (*CAR1*). More central to this work is the genetic engineering of yeast strains which are disrupted at the *CAR1* locus (Kitamoto, et al. 1991). By disrupting *CAR1*, arginase will no longer be available to degrade arginine to urea thus preventing the formation of EC in wine. While

this method does work in the laboratory (Kitamoto, et al. 1991; Yoshiuchi, Watanabe and Nishimura 2000), it has not been widely adopted in practice because arginine is one of the major nitrogen sources for *S. cerevisiae* (Ingledew, Magnus and Patterson 1987; Rossignol, et al. 2003). Moreover, usage of *Δcar1* strains is difficult due to the diploid nature of all industrial wine and Sake yeasts, and because of the high risk of contamination from wild type *CAR1*/*CAR1* yeasts; however, the problem of contamination has been dealt with in the case of Sake yeast by engineering *Δcar1* with killer character (Yoshiuchi, Watanabe and Nishimura 2000). It should be noted that *CAR1* knockouts have only been attempted in Sake yeast since it is generally regarded that yeast's inability to metabolize arginine would, in most cases, lead to stuck fermentations and possible spoilage. An antisense mediated method for the knockdown of *CAR1* has been successful in reducing Arginase activity in laboratory yeasts (Park, Shin and Woo 2001); however, this methodology has yet to be examined in industrial yeasts.

1.9.3.6 Genetic engineering (*DUR1,2*). Yeast possess the ability to degrade urea via *DUR1,2* which encodes a bi-functional adenosine tri-phosphate (ATP) and biotin dependent enzyme (urea amidolyase) that degrades urea to two molecules each of CO_2 and NH_3 in a two step reaction (Genbauffe and Cooper 1986; Genbauffe and Cooper 1991; Whitney and Cooper 1972; Whitney and Cooper 1973; Whitney, Cooper and Magasanik 1973). Urea is first carboxylated using ATP and biotin to form allophanate, after which allophanate is hydrolyzed to form CO_2 and NH_3.

Since *DUR1,2* is subject to regulation by NCR, and because regulatory mechanisms exist which allow for production of wines with high residual urea content, it is reasonable to expect that constitutive expression of *DUR1,2* should result in substantially lowered EC levels. Indeed, our group has explored this approach, and such yeasts are capable of producing wines which contain up to 89% less EC (Coulon, et al. 2006). More specifically, when the *DUR1,2* ORF was placed under the control of the yeast *PGK1* promoter and terminator signals and a single copy was integrated into the *URA3* locus of the industrial strain UC Davis 522, Chardonnay wine created by the metabolically engineered strain (522^{EC-}) contained 89.1% less EC (Coulon, et al. 2006). Analysis of the genotype, phenotype and transcriptome of 522^{EC-} suggested that the metabolically engineered strain was substantially equivalent to its parent, thus making it suitable for commercialization (Coulon, et al. 2006). Furthermore, since the urea degrading strain (522^{EC-}) contains no foreign DNA sequences, it is not classified as transgenic and thus has been

given FDA GRAS approval which should make it much more readily accepted by industry and consumers (Coulon, et al. 2006).

1.9.4 Alternative methods for EC reduction

Realistically, there are only four genes that can be manipulated to reduce EC in wine: urea production (*CAR1*), urea degradation (*DUR1,2*), urea export (*DUR4*), and urea import (*DUR3*).

Based on the literature and published data, we understand the effects of manipulating *CAR1* and *DUR1,2* expression (Coulon, et al. 2006; Yoshiuchi, Watanabe and Nishimura 2000), and while *CAR1* mutants produce almost no EC, *CAR1* is not a practical industrial target for EC reduction due to problems with stuck fermentations. Thus, manipulation of *DUR4* and *DUR3* remain as potential targets to reduce EC in wine and Sake. One option would be to knockout the urea exporter *DUR4*, thus disabling the yeast's ability to export urea into the wine. Although this sounds like an attractive option, this strategy would likely cause serious problems for winemakers. Urea is a toxic byproduct of arginine metabolism and yeast cells need to export urea in order to stay healthy. By knocking out *DUR4*, urea will accumulate in the cell and lead to an adverse physiological state resulting in stuck fermentations. The fact that existing recombinant *DUR1,2* yeasts produce any EC at all (Coulon, et al. 2006) suggests that under typical winemaking conditions more urea is produced than DUR1,2p, from a constitutively expressed copy of *DUR1,2,* can degrade.

Given the potential problems associated with *DUR4* knockouts, we focused our attention on the urea permease, *DUR3*. Constitutive expression of *DUR3* should result in yeasts which will act like urea sponges; not only will these yeasts reabsorb urea that they excreted as a byproduct of arginine metabolism, but they should also absorb a significant amount of urea that is naturally present in fermentation substrate. By combining *DUR1,2* constitutive expression with that of *DUR3*, it should be possible to create recombinant yeasts which can conduct efficient and substantially equivalent alcoholic fermentations with the production of little or no EC.

1.10 Introduction to *DUR3*: Role in the cell (urea transport, polyamines, boron)

During the early 1970's it was known that yeast cells were capable of metabolizing urea as a sole nitrogen source (Cooper and Sumrada 1975); however, there was no detailed knowledge of how it was brought into the cell. Studies using ^{14}C-urea revealed that entry of urea into the cell is bimodal (Cooper and Sumrada 1975; Sumrada, Gorski and Cooper 1976). A facilitated diffusion system brings urea into the cell in an energy independent fashion when urea is present at concentrations greater than 0.5 mM. More interestingly however, is the presence of an energy (ATP) dependent active transport system (K_m = 14 μM) which functions at low concentrations of urea and is sensitive to nitrogen catabolite repression (Cooper and Sumrada 1975; Sumrada, Gorski and Cooper 1976).

First purified and characterized in 1993, the *DUR3* (Degradation of URea) ORF encodes a 735 aa integral membrane protein which contains 15 predicted transmembrane domains (ElBerry, et al. 1993). The protein, which localizes to the plasma membrane, utilizes ATP to transport urea into the cell at low extracellular concentrations. There is some evidence to suggest that the physiological functioning state of the urea transporter is a multimeric complex, however this has not been confirmed (ElBerry, et al. 1993).

Expression of *DUR3* is regulated in a manner highly similar to other genes in the urea and allantoin degradative pathways (ElBerry, et al. 1993). Being subject to NCR, the *DUR3* promoter contains two sets of tandem GATAA consensus sequences; however, while the promoter contains efficient GATAA transcription factor binding sites (UAS_{NTR}), high level expression is strongly dependent on two upstream induction sequences (UIS) (Hofman-Bang 1999). During growth on proline media (no NCR), little *DUR3* (and *DUR1,2*) mRNA can be detected by northern blotting without the presence of a gratuitous inducer (oxalurate, an allophanate analogue) (Hofman-Bang 1999).

The activating transcription factors *DAL81* and *DAL82* act through the UIS sequences contained in the *DUR3* promoter. The consensus sequence, 5' (G/C) AAA (A/T) NTGCG (T/C) T (T/G/C) (T/G/C) 3', for *DAL81* and *DAL82* binding is shared between other allophanate induced genes (*CAR2, DAL2, DAL4, DUR1,2* and *DUR3* (Hofman-Bang 1999).

During times of strong NCR, *DUR3* is actively repressed by the negative GATAA transcription factor *DAL80*, and deletion of *DAL80* results in expression of *DUR3* even in the absence of an inducer (Hofman-Bang 1999). Conversely, during NCR de-repression, *DUR3* is actively transcribed, if an inducer is present, through the actions of the positive GATAA transcription factor *GLN3* (Hofman-Bang 1999).

In addition to the obvious role in urea uptake, *DUR3* has been shown to be involved in other important cellular processes. *DUR3* has been shown to be an important regulator of intracellular boron concentration (Nozawa, et al. 2006). Cells lacking *DUR3* show decreased intracellular boron concentration; thus, *DUR3* appears to function as an active transporter of boron into the cell (Nozawa, et al. 2006). Although evidence suggests *DUR3* plays a role in boron transport and regulation, a clear physiological role for *DUR3* in terms of boron utilization has yet to be defined.

The other important role of *DUR3* is in the uptake of polyamines (Uemura, Kashiwagi and Igarashi 2007). Polyamines, such as putrescine, spermidine, and spermine, are highly regulated peptides essential for cell growth and proliferation (Uemura, Kashiwagi and Igarashi 2007). Their function is ubiquitous to both pro- and eukaryotes. Like *E. coli*, *S. cerevisiae* possesses general polyamine transporters (*TPO1-4, UGA4, TPO5, GAP1*), as well as polyamine specific transporters (*AGP2*). Interestingly, *DUR3* has been shown to specifically uptake polyamines concurrently with urea (Uemura, Kashiwagi and Igarashi 2007). As urea is a very poor nitrogen source, and does not normally occur in significant quantities outside 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, Kashiwagi and Igarashi 2007).

Most interesting is the apparent post-translational regulation of *DUR3* polyamine uptake by the serine/threonine kinase *PTK2* (Uemura, Kashiwagi and Igarashi 2007). *PTK2* seems to positively regulate *DUR3* polyamine uptake via the phosphorylation of cytoplasmic residues Thr-250, Ser-251, and Thr-684 (Uemura, Kashiwagi and Igarashi 2007). Although *DUR3* polyamine activity and subsequent *PTK2* regulation has been preliminarily investigated in the laboratory yeast YPH499 (Uemura, Kashiwagi and Igarashi 2007), there are no known studies which have investigated the role of *DUR3* mediated urea or polyamine uptake during alcoholic fermentation. However, it is known that different strains of wine

yeast react differentially in terms of fermentation rate and biomass production in response to varying polyamine concentrations (Uemura, Kashiwagi and Igarashi 2007).

As with polyamine uptake, the *DUR3* mediated uptake of boron seems to be post-translationally regulated. Although cells lacking *DUR3* exhibit lower boron concentrations, the converse situation is not true *i.e.* cells overexpressing *DUR3* do not show significant increases in boron concentration (Nozawa, et al. 2006). Taken together, the cases of polyamine and boron uptake provide good evidence for the existence of a *DUR3* regulatory protein, presumably *PTK2*.

1.11 Proposed Research

1.11.1 Significance of Research

Given the obvious governmental health concern regarding the EC content of wines, it seems logical to pursue the goal of producing wines that contain no EC. Until recently with the advent of constitutive *DUR1,2* expression (Coulon, et al. 2006), existing methods of EC reduction were cumbersome, ineffective, expensive, and/or impractical.

The development of non-transgenic yeast strains which are capable of producing little or no EC during an efficient and substantially equivalent alcoholic fermentation would be of direct benefit to industry and consumers alike. As such, the research described herein is both an application of existing technology to a novel target, as well as a proof of concept exploration of one possible new method for EC reduction.

1.11.2 Hypotheses

1.11.2.1 The metabolically engineered Sake yeast strains $K7^{EC-}$ and $K9^{EC-}$ should reduce EC efficiently during Sake brewing trials. Given the substantially different environment of Sake mash, it is reasonable that the EC reduction of functionally enhanced Sake yeasts will be highly superior when fermenting rice mash rather than grape must. Sake yeasts have evolved to function optimally in the nutrient composition of rice mash and in the presence of 'koji', thus the efficiency of the *DUR1,2* cassette should also function optimally. Such a result should reveal the true EC reduction potential of our recombinant

yeasts and would affirm our belief that each specific yeast strain must be tested in its native environment in order to yield the most accurate results.

1.11.2.2 Constitutive co-expression of *DUR1,2* and *DUR3* in metabolically engineered yeasts should result in synergistic EC reduction. *DUR1,2*/*DUR3* yeasts should produce substantially less EC than both *DUR1,2* and parental yeasts due to their ability to absorb native urea in grape musts and to reabsorb excreted urea. Moreover, these yeasts should behave like their parental counterparts in all other aspects of fermentation i.e. growth rate, ethanol production, CO_2 production, kinetics, etc. Obtaining these results will affirm our belief that the problem with current *DUR1,2* clones is their ability to export metabolic urea before it can be completely degraded. Additionally *DUR1,2* yeasts cannot be used to degrade any urea natively present in the must/mash while *DUR1,2*/*DUR3* yeasts could.

1.11.3 Main objectives

The main objectives of this study are to:

1. Constitutively express *DUR1,2* in the Sake yeast strains K7 and K9, characterize the resultant engineered strains, and evaluate the effect of constitutive *DUR1,2* expression on EC production in Chardonnay wine
2. Characterize the EC reduction potential of Sake yeast clones $K7^{EC-}$ and $K9^{EC-}$ during small scale Sake fermentation
3. Construct a genetic cassette capable of maintaining constitutive expression of a functional *DUR3* urea permease in wine and Sake yeasts
4. Constitutively express *DUR3* both on its own and concurrently with *DUR1,2* during wine and Sake making in order to assess the effect on EC reduction
5. Characterize the role of *DUR3*, and its interplay with *DUR1,2*, in yeast urea metabolism and EC production during alcoholic fermentation.

2 MATERIALS AND METHODS

2.1 Strains, plasmids and genetic cassettes

The strains, plasmids, and genetic cassettes used in the construction and characterization of *DUR1,2* and/or *DUR3* expressing yeast strains are listed in Tables 3, 4, and 5, respectively.

Table 3. Strains used in the genetic construction and characterization of *DUR3* expressing yeast strains.

Strain	Description	Reference
E. coli Subcloning Efficiency™ DH5α™ Competent Cells	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-	Invitrogen
S. cerevisiae K7	Industrial Sake yeast strain Kyokai No. 701 (K7)	Brewing Society of Japan
S. cerevisiae K9	Industrial Sake yeast strain Kyokai No. 901 (K9)	Brewing Society of Japan
S. cerevisiae 522	Industrial wine yeast strain	UC Davis
S. cerevisiae $K7^{EC-}$	Sake yeast strain K7 containing the *DUR1,2* cassette (Coulon, et al. 2006) integrated at the *URA3* locus	This study
S. cerevisiae $K9^{EC-}$	Sake yeast strain K9 containing the *DUR1,2* cassette integrated at the *URA3* locus	This study
S. cerevisiae 522^{EC-}	Wine yeast strain 522 containing the *DUR1,2* cassette integrated at the *URA3* locus	(Coulon, et al. 2006)
S. cerevisiae $K7^{D3}$	Sake yeast strain K7 containing the *DUR3* cassette integrated at the *TRP1* locus	This study
S. cerevisiae 522^{D3}	Wine yeast strain 522 containing the *DUR3* cassette integrated at the *TRP1* locus	This study
S. cerevisiae $K7^{EC-D3}$	Sake yeast strain K7 containing the *DUR1,2* cassette integrated at the *URA3* locus and the *DUR3* cassette integrated at the *TRP1* locus	This study
S. cerevisiae 522^{EC-D3}	Wine yeast strain 522 containing the *DUR1,2* cassette integrated at the *URA3* locus and the *DUR3* cassette integrated at the *TRP1* locus	This study
A. oryzae 'Koji-Kin'	Industrial preparation of 'koji' grade *A. oryzae*	Vision Brewing http://www.visionbrewing.com

Table 4. Plasmids used in the genetic construction and characterization of *DUR3* expressing yeast strains.

Plasmid	Description	Reference
pUT332Δ*ura3*	*E. coli*/*S. cerevisiae* episomal shuttle vector containing the Tn5*ble* (phleomycin) dominant marker.	(Gatignol 1987)
pUC18	High copy number *E. coli* plasmid that contains Amp^R, *ori,* and an MCS located within a *lacZ* coding sequence thus facilitating cloning via blue/white X-Gal selection.	(Yanisch-Perron, Vieira and Messing 1985)
pUG6	High copy number *E. coli* plasmid that contains Amp^R, $kanMX^R$*,* and *ori.*	(Guldener, et al. 1996)
pHVX2	A YEplac181 based *S. cerevisiae* expression vector in which gene expression is driven from the constitutive *PGK1* promoter and terminator signals	(Volschenk, et al. 1997)
pUCTRP1	pUC18 to which the *TRP1* coding region was inserted into the *BamH1* site at the MCS	This study
pHVX2D3	pHVX2 to which the *DUR3* coding region was inserted between the *PGKp* and *PGKt* via the *Xho1* cloning site	This study
pHVXKD3	pHVX2D3 to which a *kanMX* resistance marker (from pUG6) was inserted into the Sal1 site of pHVX2D3	This study
pUCMD	pUCTRP1 based plasmid into which the *DUR3* expression cassette (5'-*PGKp-DUR3-PGKt-kanMX*-3') was PCR blunt end cloned into the middle of *TRP1* via the *EcoRV* site	This study

Table 5. Genetic cassettes used in the genetic construction and characterization of *DUR3* expressing yeast strains.

Cassette	Description	Reference
DUR1,2	Linear expression cassette containing 5'-*URA3-PGKp-DUR1,2-PGKt-URA3*-3'	(Coulon, et al. 2006)
DUR3	Linear expression cassette containing 5'-*TRP1-PGKp-DUR3-PGKt-kanMX-TRP1*-3'	This study

2.2 Culture conditions

E. coli DH5α cells were used for molecular cloning and propagation of plasmids; cells were cultured according to standard methods (Ausubel, et al. 1995). Unless otherwise indicated, all *S. cerevisiae* strains were cultured aerobically with shaking at 30°C in either liquid YPD medium (Difco, Becton Dickinson and Co., USA), or on YPD + 2% (w/v) agar (Difco, Becton Dickinson and Co., USA)

plates. YPD plates supplemented with 300 µg/mL G418 (Sigma, USA) were used to select for positive *S. cerevisiae* transformants containing the *DUR3* expression cassette.

2.3 Genetic construction of the urea degrading yeasts $K7^{EC-}$ and $K9^{EC-}$

2.3.1 Co-transformation of the *DUR1,2* cassette and pUT332

S. cerevisiae strains K7 and K9 were co-transformed with the 9191 bp *DUR1,2* cassette (Table 5) and pUT332Δ*ura3* (Gatignol 1987) combined at a 10:1 (*DUR1,2* cassette:pUT332) molar ratio. Yeast strains were transformed using the lithium acetate/polyethylene glycol/ssDNA method (Gietz and Woods 2002). Following transformation, cells were left to recover in YEG at 30°C for 2 hours before plating on YEG plates supplemented with 100 µg/mL phleomycin (Invitrogen, USA). Plates were incubated at 30°C until colonies appeared.

2.3.2 Screening of transformants for the integrated *DUR1,2* cassette

Colony PCR (Ward 1992) was used as described to detect the presence of the linear *DUR1,2* cassette integrated into the yeast genome at the *URA3* locus. Zymolyase 100 U/mL (Seikagaku Corp., Japan) was used to lyse the cells (30 µl zymolyase solution). Primers InDURURA (5'- TGGTGATATGGTTGATTCTGGTGACATA -3') and OutURAb (5'- TTCCAGCCCATATCCAACTTCCAATTTA -3') were used to generate an approximately 1500 bp fragment from the 3' end of the cassette. The primers are specific for the inside and outside of the integrated cassette in order to detect integration and correct orientation. The yeasts 522 and 522^{EC-} were used as negative and positive controls, respectively. PCR was performed with iProof™ High Fidelity DNA polymerase (BioRad, USA) using suggested reagent concentrations and 1 µl of Zymolyase treated cell suspension as a template. The PCR program was as follows: 1. Initial denaturation – 3 min at 98°C. 2. Denaturation – 10 sec at 98°C. 3. Annealing – 30 sec at 58.5°C. 4. Extension – 45 sec at 72°C. 5. Cycle to step 2, 30 times. 6. Final extension – 10 min at 72°C. Colony PCR reactions were visualized on 0.8% agarose gels stained with SYBR™ Safe (Invitrogen, USA). After identification of positive transformants, engineered strains were cultured for ~10 generations on non-selective media (YPD) in order to ensure loss of the co-transforming plasmid.

2.3.3 Genetic characterization

2.3.3.1 Southern blot analyses. Southern blotting was used to confirm integration of the *DUR1,2* cassette into the *URA3* locus. Genomic DNA from engineered strains $K7^{EC-}$ and $K9^{EC-}$ as well as their respective parent strains was digested with *BglII* (Roche, Germany) (Ausubel, et al. 1995), and separated on a 0.8% agarose gel. Following gel preparation, transfer and fixing to a positively charged Nylon membrane (Roche Diagnostics, Germany) (Ausubel, et al. 1995), the blots were probed with PCR generated fragments specific for *DUR1,2* and *URA3*. The AlkPhos^{TM} Direct Nucleic Acid Labeling and CDP-Star Detection system was used as recommended for probe detection (Amersham Biosciences, England).

The 736 bp *DUR1,2* probe was generated by PCR using genomic DNA from *S. cerevisiae* strain 522 as a template and the primers DUR1,2probe5 (5'-TTAGACTGCGTCTCCATCTTTG-3') and DUR1,2F (5'-TGCTGGCTTTACTGAAGAAGAG-3'). The 927 bp *URA3* probe was generated by PCR using 522 genomic DNA and the primers 3'URA3 (5'-TGGGAAGCATATTTGAGAAGATG-3') and OutURAb (5'-TTCCAGCCCATATCCAACTTCCAATTTA -3').

2.3.3.2 Sequence analysis. Genomic DNA isolated from strains $K7^{EC-}$ and $K9^{EC-}$ was used to amplify two separate fragments which together encompassed the entire ~10 kb linear *ura3-PGK1p-DUR1,2-PGK1t-ura3* cassette. Primers 5'OUTURA3Cas (5'-AACTAATGAGATGGAATCGGTAG-3') and DUR12rev1 (5'-TCCTGGAATGCTGTGATGG-3') amplified a 7900 bp fragment on the 5' end of the cassette, while primers PGK1forDUR (5'-TGGTTTAGTTTAGTAGAACCTCGTGAAACTTAC-3') and OutURAb (5'-TTCCAGCCCATATCCAACTTCCAATTTA -3') amplified a 7100 bp fragment on the 3' end of the cassette.

Sequencing was performed by the Nucleic Acid Protein Service Unit (NAPS) at The University of British Columbia using an Applied Biosystems PRISM 377 sequencer and Applied Biosystems BigDye v3.1 sequencing chemistry. Primers and template were supplied to NAPS in the concentrations specified by their sample submission requirements. The entire integrated *DUR1,2* cassette was sequenced via 19 different sequencing reads (Table 6) and later assembled *in silico* using Accelrys DS Gene v1.1 software. The assembled sequences were aligned against previously published sequences and those of *DUR1,2*, *URA3*, $PGK1_p$, and $PGK1_t$ obtained from SGD. If any discrepancies were found, the specific read which gave rise to the discrepancy was repeated in order to identify bona fide mutations.

Table 6. Oligonucleotide primers used in sequencing of the integrated *DUR1,2* cassette.

Primer	Primer Name	Sequence (5'→3')
P1	5'OUTURA3Cas	5'-AACTAATGAGATGGAATCGGTAG-3'
P2	5'URA3Flank	5'-AGTATTCTTAACCCAACTGCACAGA-3'
P3	5'PGK1pro1	5'-ACAAAATCTTCTTGACAAACGTCACAA3'
P4	5'PGK1pro2	5'-AATTGATGTTACCCTCATAAAGCACGT-3'
P5	PGK1forDUR	5'-TGGTTTAGTTTAGTAGAACCTCGTGAAACTTAC-3'
P6	DUR12G	5'-TACCAGAACCTGCTGTATCAG-3'
P7	DUR12rev6	5'-TCATCCGCAACTTGTTGCATAG-3'
P8	DUR12F	5'-TGCTGGCTTTACTGAAGAAGAG-3'
P9	DUR12rev5	5'-TCGGAATAAACTGCAACTGATC-3'
P10	DUR12E	5'-ACCTCTGATAATATCTCCCGAAG-3'
P11	DUR12D	5'-TTTTGGCCAATGTTGGATCATATTC-3'
P12	DUR12rev3	5'-TGTCAACTTGCCAATGGATAAAGTAG-3'
P13	DUR12C	5'-TTGTAATGAACCTTCCACTTCTC-3'
P14	DUR12B	5'-ACACATGCCAAAGTCTTCGAG3'
P15	DUR12A	5'-ATTTCCAAAAACGCCCAGAATAC-3'
P16	DUR12for3end	5'-TCATCAAGAATACTTGAGATGGATC-3'
P17	InDURURA	5'-TGGTGATATGGTTGATTCTGGTGACATA-3'
P18	3'URA3	5'-TGGGAAGCATATTTGAGAAGATG-3'
P19	OutURAb	5'-TTCCAGCCCATATCCAACTTCCAATTTA-3'

2.3.3.3 Analysis of *DUR1,2* gene expression by qRT-PCR. Single colonies of parental strains as well as engineered strains from freshly streaked YPD plates 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 again 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 sterile 250 mL Schott bottles filled with 200 mL filter sterilized (0.22 µm, Millipore, USA) Calona Chardonnay juice to a final OD_{600} = 0.1. Bottles were aseptically sealed with vapour locks (sterilized with 70% v/v ethanol) filled with sterile water. Sealed bottles were incubated at 20°C for 24 hours.

Total RNA from 24 hour fermentations was extracted using the hot phenol method (Ausubel, et al. 1995); RNA was cleaned up post extraction using a total RNeasy Midi Kit (Qiagen), and quantified on a Pharmacia Ultrospec 3000 UV/Vis spectrophotometer. Clean total RNA (1 µg) was used for cDNA synthesis (iScript™, BioRad, USA) according to the manufacturer's instructions. iTAQ™ SYBR® Green

Supermix with ROX (BioRad, USA) was used in conjunction with an Applied Biosystems 7500 Real Time PCR machine in order to determine the relative levels of gene expression in the strains studied. Real time primers for both *DUR1,2* and *ACT1* were automatically optimized and designed using Applied Biosystem's Primer Express™ v2.0 software. The *DUR1,2* amplification product was amplified using the primers DUR12RTfwd (5'-CTCTGGTCCAATGGACGCATA -3') DUR12RTrev (5'-GATGGATGGACCAGTCAACGTT-3'). *ACT1* was amplified using the primers act1forward (5'-GTTTCCATCCAAGCCGTTTTG-3') and act1reverse (5'-GCGTAAATTGGAACGACGTGAG-3'). For each strain, *DUR1,*2 and *ACT1* expression was analyzed six times and results were averaged. RQ data were analyzed using the Applied Biosystems RQ Study software v1.2.2.

2.3.3.4 Global gene expression analysis. Total RNA from strains K7 and $K7^{EC-}$ was extracted after 24 hours of fermentation (Section 2.3.3.3), via the hot phenol method (Ausubel, et al. 1995), quantified on a NanoDrop ND-1000 spectrophotometer and visualized on a 0.8% agarose gel. A 'GeneChip® One-Cycle Target Labeling and Control' kit (Affymetrix, USA) 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 high quality total RNA. Microarray analyses were done in duplicate, each with independently grown cell cultures.

Four oligonucleotide yeast genome arrays, two per strain, (YGS98; Affymetrix, USA) were used for hybridization of fragmented labelled cRNA. Preparation of hybridization solution, hybridization, washing, staining, and scanning of the microarrays were performed as per the manufacturer's instructions (Eukaryotic Array Gene Chip Expression Analysis and Technical Manual; Affymetrix, USA). The EukGE-WS2v4 fluidics protocol of Affymetrix MASv5.0 software was used for array staining and washing procedures while arrays were scanned using a G2500A GeneArray Scanner (Agilent Technologies, USA).

Data were analyzed with MASv5.0 and DMT software (Affymetrix, USA) running on default settings (Affymetrix Statistical Algorithm Reference Guide). Statistically significant and reproducible results were obtained by only including genes which responded similarly in all four cross comparisons and with change p-values of ≤0.005 (increasers) or ≥0.995 (decreasers). Reported fold change values are derived from the average (n=4) of the Signal Log (base 2) Ratio (SLR). Array annotations were linked to

their gene ontology (GO) annotations using the 'gene_association.sgd.tab' table (http://www.yeastgenome.org/gene_list.shtml).

2.3.4 Phenotypic characterization

2.3.4.1 Analysis of fermentation rate in Chardonnay must. Single colonies of parental strains (K7 and K9) as well as appropriate engineered strains from freshly streaked YPD plates, 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 again 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 sterile 250 mL Schott bottles filled with 200 mL unfiltered Chardonnay juice obtained from Calona Vineyards, Kelowna, BC, Canada to a final OD_{600} = 0.1. Bottles were aseptically sealed with sterilized (70% v/v ethanol) vapour locks filled with sterile water. Sealed bottles were incubated at 20°C, and weighed daily to monitor CO_2 production. Data were plotted in Excel to generate fermentation profiles.

2.3.4.2 Analysis of fermentation rate in Sake mash. Koji rice was prepared in 400 g batches from short grain Japanese Kokoho Rose (Safeway, Canada). Rice was rinsed with tap water at room temperature until the water ran clear; the rice was subsequently soaked for 1.5 hours at room temperature in enough water to cover the rice. The rice was then drained in a kitchen sieve for 20 min. The rice in the sieve was then placed over a pot of boiling water and covered with a bamboo steamer lid. The sieve was arranged in such a way that the rice was not in direct contact with boiling water. After steaming until soft and slightly transparent, the cooked rice was placed in a stainless steel bowl and cooled to ~30°C. Upon reaching 30°C, the cooled rice was inoculated with 1.5 g of koji seeds (Vision Brewing, Washington, USA) mixed with 1 teaspoon of all purpose white flour. The rice was mixed well, covered with a piece of moist Whatman No. 3 paper, and the bowl was sealed with plastic film. The covered bowl was incubated, with occasional mixing, at 30°C for 48 hours, or until the rice grains were covered with fine white fibres and the entire mixture had a cheese-like aroma. The koji was then transferred to sterile 500 mL centrifuge bottles and stored at -30°C until use.

Single colonies of parental strains (K7 and K9) as well as appropriate engineered strains from freshly streaked YPD plates, were inoculated into 5mL YPD and grown overnight at 30°C on a rotary

wheel. Cells were subcultured into 50 mL YPD (final OD_{600} = 0.05) and again 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.

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 (steamed as per 'koji' preparation above), and 100 mL of water containing 0.125 g/L 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 CO_2 production. Data were plotted in Excel to generate fermentation profiles.

2.3.4.3 Analysis of glucose/fructose utilization and ethanol production. Following fermentation, 1 mL of fresh unheated Sake was transferred to a sterile 1.5 mL microcentrifuge tube and centrifuged for 10 min at max speed (13K RPM) to pellet cells and any particulate. Supernatant (500 µL) was transferred to a new autosampler screw cap vial (Agilent, USA).

A 20% (v/v) EtOH standard was made from 100% EtOH (Sigma, USA) mixed with sterile MilliQ water (Millipore, USA), and 1 mL of the standard was transferred to new autosampler screw cap vial. A standard curve corresponding to 4, 8, 12, 16, and 20% (v/v) EtOH was plotted after duplicate injections of 2, 4, 6, 8, and 10 µL of the 20% standard as outlined below.

A 3 g/L glucose/fructose standard was made from D-Glucose (Fisher Scientific, USA) and D-Fructose (Fisher Scientific, USA) mixed with sterile MilliQ water (Millipore, USA), and 1 mL of the standard was transferred to new autosampler screw cap vial. A standard curve corresponding to 0.6, 1.2, 1.8, 2.4, and 3.0 g/L glucose/fructose was plotted after duplicate injections of 2, 4, 6, 8, and 10 µL of the 20% standard as outlined below.

Samples were analyzed on an Agilent 1100 series liquid chromatograph running Chemstation Rev A.09.03 [1417] software (Agilent Technologies, USA). The LC was fitted with a Supelcogel C-610H main column [column temperature: 50°C, 30 cm x 7.8 mm ID] (Supelco, USA) that was protected by a Supelguard C-610H [5cm x 4.6 mm ID] (Supelco, USA) 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, fructose was eluted at ~8 min and glucose was eluted at ~9.5 min, and all compounds were detected by a refractive index detector running in positive mode. The concentration of each compound was determined automatically by Chemstation software as based on the standard curves.

2.3.5 Functionality analyses

2.3.5.1 Reduction of EC in Chardonnay wine. Chardonnay wine was produced with K7, K7^{EC-}, K9, and K9^{EC-} as in Section 2.3.4.1. At the end of fermentation, cells were removed by centrifugation (5000 rpm, 4°C, 5 min), and ~ 50 mL of wine was decanted into sterile 50 mL Schott bottles. Bottles were incubated in a 70°C water bath for exactly 48 hours to maximize EC production, and then stored at 4°C until GC/MS analysis (Section 2.3.5.3).

2.3.5.2 Reduction of EC in Sake wine. Sake wine was produced with K7, K7^{EC-}, K9, and K9^{EC-} as in Section 2.3.4.2. At the end of fermentation, cells and rice were removed by centrifugation (5000 rpm, 4°C, 5 min), and ~ 50 mL of wine was decanted into sterile 50 mL Schott bottles. Bottles were incubated in a 70°C water bath for exactly 48 hours to maximize EC production, and then stored at 4°C until GC/MS analysis (Section 2.3.5.3).

2.3.5.3 Quantification of EC in wine by solid phase microextraction and GC/MS. A 10 mL sample of heated (70°C - 48 hours) wine was pipetted into a 20 mL sample vial, to which a magnetic stirring bar and 3 g of NaCl were added. The vial was capped with a PTFE/silicone septum, placed on a stirrer at 22°C, and allowed to equilibrate, while stirring, for 15 min. A Solid Phase Microextraction (SPME) fibre [65 µm Carbowax/Divinylbenzene (CW/DVB)] was conditioned at 250°C for 30 min before use. After sample equilibration, the fibre was inserted into the head space. After 30 min, the fibre was removed from the sample vial and inserted into the injection port for 15 min. A blank run was performed before each sample run. Quantification was done as follows: an ethyl carbamate (Sigma-Aldrich) standard stock solution (0.1 mg/mL) was prepared in distilled H_20 containing 12% (v/v) ethanol and 1 mM tartaric acid at pH 3.1. Calibration standards were prepared with ethyl carbamate concentrations of 5, 10, 20, 40, 90 µg/L. The standard solutions were stored in the refrigerator at 4°C.

Ethyl carbamate in wine was quantified using an Agilent 6890N GC interfaced to a 5973N Mass Selective Detector. A 60 m x 0.25 mm ID, 0.25 µm thickness DBWAX fused silica open tubular column

(J&W Scientific, Folsom, CA, USA) was employed. The carrier gas was ultra high purity helium at a constant flow of 36 cm/s. The injector and transfer line temperature was set at 250°C. The oven temperature was initially set at 70°C for 2 min then raised to 180°C at 8°C /min and held for 3 min. The temperature was then programmed to increase by 20°C /min to 220°C where it was held for 15 min. The total run time was 35.75 min. The injection mode was splitless for 5 min (purge flow: 5 mL/min, purge time: 5 min). The MS was operated in Selected Ion Monitoring (SIM) mode with electron impact ionization; MS quad temperature 150°C and MS source temperature 230°C. The solvent delay was 8 min. Specific ions 44, 62, 74, 89 were monitored with a dwell time of 100 msec. Mass 62 was used for quantification against the mass spectrum of the authentic ethyl carbamate standard. Wines were analyzed by three separate injections and the data were averaged.

2.4 Genetic construction of the urea importing yeasts $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$

2.4.1 Construction of the *DUR3* linear cassette

In order to express *DUR3* constitutively, a cassette similar to the *DUR1,2* cassette previously made by our group (Coulon, et al. 2006) was constructed. All cloning steps and reactions, unless otherwise stated, were performed according to standard molecular biology standards methods (Ausubel, et al. 1995). All PCR reactions, unless otherwise indicated, were performed using iProof™ High Fidelity DNA polymerase (BioRad, USA) as per the manufacturer's instructions.

2.4.1.1 Construction of pHVX2D3. In order to place *DUR3* under the control of the constitutive *PGK1* promoter and terminator signals, the *DUR3* ORF was cloned into pHVX2 (Volschenk, et al. 1997) (Figure 10). The *DUR3* ORF was amplified from 522 genomic DNA using the following primers which contained *Xho1* restriction enzyme sites built into their 5' ends:

1. DUR3forXho1 (5'-AAAACTCGAGATGGGAGAATTTAAACCTCCGCTAC-3')
2. DUR3revXho1 (5'-AAAACTCGAGCTAAATTATTTCATCAACTTGTCCGAAATGTG-3').

Following PCR, 0.8% agarose gel visualization, and PCR cleanup (Qiagen, USA – PCR Purification Kit), both the PCR product (insert) and pHVX2 (vector) were digested with *Xho1* (Roche, Germany). After the digested vector was treated with SAP (Fermentas, USA) to prevent re-circularization, the insert and linearized-SAP treated vector were ligated overnight at 22°C (T4 DNA Ligase – Fermentas, USA); the

ligation mixture (5 µL) was used to transform DH5α™ competent cells (Invitrogen, USA) that were subsequently grown on 100 µg/mL Ampicillin (Fisher, USA) supplemented LB (Difco, USA) plates. Plasmids from a random selection of transformant colonies were harvested (Qiagen, USA – QIAprep Spin Miniprep kit) and digested by *EcoR1* (Roche, Germany); PCR, using inside-outside primers, was done to identify plasmids with the desired insert.

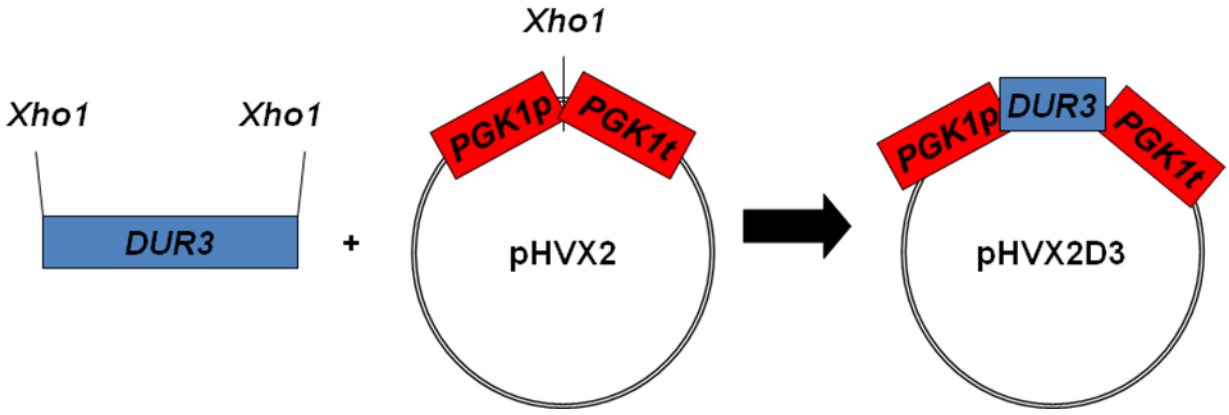


Figure 10. Schematic representation of cloning strategy for creation of pHVX2D3. The *DUR3* ORF was PCR amplified from 522 genomic DNA and ligated into the *Xho1* site of pHVX2.

2.4.1.2 Construction of pHVXKD3. A *kanMX* marker was obtained from pUG6 (Guldener, et al. 1996) via double digestion with *Xho1* and *Sal1* (Fermentas, USA). Following digestion, the 1500 bp *kanMX* band was gel purified (Qiagen, USA – Gel Extraction Kit) and ligated into the *Sal1* site of linearized-SAP treated pHVX2D3. The ligation mixture (5 µL) was used to transform DH5α™ competent cells which were grown on LB-Ampicillin (100 µg/mL). Recombinant plasmids (Figure 11) were identified by *HindIII* (Roche, Germany) digestion of plasmids isolated from 24 randomly chosen colonies.

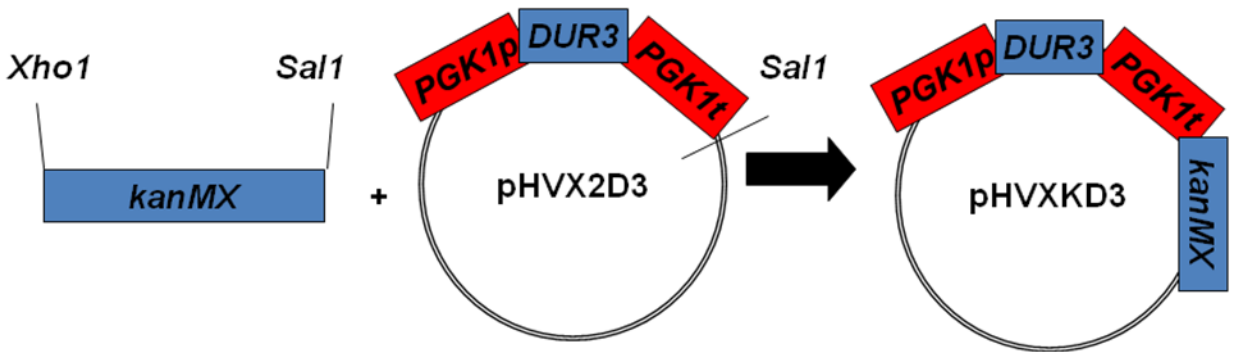


Figure 11. Schematic representation of cloning strategy for creation of pHVXKD3. The *kanMX* marker was obtained from *Xho1*/*Sal1* digestion of pUG6 and ligated into the *Sal1* site of pHVXKD3.

2.4.1.3 Construction of pUCTRP1. The *TRP1* coding region was PCR amplified from 522 genomic DNA using *TRP1* specific primers, each containing *BamH1* (bold) and then *Apa1* (underline) sites at their 5' ends: BamH1Apa1TRP1ORFfwd (5'-AAAAAA**GGATCC**AAAAAAGGGCCCATGTCTGTTATTAATTTCACAGG-3'); BamH1Apa1TRP1ORFrev (5'-AAAAAA**GGATCC**AAAAAAGGGCCCCTATTTCTTAGCATTTTTGACG-3').

Following amplification, cleanup, and quantification, the ~750 bp fragment was ligated into the *BamH1* (Roche, Germany) site of linearized-SAP treated pUC18 (Figure 12). Recombinant plasmids were identified primarily through blue/white screening (growth on LB-Ampicillin supplemented with 50 µg/mL Xgal) and subsequently confirmed through *HindIII*/*EcoR1* digestion.

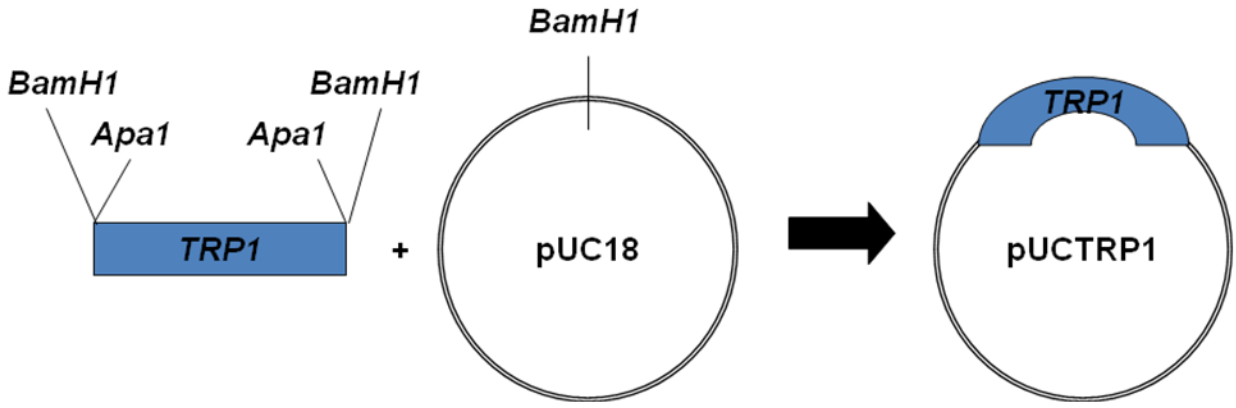


Figure 12. Schematic representation of cloning strategy for creation of pUCTRP1. The *TRP1* ORF was PCR amplified from 522 genomic DNA and ligated into the *BamH1* site of pUC18.

2.4.1.4 Construction of pUCMD. The *PGK1*p-*DUR3*-*PGK1*t-*kanMX* cassette located within pHVXKD3 was amplified from pHVXKD3 plasmid DNA using cassette specific primers: 1. pHVXKfwdlong (5'- CTGGCACG ACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG-3'); pHVXKrevlong (5'- CTGGCGAAAGGGGGATGTGCTGCAA GGCGATTAAGTTGGG-3'). Following amplification, cleanup, and quantification, the ~6500 bp blunt end PCR generated fragment was treated with polynucleotide kinase (New England Biolabs, USA) in order to facilitate ligation (O/N at 22°C) into the blunt *EcoRV* (Fermentas, USA) site of linearized-SAP treated pUCTRP1.

Recombinant plasmids (Figure 13) were initially identified using E-lyse analysis (Eckhardt 1978) and later confirmed via *Apa1* (Stratagene, USA) /S*al1* digestion. Briefly, E-lyse efficiently screens large numbers of colonies for the presence of plasmid DNA by lysing the colonies within the wells of an agarose gel, followed by electrophoresis (Eckhardt 1978). More specifically, after patching onto selective media, small aliquots of colonies were suspended in 5 µL TBE buffer and then mixed with 10 µL SRL buffer (25% v/v sucrose, 50 µg/mL RNaseA, 1 mg/mL lysozyme). After mixing by pipetting, cell suspensions were loaded into the wells of a 0.2% (w/v) SDS - 0.8% (w/v) agarose gel. After the cell suspension in the wells had become clear indicating cell lysis (~ 30 min), the DNA was electrophoresed at 20 V for 45 min, then at 80 V for 45 min. Finally the gel was stained as required with SYBR™ Safe (Invitrogen, USA).

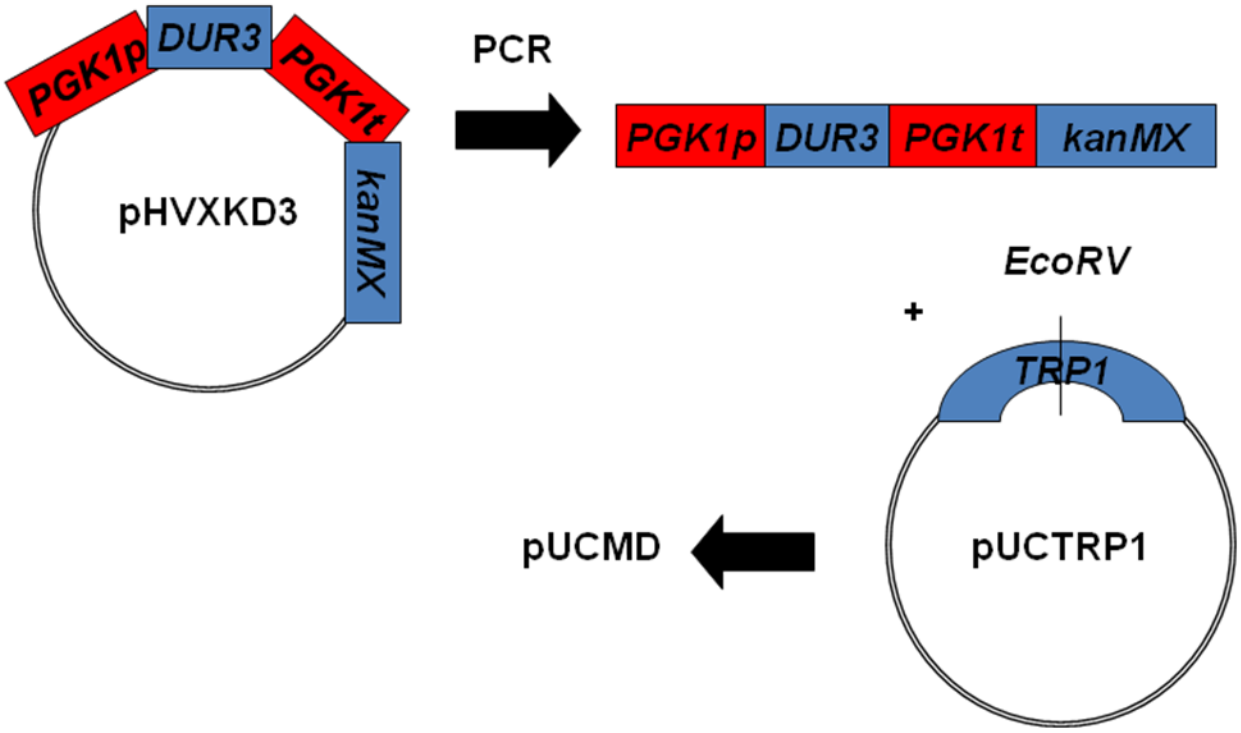


Figure 13. Schematic representation of cloning strategy for creation of pUCMD. The linear *PGK1*p-*DUR3*-*PGK1*t-*kanMX* construction was PCR amplified from pHVXKD3 and blunt end cloned into the *EcoRV* site of pUCTRP1 to yield pUCMD.

2.4.2 Sequence analysis of the *DUR3* cassette in pUCMD

Plasmid pUCMD (Figure 14) was isolated from *E. coli* (Qiagen, USA – QIAprep Spin Miniprep kit) and used directly as a sequencing template. Sequencing was performed by the Nucleic Acid Protein Service Unit (NAPS) at The University of British Columbia using an Applied Biosystems PRISM 377 sequencer and Applied Biosystems BigDye v3.1 sequencing chemistry. Primers and template were supplied to NAPS in the concentrations specified by their sample submission requirements. The entire *DUR3* cassette, beginning from the 5' *TRP1* flanking sequence and ending with the 3' *TRP1* flanking sequence, was sequenced via 16 different sequencing reads (Table 6) and later assembled *in silico* using Accelrys DS Gene v1.1 software. The assembled sequences were aligned against previously published sequences and those of *DUR3*, *TRP1*, $PGK1_p$, and $PGK1_t$ obtained from SGD. If any discrepancies were found, the specific read which gave rise to the discrepancy was repeated in order to identify bona fide mutations.

Table 7. Oligonucleotide primers used in sequencing of *DUR3* cassette in pUCMD.

Primer	Primer Name	Sequence (5'→3')
P1	BamH1TRP1Apa1Fwd	5'-AAAAAAGGATCCAAAAAAGGGCCCATGTCTGTTATTAATTTCACAGG-3'
P2	pHVXKlongfwd	5'-CTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAG-3'
P3	pHVXKfwd	5'-CTGGCACGACAGGTTTCCCGACTGG-3'
P4	pDUR3tfwd	5'-TTTCCGCGGAGCTTTCTAACTGATCTATCC-3'
P5	DUR3Xho1fwd	5'-AAAACTCGAGATGGGAGAATTTAAACCTCCGCTAC-3'
P6	DUR3Xho1rev	5'-AAAACTCGAGCTAAATTATTTCATCAACTTGTCCGAAATGTG-3'
P7	pDUR3trev	5'-TTTCCGCGGTGCGGTGTGAAATACC-3'
P8	kanMXORFrev	5'-TTAGAAAAACTCACTGAGCATCAAATGAAACTGC-3'
P9	kanMXORFfwd	5'-ATGGGTAAGGAAAAGACTCACGTTTCGAGG-3'
P10	pHVXKlongrev	5'-CTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGG-3'
P11	BamH1TRP1Apa1rev	5'-AAAAAAGGATCCAAAAAAGGGCCCCTATTTCTTAGCATTTTTGACG-3'
P12	DUR3RTfwd	5'-GATCGGCCATGGTTGCTACTT-3'
P13	RevPGKtPst1	5'-TTTTCTGCAGAAGCTTTAACGAACGCAGAATT-3'
P14	PGKpro1	5'-ACAAAATCTTCTTGACAAACGTCACAA3'
P15	PGKpro2	5'-AATTGATGTTACCCTCATAAAGCACGT-3'
P16	PGKforDUR	5'-TGGTTTAGTTTAGTAGAACCTCGTGAAACTTAC-3'

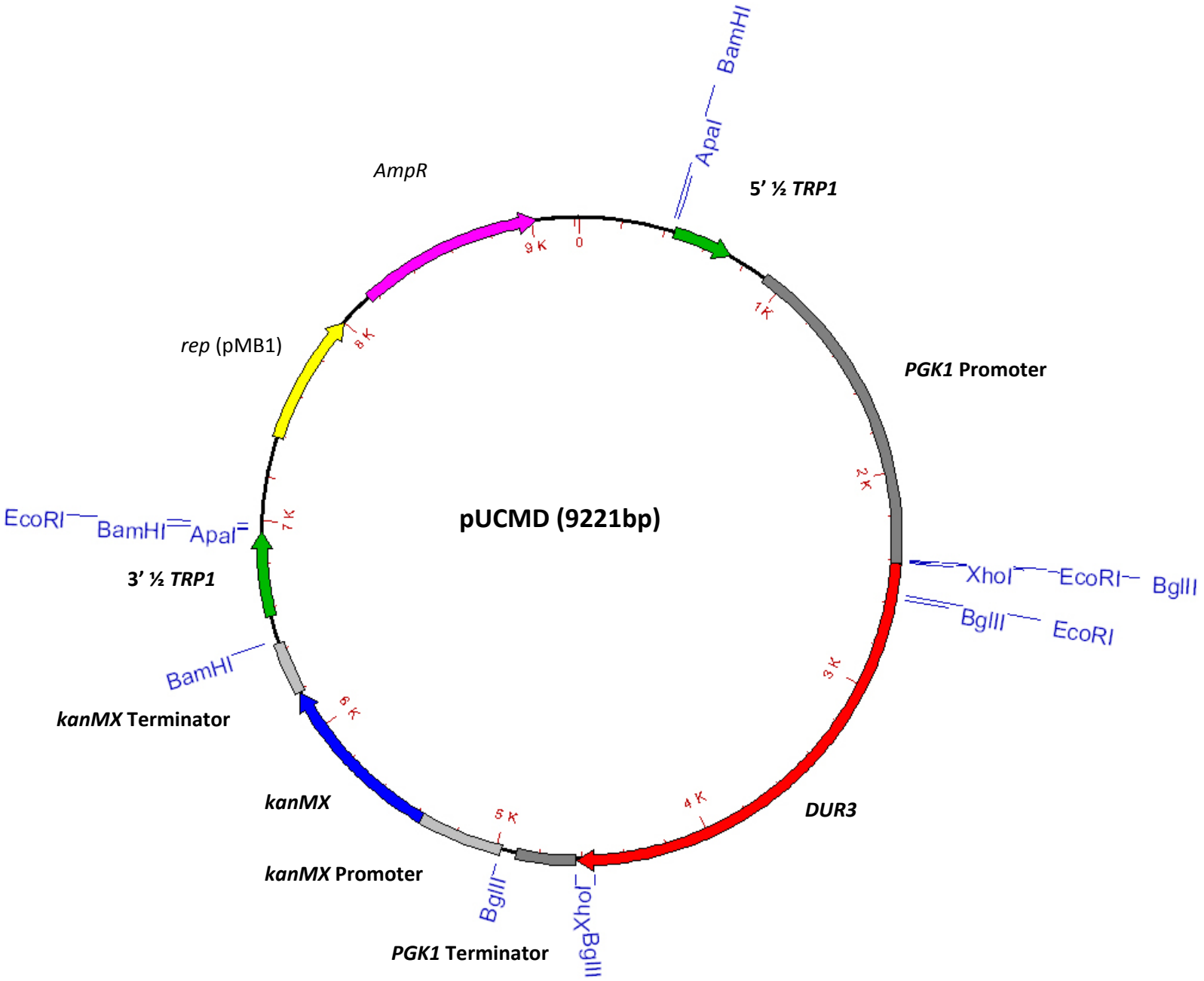


Figure 14. Schematic representation of pUCMD. The *DUR3* linear cassette stretches between *Apa1* sites encompassing 5'1/*2TRP1-PGK1p-DUR3-PGK1t-kanMXp-kanMX-kanMXt*-3'1/2*TRP1*.

2.4.3 Transformation of the linear *DUR3* cassette into *S. cerevisiae* and selection of transformants

The 6536 bp *DUR3* cassette was cut from pUCMD using *Apa1* (Stratagene, USA) (Ausubel, et al. 1995) and visualized on a 0.8% agarose gel. From the gel, the expected 6536 bp band was resolved and extracted (Qiagen, USA – Gel extraction kit). After extraction, clean up, and quantification using a Nanodrop ND-1000 spectrophotometer (Nanodrop, USA), 250 ng of linear cassette was used to transform *S. cerevisiae* strains K7, $K7^{EC-}$, 522, and 522^{EC-}. Yeast strains were transformed using the lithium acetate/polyethylene glycol/ssDNA method (Gietz and Woods 2002). Following transformation, cells were left to recover in YPD at 30°C for 2 hours before plating on to YPD plates supplemented with 300 µg/mL G418 (Sigma, USA). Plates were incubated at 30°C until colonies appeared.

2.4.3.1 Confirmation of integration via colony PCR. Colony PCR was used as previously described to detect the presence of the linear *DUR3* cassette integrated into the yeast genome at the *TRP1* locus (Ward 1992). Zymolyase 100 U/mL (Seikagaku Corp., Japan) was used to lyse the cells (30 µl zymolyase solution). Primers kanMXORFfwd (5'-ATGGGTAAGGAAAAGACTCACGTTTCGAGG-3') and kanMXORFrev (5'-TTAGAAAAACTCATCGAGCATCAAATGAAACTGC-3') were used to generate an 809 bp fragment from the 3' end of the cassette. 522 genomic DNA was used as a negative control and pUCMD as a positive control. PCR was performed with iProof™ High Fidelity DNA polymerase (BioRad, USA) using suggested reagent concentrations and 1 µl of Zymolyase treated cell supernatant as template. The PCR program was as follows: 1. Initial denaturation – 3 min at 98°C. 2. Denaturation – 10 sec at 98°C. 3. Annealing – 20 sec at 62.4°C. 4. Extension – 30 sec at 72°C. 5. Cycle to step 2, 30 times. 6. Final extension – 10 min at 72°C. Colony PCR reactions were visualized on 0.8% agarose gels stained with SYBR™ Safe (Invitrogen, USA).

2.4.4 Genetic characterization

2.4.4.1 Southern blot analyses. Southern blotting was used to confirm integration of the *DUR3* cassette into the *TRP1* locus. Genomic DNA from engineered strains $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3}, as well as their respective parent strains, was digested with *EcoRI* (Roche, Germany) (Ausubel, et al. 1995), and separated on a 0.8% agarose gel. Following gel preparation, transfer and fixing to a positively charged Nylon membrane (Roche Diagnostics, Germany) (Ausubel, et al. 1995), the blots 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 detection (Amersham Biosciences, England).

The 661 bp *DUR3* probe was generated using genomic DNA from *S. cerevisiae* strain 522 as a template and the primers DUR3probefwd (5'- CAGCAGAAGAATTCACCACCGCCGGTAGATC-3') and DUR3proberev (5'- CAATCAGGTTAATAATTAATAAAATACCAGCGG-3'). The 461 bp *TRP1* probe was generated using genomic DNA from 522 as a template and the primers TRP1probefwd (5'- TTAATTTCACAGGTAGTTCTGGTCCATTGG-3') and TRP1proberev (5'- CAATCCAAAAGTTCACCTGTCCCACCT GCTTCTG-3').

2.4.4.2 Analysis of gene expression by northern blotting. Fermentations with K7, $K7^{EC-}$ $K7^{D3}$, and $K7^{EC-D3}$ in filter sterilized Calona Chardonnay must were conducted as in Section 2.3.3.3. After 24 hours, cells were harvested by centrifugation (5000 rpm, 4°C, 4 min), snap frozen in liquid nitrogen (3 min), and stored at -80°C until RNA extraction. Total RNA was extracted using a hot phenol method (Ausubel, et al. 1995), quantified on a NanoDrop ND-1000 spectrophotometer and visualized on a 0.8% agarose gel.

Northern blot analysis was performed as described (Ausubel, et al. 1995). Briefly, 30 µg total RNA was separated on a 1% agarose-formaldehyde denaturing gel and transferred to a positively charged Nylon membrane (Roche Diagnostics, Germany). Blots were probed with PCR generated fragments specific for *DUR3* and the loading control *HHF1*. The AlkPhos™ Direct Nucleic Acid Labeling and CDP-Star Detection system was used as recommended for probe detection (Amersham Biosciences, England).

The 661 bp *DUR3* probe used for northern blotting was the same as the probe used for Southern blotting (Section 2.4.4.1). The ~500 bp *HHF1* probe was supplied by another member of our lab (Coulon, et al. 2006).

2.4.4.3 Analysis of *DUR3* gene expression by qRT-PCR. Gene expression analysis of K7, $K7^{EC-}$ $K7^{D3}$, and $K7^{EC-D3}$ was performed as described in Section 2.3.3.3 using total RNA from 24 hour fermentations (Section 2.4.4.2). The *DUR3* real time PCR product was generated using the primers DUR3RTfwd (5'-GATCGGCCATGGTTGCTACTT-3') and DUR3RTrev (5'-GCGATAGTGTTCATCCCGGTT-3').

2.4.4.4 Global gene expression analysis. Following 24 hour fermentations (Section 2.4.4.2), total RNA from strains K7 and $K7^{D3}$ was used for transcriptome analysis as described in Section 2.3.3.4.

2.4.5 Analysis of urea uptake using ^{14}C-urea

In order to assess the effect of *DUR3* constitutive expression on urea uptake activity, a ^{14}C-urea uptake assay was performed as previously described (Cooper and Sumrada 1975). Briefly, appropriate strains were grown (30°C – 180 RPM) in minimal media (1.7 g/L YNB w/o amino acids w/o ammonium sulfate, 20 g/L glucose, 1 g/L ammonium sulfate or 1 g/L L-proline) to approximately $1x10^7$ cells/mL. An 11 mL sample of cell culture was transferred to a 250 mL Erlenmeyer flask containing 80 µL of 36.6 mM ^{14}C-urea (Sigma, USA – 6.8 mCi/mmol). Cells were then cultured (30°C – 180 RPM) for 20 min and 1 mL samples were taken every 2 min. The 1 mL samples were applied to 0.22 µm nylon filters (Millipore, USA) and washed with 25 mL aliquots of cold minimal media to which 10 mM urea (Fisher, USA) had been added. Filters were placed in scintillation vials, filled with scintillation fluid (Fisher, USA) and left overnight to equilibrate. Samples were then counted in a recently calibrated Beckman LS6000IC liquid scintillation counter using the counter's factory '14C quench' mode. DPM values were then converted into nano-mole urea transported (Cooper and Sumrada 1975) and plotted against time in Excel.

2.4.6 Phenotypic characterization

2.4.6.1 Analysis of fermentation rate in Chardonnay must. Fermentations of unfiltered Calona Chardonnay must with K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} were performed as described in Section 2.3.4.1.

2.4.6.2 Analysis of fermentation rate in Sake mash. Fermentations of Sake rice mash with K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} were performed as described in Section 2.3.4.2.

2.4.6.3 Analysis for ethanol content. Ethanol production by K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} in Chardonnay and Sake wine was quantified as described in Section 2.3.4.3.

2.4.7 Functionality of metabolically enhanced yeasts

2.4.7.1 Reduction of EC in Chardonnay wine. Chardonnay wine was produced with K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} as described in 2.3.4.1. Quantification of EC reduction was performed as described in Section 2.3.5.1.

2.4.7.2 Reduction of EC in Sake wine. Sake wine was produced with K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} as described in 2.3.4.2. Quantification of EC reduction was performed as described in Section 2.3.5.2.

2.5 Statistical analyses

Two factor ANOVA analyses were used to evaluate the variations in glucose, fructose, and ethanol measured in Chardonnay and Sake wine produced by parental and engineered yeast strains. Fisher's LSD (Least Significant Difference) test was used after ANOVA to determine which means were statistically significant ($p<0.05$). All statistical calculations were performed in Excel 2007 (Microsoft, USA).

3 RESULTS

3.1 Constitutive expression of *DUR1,2* in Sake yeast strains K7 and K9

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

In order to constitutively express *DUR1,2*, the Sake yeast strains K7 and K9 were transformed with the linear *DUR1,2* cassette (Figure 15). After colony PCR screening of approximately 1500 yeast transformants for the integration of the *DUR1,2* cassette, two metabolically engineered strains, $K7^{EC-}$ and $K9^{EC-}$, were obtained. The designation 'EC-' denotes integration of the linear *DUR1,2* cassette into the *URA3* locus.

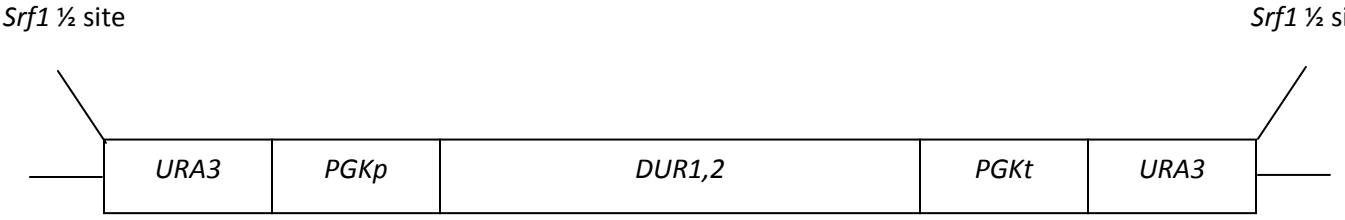


Figure 15. Schematic representation of the linear *DUR1,2* cassette.

3.1.2 Genetic characterization of $K7^{EC-}$ and $K9^{EC-}$

3.1.2.1 Correct integration of the *DUR1,2* linear cassette into the genomes of $K7^{EC-}$ and $K9^{EC-}$. In order to confirm the correct integration of the *DUR1,2* cassette, a Southern blot was performed using PCR generated *DUR1,2* and *URA3* probes and the *BglII* digested genomic DNA of $K7^{EC-}$ and $K9^{EC-}$.

When probed with *DUR1,2*, two signals were detected for $K7^{EC-}$ and $K9^{EC-}$ corresponding to 5.0 kb and 9.0 kb DNA fragments, and one signal was detected for K7 and K9 corresponding to a 5.0 kb DNA fragment (Figure 16a). The 5.0 kb fragment matches the expected fragment size for the native *DUR1,2* locus while the 9.0 kb fragment matches the expected fragment size for the presence of the *DUR1,2* cassette integrated into the *URA3* locus (Figure 16b).

Probing with *URA3* gave rise to two signals for K7^{EC-} and K9^{EC-}, corresponding to 3.8 kb and 4.4 kb DNA fragments, and one signal for K7 and K9 corresponding to a 4.4 kb fragment (Figure 17a). The 3.8 kb fragment matches the expected fragment size for the recombinant *URA3* locus, and the 4.4 kb fragment matches the expected fragment size for a non-disrupted *URA3* locus (Figure 17b).

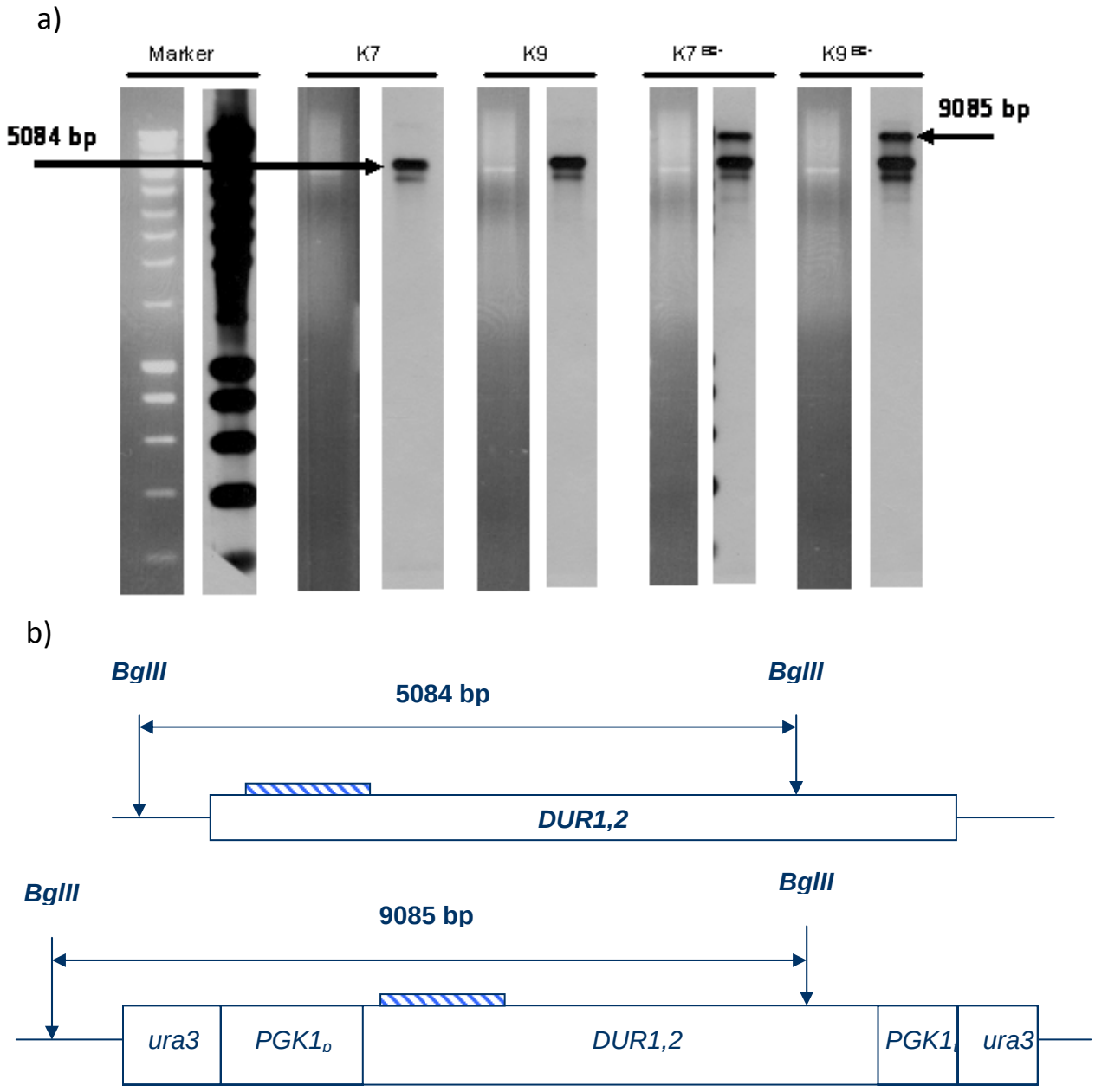


Figure 16. Integration of the *DUR1,2* cassette into the *URA3* locus of K7^{EC-} and K9^{EC-} was confirmed by Southern blot analyses using a *DUR1,2* probe (a). All faint bands that do not correspond to a size in the schematic representation were deemed to be non-specific. For each sample, the agarose gel is shown on the left while the exposed film is on the right. b) Schematic representation of the signals expected during Southern blot analyses (*DUR1,2* probe) of recombinant yeasts containing the recombinant *DUR1,2* cassette integrated into the *URA3* locus. The *DUR1,2* probe is illustrated with blue hatched lines (b).

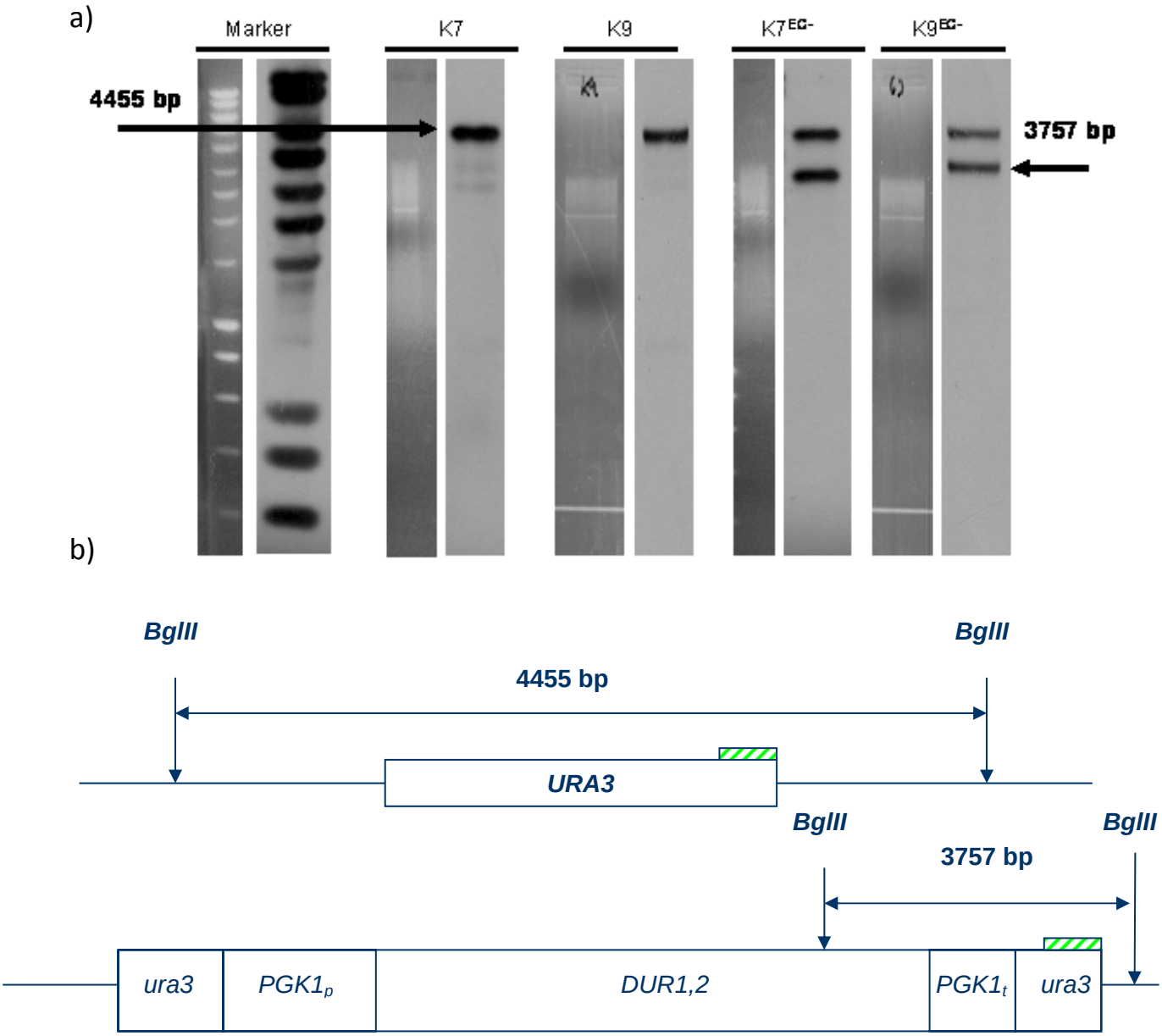


Figure 17. Disruption of the *URA3* locus by integration of the *DUR1,2* cassette in $K7^{EC-}$ and $K9^{EC-}$ was confirmed by Southern blot analyses using a *URA3* probe (a). All faint bands that do not correspond to a size in the schematic representation were deemed to be non-specific. For each sample, the agarose gel is shown on the left while the exposed film is on the right. b) Schematic representation of the signals expected during Southern blot analysis (*URA3* probe) of recombinant yeasts containing the recombinant *DUR1,2* cassette integrated into the *URA3* locus. The *URA3* probe is illustrated with green hatched lines (b).

3.1.2.2 Sake strains K7^{EC-} and K9^{EC-} do not contain the *bla* and Tn5*ble* antibiotic resistance markers. After transformation, K7^{EC-} and K9^{EC-} were successively sub-cultured on a non-selective medium in order to eliminate pUT332, whose only purpose was to facilitate early screening for transformants. A Southern blot using probes specific for *bla* (Ampicillin resistance) and Tn5*ble* (Phleomycin resistance) revealed that these genes were absent from K7^{EC-} and K9^{EC-}, as well as the parental strains K7 and K9 (Figure 18).

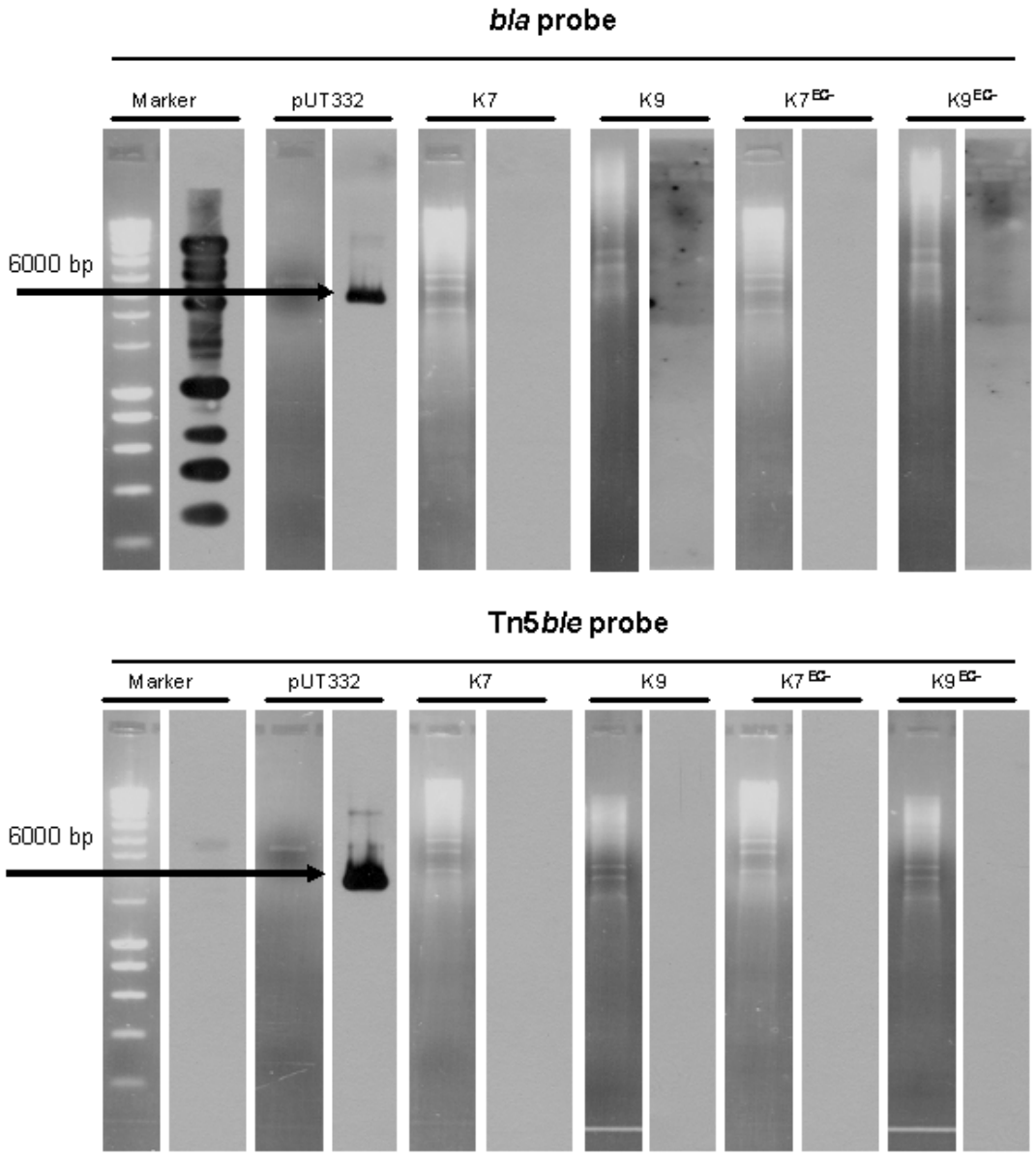


Figure 18. The genetically engineered strains K7^{EC-} and K9^{EC-} do not contain the *bla* and Tn5*ble* antibiotic resistance markers. The plasmid pUT332 was used as a positive control for both *bla* and Tn5*ble*. For each sample, the agarose gel is shown on the left while the exposed film is on the right.

3.1.2.3 Sequence of the *DUR1,2* cassette integrated into the genomes of K7^{EC-} and K9^{EC-}. To verify the sequence of the *DUR1,2* cassette integrated into the *URA3* locus, single strand DNA sequencing of the cassette in K7^{EC-} and K9^{EC-} was completed. *In silico* sequence assembly and subsequent analysis revealed one nucleotide in the cassette sequence of K9^{EC-} that did not match the previously reported recombinant *DUR1,2* sequences and/or SGD's S288C *DUR1,2* sequence (Table 8). 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 due to a genetic polymorphism between the Sake strain K9 and the laboratory strain S288C. Comparison of the recombinant *DUR1,2* ORF sequence in K7^{EC-} and K9^{EC-} and that of SGD revealed no nucleotide changes or amino acid substitutions. A detailed description of the DNA sequences that comprise the *DUR1,2* cassette is given in Table 9.

Table 8. Discrepancies between the integrated *DUR1,2* cassette of K9^{EC-} and published sequences.

Nucleotide position	Description
821	Difference in the 5' region of the *URA3* open reading frame of K9^{EC-} C→T

Table 9. Detailed description of the DNA sequences that comprise the *DUR1,2* cassette.

Nucleotide position	Description
1-4	**5' *SRF1 ½* site**
5-950	***URA3* sequence**
5-508	5' non coding sequence
509-950	5' part of *URA3* ORF
951-2445	***PGK1* promoter**
2446-7953	***DUR1,2* ORF**
7954-8235	***PGK1* terminator**
8236-9187	***URA3* sequence**
8236-8640	3' part of *URA3* ORF
8641-9187	3' non coding sequence
9188-9191	**3' *SRF1 ½* site**

In silico analysis of the integrated *DUR1,2* cassette revealed that two new ORFs were created during construction of the *DUR1,2* cassette; these ORFs were composed entirely of *S. cerevisiae* sequences (Figure 19). Novel ORF1 (447 bp) is located at nucleotide position 509-955 while novel ORF2 (792 bp) is located at position 767-1558.

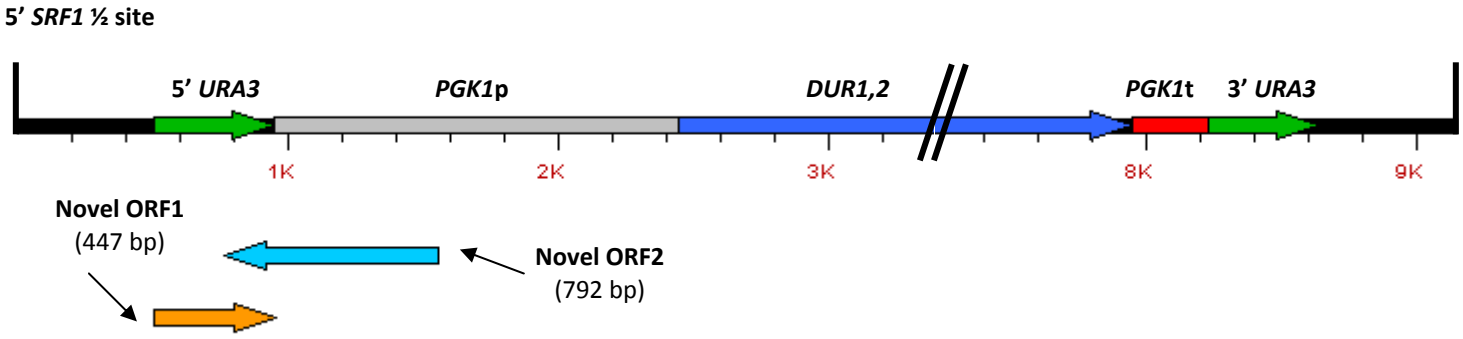


Figure 19. A schematic representation of new ORFs of more than 100 codons generated during construction of the *DUR1,2* cassette. Two new ORFs, entirely composed of *S. cerevisiae* sequences, were created.

3.1.2.4 Confirmation of constitutive expression of *DUR1,2* in $K7^{EC-}$ and $K9^{EC-}$ by qRT-PCR. Total RNA from yeast cells in 24 hour fermentations of Chardonnay must was used to confirm and quantify constitutive expression of *DUR1,2* in $K7^{EC-}$ and $K9^{EC-}$. Integration of the *DUR1,2* cassette in $K7^{EC-}$ and $K9^{EC-}$ up regulated *DUR1,2* expression by 9.13-fold and 12.77-fold, respectively, compared to the parental strains K7 and K9 (Figure 20). Expression of *DUR1,2* in $K7^{EC-}$ and $K9^{EC-}$ was detected in non-inducing (NCR) conditions indicating that the *PGK1* promoter and terminator signals are effective at overcoming repression by NCR during fermentation.

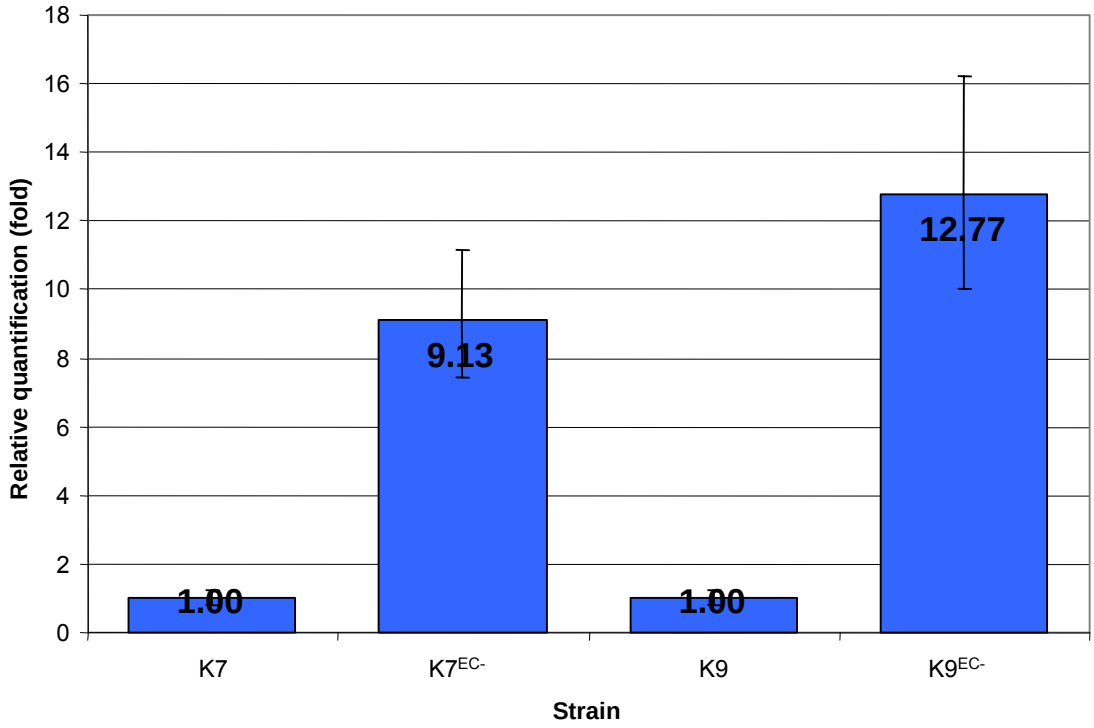


Figure 20. Gene expression analysis (qRT-PCR) of K7, $K7^{EC-}$, K9, and $K9^{EC-}$ indicates functionality of the *DUR1,2* cassette and constitutive expression of *DUR1,2* in non-inducing (NCR) conditions. Total RNA was extracted from yeast cells harvested after 24 hour fermentation (20°C) in filter sterilized Calona Chardonnay must that was inoculated to a final OD_{600} = 0.1. Total RNA was subsequently reverse transcribed, and the resultant cDNA was amplified in the presence of SYBR green dye. *DUR1,2 g*ene expression was standardized to *ACT1* expression and data for strains $K7^{EC-}$ and $K9^{EC-}$ were calibrated to their respective parental strain K7 and K9. Fermentations were conducted in triplicate and the data averaged; error bars represent 95% confidence intervals.

3.1.2.5 Effect of the integrated *DUR1,2* cassette on the transcriptomes of $K7^{EC-}$ and $K9^{EC-}$. Total RNA from yeast cells in 24 hour fermentations of Chardonnay must was used analyze the impact of the integrated *DUR1,2* cassette on global gene expression in $K7^{EC-}$. Reported changes in gene expression were cut off at a minimum 4-fold change in expression ($SLR_{Avg} \geq 2$ or $SLR_{Avg} \leq -2$) to ensure elimination of experimental noise inherent in microarray analysis; this cut-off is supported by the previously published statistical examination of a wine yeast's transcriptome during fermentation (Marks, et al. 2008).

Integration of the *DUR1,2* cassette into the *URA3* locus of $K7^{EC-}$ had a minimal effect on the yeast's transcriptome. *DUR1,2* was upregulated by 6.35-fold in $K7^{EC-}$; *URA3* was downregulated by 2.35-

fold but was not included in Table 10 as it fell below the 4-fold cut off. Besides *DUR1,2*, two genes were upregulated greater than 4-fold in K7^{EC-} (Table 10); seven genes were downregulated more than 4-fold in K7^{EC-}. No metabolic pathways were affected by the presence of the integrated *DUR1,2* cassette; however, integration of the *DUR1,2* cassette downregulated three unrelated genes involved in meiosis/sporulation (*RME1*, *SSP1*, *SDS3*) indicating a possible negative effect on sporulation efficiency (Table 10).

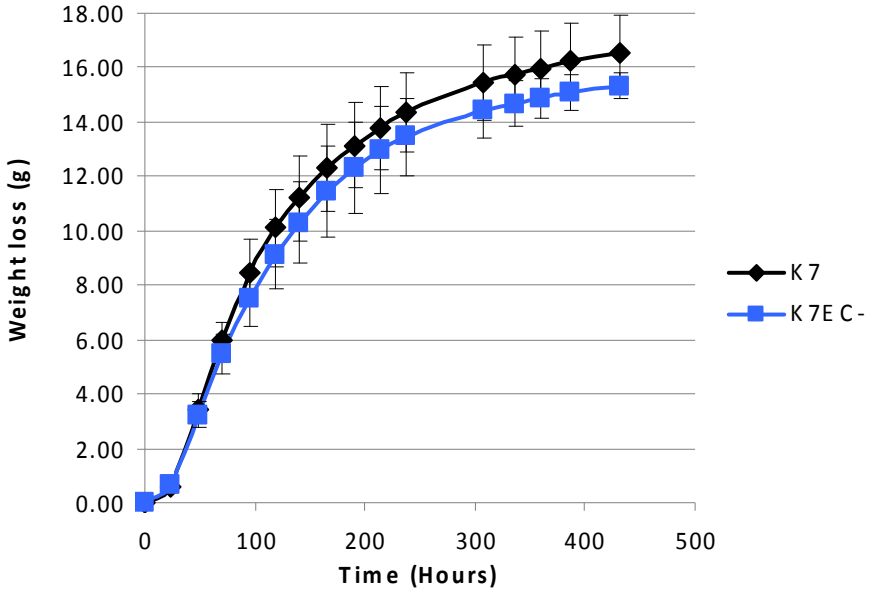
Table 10. Effect of the integrated *DUR1,2* cassette in the genome of K7 on global gene expression patterns in *S. cerevisiae* K7^{EC-} (≥ 4-fold change). Reported changes are relative to the parental strain K7. Total RNA from K7 and K7^{EC-}, harvested at 24 hours into fermentation of filter sterilized Chardonnay must, were used for hybridization to microarray. Fermentations were conducted in duplicate and the data were averaged (p≤0.005).

Genes expressed at higher levels in K7^{EC-}		
Fold Change	**Gene Symbol**	**Biological Process**
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^{EC-}		
Fold Change	**Gene Symbol**	**Biological Process**
-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 deactylase 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

3.1.3 Phenotypic characterization of K7^{EC-} and K9^{EC-}

3.1.3.1 Fermentation rate of K7^{EC-} and K9^{EC-} in Chardonnay must. Fermentation profiles of the parental and metabolically engineered Sake yeasts are shown in Figures 21a,b. As is common in grape must, fermentations were robust and rapid (~400 hours). The fermentation profiles shown in Figures 21a,b indicate substantial equivalence amongst the parent and engineered strains as far as fermentation rate is concerned.

a)



b)

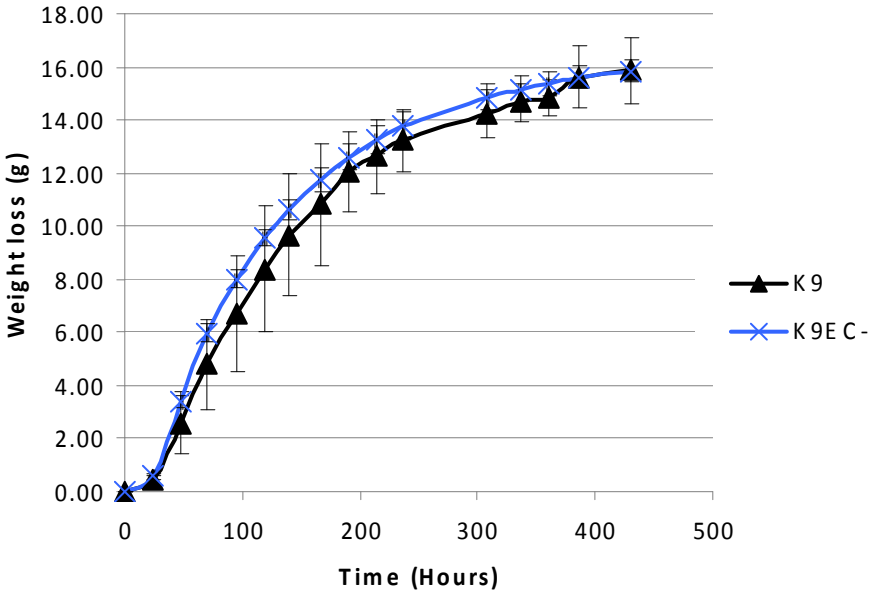
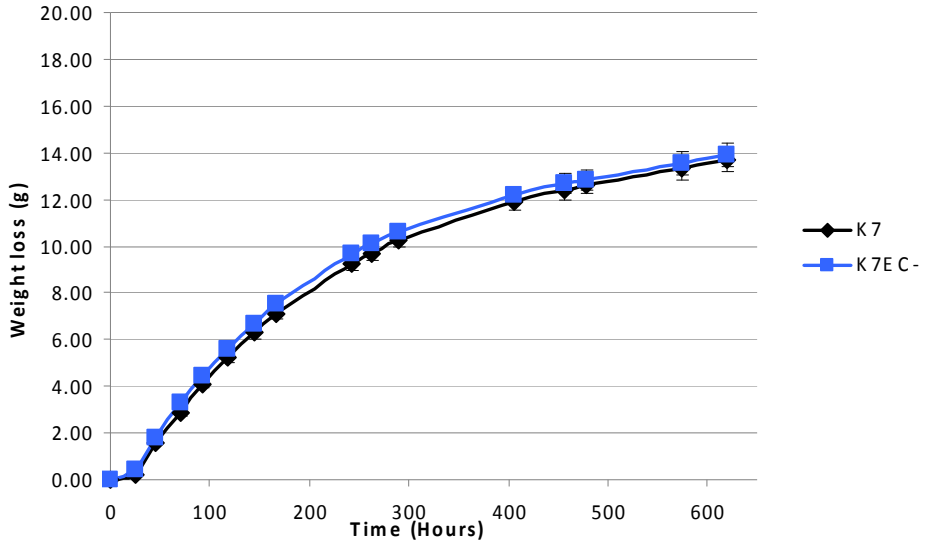


Figure 21. Fermentation profiles (weight loss) of parental and *DUR1,2* engineered Sake strains in Chardonnay wine. Chardonnay wine was produced by inoculating Sake yeast strains (a) K7 and $K7^{EC-}$ and (b) K9 and $K9^{EC-}$ into unfiltered Calona Chardonnay must (final OD_{600} = 0.1). Fermentations were incubated to completion (~400 hours) at 20°C. Fermentations were conducted in triplicate and the data were averaged; error bars indicate one standard deviation.

3.1.3.2 Fermentation rate of $K7^{EC-}$ and $K9^{EC-}$ in Sake mash. Fermentation profiles of the parental and metabolically engineered Sake yeasts are shown in Figures 22a,b. In contrast to the fermentations of

Chardonnay must in Section 3.1.3.1, Sake fermentations were less robust, and slower (~600 hours to completion). The fermentation profiles shown in Figures 22a,b indicate substantial equivalence amongst the parent and engineered strains as far as fermentation rate is concerned.

a)



b)

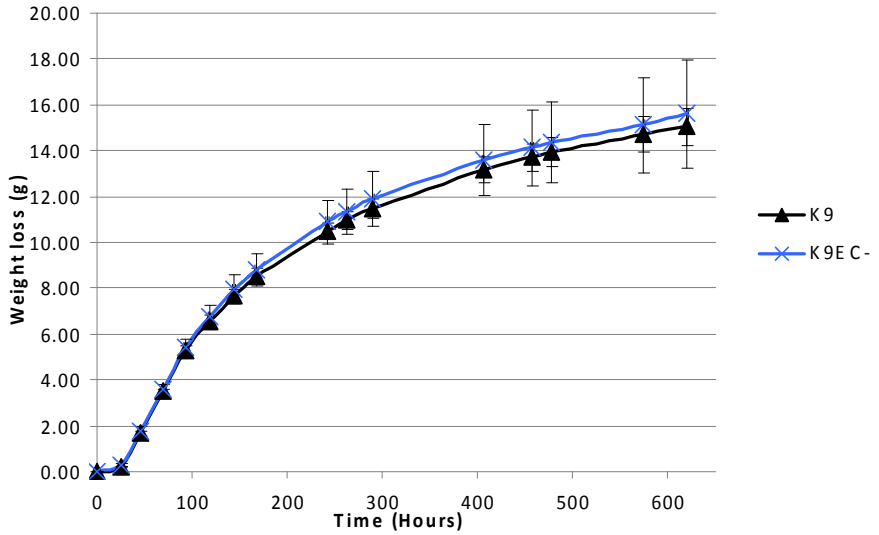


Figure 22. Fermentation profiles (weight loss) of parental and *DUR1,2* engineered Sake strains in Sake wine. Sake wine was produced by inoculation (final OD_{600} = 0.1) of Sake yeast strains (a) K7 and $K7^{EC-}$ and (b) K9 and $K9^{EC-}$ into white rice (Kokako Rose) and koji mash (Vision Brewing). Fermentations were incubated to completion (~600 hours) at 18°C. Fermentations were conducted in triplicate and data averaged; error bars indicate one standard deviation.

3.1.3.3 Utilization of glucose and fructose and production of ethanol by $K7^{EC-}$ and $K9^{EC-}$ in Sake wine.

The effect of the *DUR1,2* cassette on glucose and fructose utilization as well as ethanol production in

strains K7^{EC-} and K9^{EC-} was investigated. Glucose, fructose and ethanol were quantified by LC analysis at the end of fermentation. Compared to their respective parental strains, K7^{EC-} and K9^{EC-} produced Sake wine with substantially equivalent amounts of residual glucose, residual fructose and ethanol (Table 11).

Table 11. Utilization of glucose and fructose and production of ethanol in Sake wine by parental yeast strains (K7 and K9), their metabolically engineered counterparts (K7^{EC-} and K9^{EC-}). Sake wine was produced by inoculation of Sake yeast strains, K7, K7^{EC-}, K9 and K9^{EC-} in white rice (Kokako Rose) and koji mash (Vision Brewing). Fermentations (Figures 22a,b) were incubated to completion (~600 hours) at 18°C. Glucose and fructose (g/L) and ethanol (v/v) were quantified at the end of fermentation. Data were analyzed for statistical significance (p≤0.05) using two factor ANOVA analysis.

	Glucose					
	K7	**K7^{EC-}**	***p****	**K9**	**K9^{EC-}**	***p****
Replicate 1	0.059	0.056	--	0.057	0.102	--
Replicate 2	0.066	0.056	--	0.114	0.103	--
Replicate 3	0.059	0.051	--	0.072	0.090	--
Residual glucose average (n=3)	**0.06**	**0.05**	**ns**	**0.08**	**0.10**	**ns**
STDEV	0.00	0.00	--	0.03	0.01	--

	Fructose					
	K7	**K7^{EC-}**	***p****	**K9**	**K9^{EC-}**	***p****
Replicate 1	0.736	0.733	--	0.333	0.242	--
Replicate 2	0.841	0.752	--	0.271	0.361	--
Replicate 3	0.374	0.665	--	0.338	0.303	--
Residual fructose average (n=3)	**0.65**	**0.72**	**ns**	**0.31**	**0.30**	**ns**
STDEV	0.25	0.05	--	0.04	0.06	--

	Ethanol					
	K7	**K7^{EC-}**	***p****	**K9**	**K9^{EC-}**	***p****
Replicate 1	12.23	12.49	--	12.71	13.22	--
Replicate 2	12.27	12.64	--	13.59	12.71	--
Replicate 3	11.95	11.93	--	13.05	12.17	--
Ethanol average (n=3)	**12.15**	**12.35**	**ns**	**13.12**	**12.70**	**ns**
STDEV	0.17	0.37	--	0.44	0.53	--

* si, ns: significant at p≤0.05, or non-significant

3.1.4 Constitutive expression of *DUR1,2* in Sake yeast strains K7^{EC-} and K9^{EC-} reduces EC in Chardonnay wine by approximately 30%

In order to assess the effect of the *DUR1,2* cassette on EC reduction in Chardonnay wine, K7^{EC-} and K9^{EC-} were used to ferment Chardonnay must, and EC content was measured in the resultant wine.

Fermentation profiles of the parental and metabolically engineered Sake yeasts are shown in Figures 21a,b.

In contrast to the usually high amounts of EC found in Sake wine, during laboratory scale wine fermentations the parental Sake strains K7 and K9 produced relatively low amounts of EC, 64.87 and 56.16 ppm, respectively (Table 12); the engineered strains $K7^{EC-}$ and $K9^{EC-}$ reduced by EC in the Chardonnay wine by 29.88% and 0%, respectively.

Table 12. Reduction of EC by functionally enhanced yeast strains $K7^{EC-}$ and $K9^{EC-}$ during wine making. The concentration of EC (µg/L) in Chardonnay wine produced by Sake yeast strains K7, $K7^{EC-}$, K9, and $K9^{EC-}$ was quantified by GC/MS. Strains were inoculated (final OD_{600} = 0.1) into unfiltered Calona Chardonnay must and fermentations were incubated to completion (~350 hours) at 20°C. Fermentation profiles are given in Figure 21.

Yeast strain	**K7**	**$K7^{EC-}$**	**K9**	**$K9^{EC-}$**
Replicate 1	69.05	46.3	56.13	55.18
Replicate 2	63.62	42.94	--	--
Replicate 3	61.93	47.21	--	--
Average (n=3)	**64.87**	**45.48**	**56.16**	**55.18**
STDEV	3.72	2.25	--	--
RSD (%)	5.73	4.94	--	--
Reduction (%)	**--**	**29.88**	**--**	**~0**

3.1.5 Constitutive expression of *DUR1,2* in Sake yeast strains $K7^{EC-}$ and $K9^{EC-}$ reduces EC in Sake wine by approximately 68%

To assess the effect of the *DUR1,2* cassette on EC reduction in Sake wine, $K7^{EC-}$ and $K9^{EC-}$ were used to ferment Sake rice mash (rice and koji), and EC content was measured in the resultant Sake wine. Fermentation profiles of the parental and metabolically engineered Sake yeasts are shown in Figures 22a,b.

During laboratory scale Sake wine fermentations the parental Sake strains K7 and K9 produced significantly more EC (211.19 and 344.16 ppm, respectively - Table 13) than during wine fermentations (Table 12). In Sake wine, the engineered strains $K7^{EC-}$ and $K9^{EC-}$ reduced by EC by 67.54% and 68.33%, respectively (Table 13), making $K7^{EC-}$ and $K9^{EC-}$ much more effective at EC reduction in Sake wine than in

Chardonnay wine. Given the substantial difference in EC production and reduction by identical strains, it seems imperative that yeast strains be evaluated in their native environment to accurately assess the efficacy of the *DUR1,2* cassette.

Table 13. Reduction of EC by functionally enhanced yeast strains $K7^{EC-}$ and $K9^{EC-}$ during Sake brewing. The concentration of EC (μg/L) in Sake wine, produced by inoculation (final OD_{600} = 0.1) of Sake yeast strains K7, $K7^{EC-}$, K9, and $K9^{EC-}$ into white rice (Kokako Rose) and koji mash (Vision Brewing), was quantified by GC/MS. Fermentations were incubated to completion (~600 hours) at 18°C and fermentation profiles are given in Figure 22.

Yeast strain	**K7**	**$K7^{EC-}$**	**K9**	**$K9^{EC-}$**
Replicate 1	224.11	72.5	241.73	108.92
Replicate 2	198.7	79.28	265.01	105.96
Replicate 3	210.75	53.85	525.74	112.15
Average (n=3)	**211.19**	**68.54**	**344.16**	**109.01**
STDEV	12.71	13.17	157.68	3.10
RSD (%)	6.02	19.22	45.82	2.84
Reduction (%)	**--**	**67.54**	**--**	**68.33**

3.2 Constitutive expression of *DUR3* in the Sake yeast strain K7 and the wine yeast strain 522

3.2.1 Sequence of pUCMD

A multicopy episomal plasmid containing the *DUR3* ORF inserted between the *PGK1* promoter and terminator signals flanked by *TRP1* sequences was constructed; *kanMX* served as a selective marker in pUCMD (Figure 14). Single strand sequencing revealed this plasmid contained the desired DNA fragments in the correct order and orientation. Furthermore, *in silico* assembly of the *DUR3* coding region in pUCMD revealed that the *DUR3* ORF was identical in amino acid sequence and length to that published on SGD.

Aligning the sequence data of the *DUR3* cassette with the expected sequence revealed nine single nucleotide changes along the length of the cassette (Table 14), which are highlighted in a DNA sequence alignment between of S288C and pUCMD (Figure 23). A detailed description of the DNA sequences that comprise the *DUR3* cassette is given in Table 15.

Table 14. Discrepancies between the *DUR3* cassette in pUCMD and published sequences. Nucleotide mismatches are designated as 'X→ Y', meaning that the predicted nucleotide 'X' has been sequenced as 'Y'. The DNA alignment of S288C and pUCMD is given in Figure 23.

Nucleotide position	Region of cassette	Description
928	*PGK1* promoter	C→T
999	*PGK1* promoter	C→T
1230	*PGK1* promoter	C→T
1491	*PGK1* promoter	C→T
1494	*PGK1* promoter	G→A
3524	*DUR3* ORF	G→C: Silent
3821	*DUR3* ORF	T→C: Silent
3970	*DUR3* ORF	G→A: Silent
4160	*DUR3* ORF	A→C: Silent

Table 15. Detailed description of the DNA sequences that comprise the *DUR3* cassette.

Nucleotide position	Description
1-6	5' *Apa1* restriction site
7-291	*5' TRP1* sequence
292-475	pHVXKD3 vector sequence from cloning strategy
476-1976	*PGK1* promoter
1977-4184	*DUR3* ORF
4185-4467	*PGK1* terminator
4468-4535	pHVXKD3 vector sequence from cloning strategy
4536-4933	*kanMX* promoter
4934-5743	*kanMX* ORF
5744-5992	*kanMX* terminator
5993-6120	pHVXKD3 vector sequence from cloning strategy
6121-6510	3' *TRP1* sequence
6511-6516	3' *Apa1* restriction site

Alignment of DNA sequences: S288C and pUCMD

Upper line: S288C, from 1 to 6515
Lower line: pUCMD, from 1 to 6515

Data identity= 99%

```
901   TAGCATACAATTAAAACATGGCGGGCACGTATCATTGCCCTTATCTTGTGCAGTTAGACG
      |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||
901   TAGCATACAATTAAAACATGGCGGGCATGTATCATTGCCCTTATCTTGTGCAGTTAGACG

961   CGAATTTTTCGAAGAAGTACCTTCAAAGAATGGGGTCTCATCTTGTTTTGCAAGTACCAC
      |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
961   CGAATTTTTCGAAGAAGTACCTTCAAAGAATGGGGTCTTATCTTGTTTTGCAAGTACCAC

      ------------------------------------------------------------
      --------------CONTINUATION OF PGK1 PROMOTER----------------
      ------------------------------------------------------------

1201  TCAAGACGCACAGATATTATAACATCTGCACAATAGGCATTTGCAAGAATTACTCGTGAG
      |||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||
1201  TCAAGACGCACAGATATTATAACATCTGCATAATAGGCATTTGCAAGAATTACTCGTGAG

      ------------------------------------------------------------
      --------------CONTINUATION OF PGK1 PROMOTER----------------
      ------------------------------------------------------------

1441  CCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGACACGCTCGAC
      |||||||||||||||||||||||||||||||||||||||||||||||||| || ||||||
1441  CCGTCGCTCGTGATTTGTTTGCAAAAAGAACAAAACTGAAAAAACCCAGATACACTCGAC

1501  TTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGTCCTAGCGACG
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1501  TTCCTGTCTTCCTATTGATTGCAGCTTCCAATTTCGTCACACAACAAGGTCCTAGCGACG

      ------------------------------------------------------------
      ----CONTINUATION OF PGK1 PROMOTER AND START OF DUR3 ORF-----
      ------------------------------------------------------------

3481  CTTTGCTATCACCAGCCATTTTTATTCCTATTTTAACGTATGTGTTTAAGCCACAAAATT
      |||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||
3481  CTTTGCTATCACCAGCCATTTTTATTCCTATTTTAACGTATGTCTTTAAGCCACAAAATT

      ------------------------------------------------------------
      --------------CONTINUATION OF DUR3 ORF---------------------
      ------------------------------------------------------------

3781  TACAAAATGAATTAGACGAAGAACAAAGAGAACTAGCACGTGGTTTAAAAATTGCATACT
      |||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||
3781  TACAAAATGAATTAGACGAAGAACAAAGAGAACTAGCACGCGGTTTAAAAATTGCATACT

3841  TCCTATGTGTTTTTTTCGCTTTGGCATTTTTGGTAGTTTGGCCCATGCCCATGTATGGTT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3841  TCCTATGTGTTTTTTTCGCTTTGGCATTTTTGGTAGTTTGGCCCATGCCCATGTATGGTT

3901  CCAAATATATCTTCAGTAAAAAATTCTTTACCGGTTGGGTTGTTGTGATGATCATCTGGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
3901  CCAAATATATCTTCAGTAAAAAATTCTTTACCGGTTGGGTTGTTGTGATGATCATCTGGC

3961  TTTTTTTCAGTGCGTTTGCCGTTTGTATTTATCCACTCTGGGAAGGTAGGCATGGTATAT
```

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      |||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
3961  TTTTTTTCAGTGCATTTGCCGTTTGTATTTATCCACTCTGGGAAGGTAGGCATGGTATAT

4021  ATACCACTTTGCGAGGCCTTTACTGGGATCTATCTGGTCAAACTTATAAATTAAGGGAAT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4021  ATACCACTTTGCGAGGCCTTTACTGGGATCTATCTGGTCAAACTTATAAATTAAGGGAAT

4081  GGCAAAATTCGAACCCACAAGATCTGCATGTAGTAACAAGCCAAATTAGTGCGAGAGCAC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4081  GGCAAAATTCGAACCCACAAGATCTGCATGTAGTAACAAGCCAAATTAGTGCGAGAGCAC

4141  ATAGACAATCATCACATTTCGGACAAGTTGATGAAATAATTTAGCTCGAGGATTGAATTG
      |||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
4141  ATAGACAATCATCACATTTCGGCCAAGTTGATGAAATAATTTAGCTCGAGGATTGAATTG

4201  AATTGAAATCGATAGATCAATTTTTTTCTTTTCTCTTTCCCCATCCTTTACGCTAAAATA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
4201  AATTGAAATCGATAGATCAATTTTTTTCTTTTCTCTTTCCCCATCCTTTACGCTAAAATA
```

Figure 23. DNA sequence alignment of S288C and pUCMD revealed nine discrepancies along the length of *DUR3* cassette. The sequence obtained from S288C is shown in the upper line and the sequence of the *DUR3* cassette in pUCMD is shown in the lower line. Only a partial sequence of the *DUR3* cassette is displayed. The *PGK1* promoter is shown in green, the *DUR3* ORF is shown in black, and the *PGK1* terminator is shown in blue. Nucleotide mismatches are highlighted in bold and red, and are underlined. In the *DUR3* ORF, mismatches are oriented within their respective codons by underlining; the stop codon is italicized.

In silico analysis of the integrated *DUR3* cassette revealed that two new ORFs were created during construction of the *DUR3* cassette; these ORFs were composed of a mixture of *S. cerevisiae* sequence and pHVXK vector sequence (Figure 24). Novel ORF1 (324 bp) is located at nucleotide position 7-330 while novel ORF2 (621 bp) is located at position 463-1083.

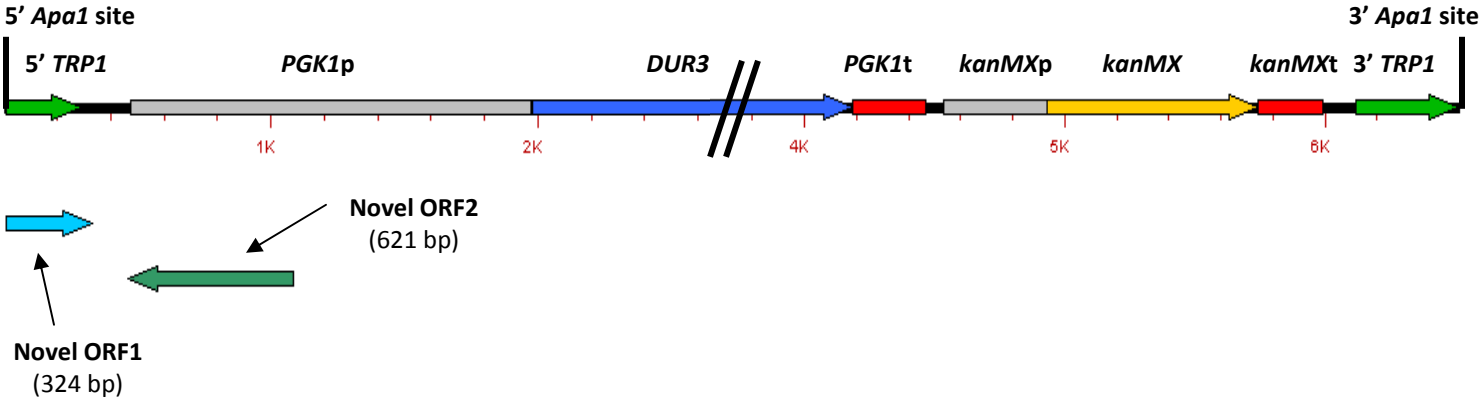


Figure 24. A schematic representation of new ORFs of more than 100 codons generated during construction of the *DUR3* cassette. Two new ORFs, composed of a mixture of *S. cerevisiae* sequence and pHVXK vector sequence, were created.

3.2.2 Integration of the linear *DUR3* cassette into the genomes of yeast strains K7, K7^{EC-}, K9, and K9^{EC-}

To constitutively express *DUR3,* the linear *DUR3* cassette (Figure 25) was transformed into Sake yeast strains K7 and K7^{EC-}, and the wine strains 522 and 522^{EC-}. Following positive selection on a G418 medium and sub-culturing, the eight strains listed in Table 16 were obtained.

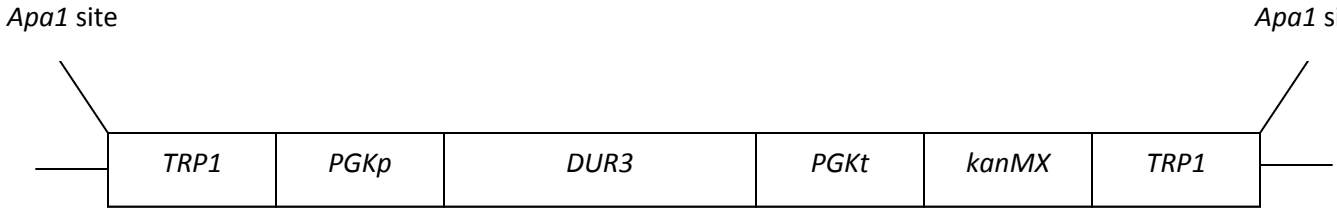


Figure 25. Schematic representation of the linear *DUR3* cassette.

Table 16. Recombinant yeast strains created by integration of the *DUR3* cassette into the *TRP1* locus. The designation 'EC-' corresponds to integration of the *DUR1,2* cassette while 'D3' corresponds to integration of the *DUR3* cassette. 'EC-D3' designates integration of both cassettes.

Genetic modification	Parent strain	
	K7	**522**
Wild type	K7	522
DUR1,2	K7^{EC-}	522^{EC-}
DUR3	K7^{D3}	522^{D3}
DUR1,2/DUR3	K7^{EC-D3}	522^{EC-D3}

3.2.3 Genetic characterization of K7^{D3}, K7^{EC-D3}, 522^{D3}, and 522^{EC-D3}

3.2.3.1 Correct integration of the *DUR3* cassette into the genomes of K7^{D3}, K7^{EC-D3}, 522^{D3}, and 522^{EC-D3}.

A Southern blot, using *EcoR1* digested genomic DNA and PCR created *DUR3* and *TRP1* probes (Figure 26), was performed on each of the recombinant yeast strains to assess proper integration of the *DUR3* cassette into the genome. The *DUR3* and *TRP1* probes are schematically represented in Figures 27a,b.

In each of the recombinant yeast strains, two signals corresponding to 2.4 kb and 4.7 kb, were detected when probed for *DUR3* (Figure 26). The 2.4 kb fragment matches the expected fragment size for the native *DUR3* locus while the 4.7 kb fragment matches the expected size for the recombinant *TRP1-PGK1p-DUR3-PGK1t-kanMX-TRP1* locus. In strains that did not carry the recombinant *DUR3* cassette, only the 2.4 kb signal was detected.

When probed for *TRP1*, three signals, corresponding to 1.5 kb, 3.0 kb and 4.7 kb, were detected in each of the strains containing the recombinant *DUR3* cassette (Figure 26). The 1.5 kb fragment matches the expected fragment size for a non-disrupted *TRP1* locus, while the 3.0 kb and 4.7 kb fragments are in accordance with the presence of the recombinant *DUR3* cassette integrated into the *TRP1* locus.

Unexpectedly, when any of the K7 derived strains, except for $K7^{EC\text{-}D3}$, were probed for *DUR3*, a novel signal was detected at ~3.5 kb (Figure 26). This fragment corresponds to the disappearance of the 2.4 kb band that represents the native *DUR3* locus. This result was confirmed by sequencing to be caused by a restriction fragment length polymorphism (RFLP) between 522 and K7. The *DUR3* locus in K7 is mutated such that it no longer contains the *EcoR1* site within the coding region (Figure 28) and, as a

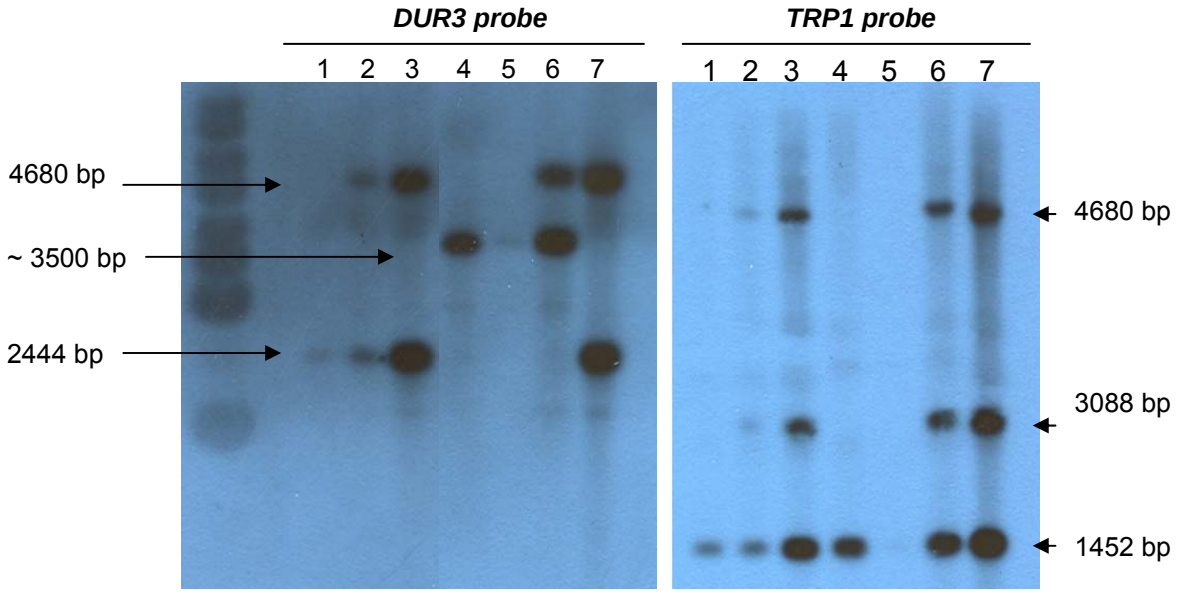


Figure 26. Integration of the *DUR3* cassette into the *TRP1* locus of 522^{D3}, $522^{EC\text{-}D3}$, $K7^{D3}$, and $K7^{EC\text{-}D3}$ was confirmed by Southern blot analysis using *DUR3* and *TRP1* probes. Approximately 1 µg of *EcoR1* digested genomic DNA from 522 (lane #1), 522^{D3} (lane #2), $522^{EC\text{-}D3}$ (lane #3), K7 (lane #4), $K7^{EC\text{-}}$ (lane #5), $K7^{D3}$ (lane #6), and $K7^{EC\text{-}D3}$ (lane #7) was probed with either *DUR3* or *TRP1*. In lane #5 less DNA was accidentally loaded thus accounting for the faint band pattern. All faint bands that did not correspond to a size in the schematic representation were deemed to be non-specific binding**.**

result, the native *DUR3* locus in K7 produced a larger signal (~3500 bp). By integrating a copy of *DUR3* derived from 522, which contains the internal *EcoR1* site, a recombinant locus was created which gives rise to the same band pattern seen in 522. Although this RFLP should have been observed in $K7^{EC\text{-}D3}$ it was not and therefore warrants further investigation.

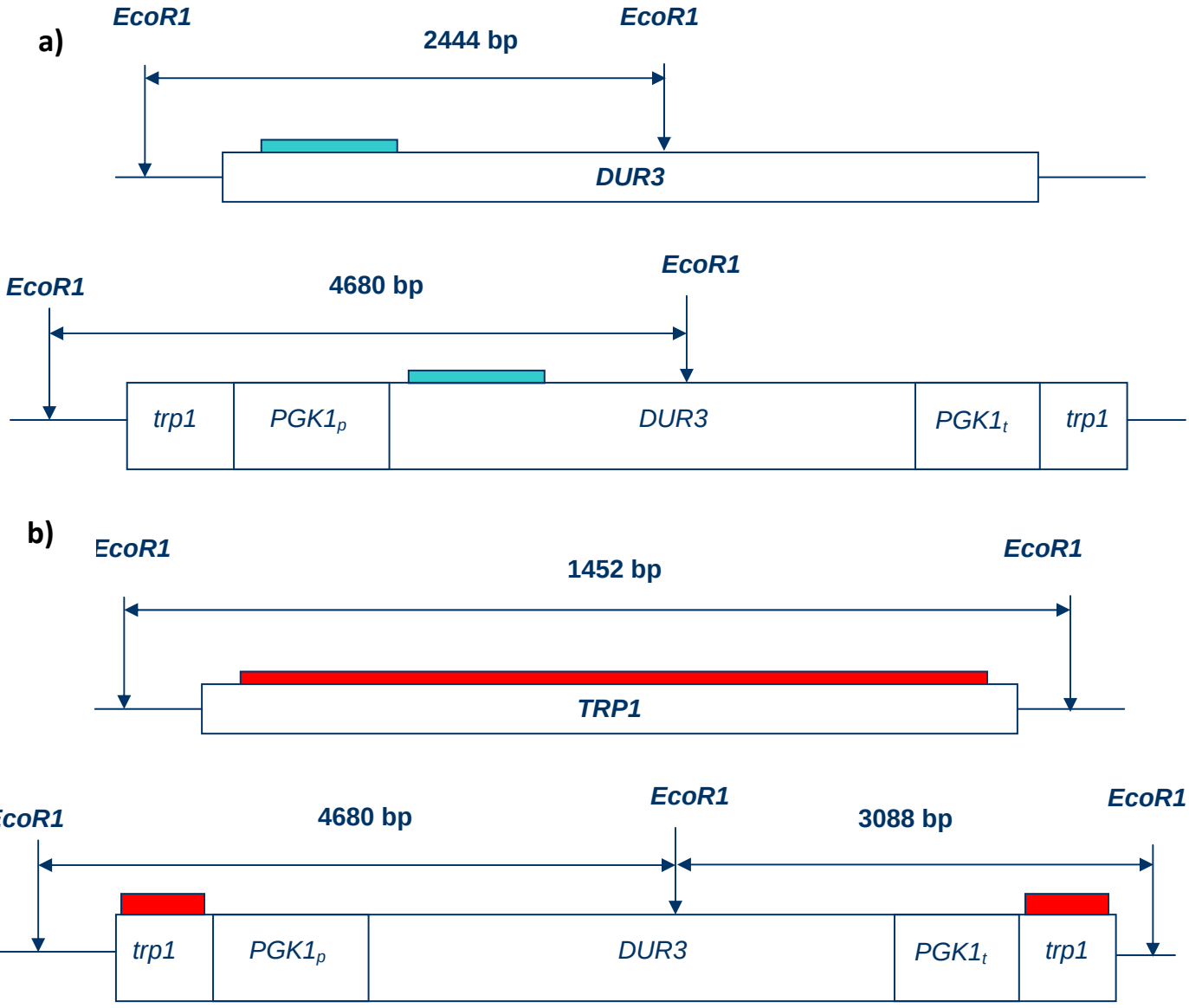


Figure 27. Schematic representation of the signals expected during Southern blot analysis of recombinant yeasts containing the recombinant *DUR3* cassette integrated into the *TRP1* locus. Expected signals during Southern blotting with the *DUR3* probe (drawn in green) (a). Expected signals during Southern blotting with the *TRP1* probe (drawn in red) (b).

Alignment of DNA sequences: S288C, 522, K7 (1-240 bp *DUR3*)

```
S288C       ATGGGAGAATTTAAACCTCCGCTACCTCAAGGCGCTGGGT     40
522         ....................cAtCcaggGGCGCTGGGa     18
K7          ............cAACaaaCcCcctactcAGGCGCTGGGT     28

S288C       ACGCTATTGTATTGGGCCTAGGGGCCGTATTTGCAGGAAT     80
522         .CGCTATTGTATTGGGCCTAGGGGCCGTATTTGCAGGAAT     57
K7          .CGCTATTGTATTGGGCCTAGGGGCCGTATTTGCAGGAAT     67

S288C       GATGGTTTTGACCACTTATTTACTGAAACGTTATCAAAAG    120
522         GATGGTTTTGACCACTTATTTACTGAAACGTTATCAAAAG     97
K7          GATGGTTTTGACCACTTATTTACTGAAACGTTATCAAAAG    107

S288C       GAAATCATCACAGCAGAAGAATTCACCACCGCCGGTAGAT    160
522         GAAATCATCACAGCAGAAGAATTCACCACCGCCGGTAGAT    137
K7          GAAATCATCACAGCAGAAGAATTtACCACCGCCGGcAGAT    147

S288C       CTGTAAAAACCGGCTTAGTGGCTGCAGCCGTGGTTTCTAG    200
522         CTGTAAAAACCGGCTTAGTGGCTGCAGCCGTGGTTTCTAG    177
K7          CTGTAAAAACCGGCTTAGTGGCTGCcGCCGTGGTTTCTAG    187

S288C       TTGGATCTGGTGTTCTACATTGTTAACGTCGTCAACAAAG    240
522         TTGGATCTGGTGTTCTACATTGTTAACGTCGTCAACAAAG    217
K7          TTGGATCTGGTGTTCTACATTGTTAACGTCGTCAACAAAG    227
```

Figure 28. Alignment of the DNA sequences of S288C, 522, and K7 confirmed the presence of a mutant *EcoR1* site in the *DUR3* coding region of K7. Only a partial sequence of *DUR3* is displayed. The *EcoR1* site in question is displayed in red; other mismatches revealed by sequencing are highlighted in blue.

3.2.3.2 Confirmation of constitutive expression of *DUR3* in K7^{D3} and K7$^{EC\text{-}D3}$ by northern blotting. In order to assess the constitutive expression of *DUR3* from the *DUR3* cassette in K7^{D3} and K7$^{EC\text{-}D3}$, a northern blot was performed using PCR generated probes for *HHF1* (Histone H4) and *DUR3* on freshly harvested total RNA from K7, K7$^{EC\text{-}}$, K7^{D3}, and K7$^{EC\text{-}D3}$. Identical signals matching the predicted transcript size for *HHF1* (312 bp) were observed in all of the strains tested (Figure 29), indicating an abundance of un-degraded mRNA in the samples. Signals, which matched the predicted transcript size (2208 bp) for *DUR3*, were observed in strains K7^{D3} and K7$^{EC\text{-}D3}$ when probed for *DUR3* (Figure 29), thus confirming constitutive expression of *DUR3* in non-inducing conditions as a result of integration of the *DUR3* cassette. No *DUR3* signal was detected in K7 or K7$^{EC\text{-}}$ confirming that *DUR3* mRNA is absent during fermentation due to repression by NCR.

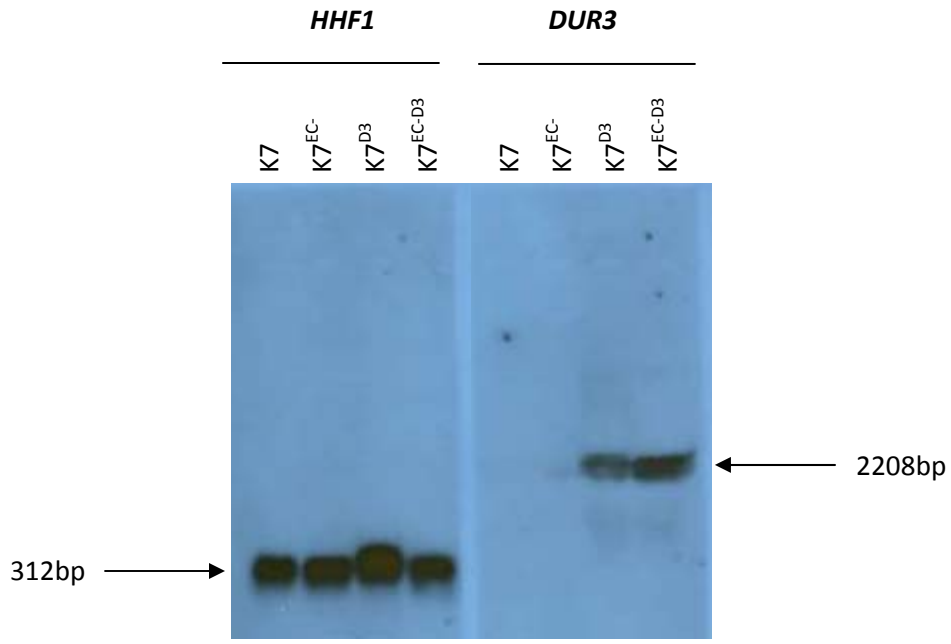


Figure 29. Constitutive expression of *DUR3* (2208 bp) was confirmed by northern blot analysis of K7, $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$. Total RNA (30 µg) was harvested from 24 hour fermentations of Calona Chardonnay must inoculated to a final OD_{600} = 0.1, separated on a 1% agarose-formaldehyde gel and probed for both the loading control *HHF1* and *DUR3*.

3.2.3.3 Quantification of constitutive *DUR3* expression in $K7^{D3}$ and $K7^{EC-D3}$ by qRT-PCR. Expression of *DUR3* and *DUR1,2* was verified and quantified by qRT-PCR analysis of cDNA reverse transcribed from the total RNA of K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$. Analyses were performed on the same RNA samples as in Section 3.2.3.2.

The *PGK1* promoter and terminator signals resulted in high level expression, under non-inducing (NCR) conditions, of both the *DUR1,2* and *DUR3* genes in the integrated cassettes (Figure 30); *DUR1,2* was upregulated 11.8-fold in $K7^{EC-}$ while *DUR3* was upregulated 22.1-fold in $K7^{D3}$. High level expression of both *DUR1,2* and *DUR3* (17.3-fold and 11.5-fold, respectively) was sustained in $K7^{EC-D3}$, when both *DUR1,2* and *DUR3* 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 (Figure 30). Constitutive expression of *DUR1,2* in $K7^{EC-}$

induced expression of *DUR3* by 3.78-fold. Likewise, constitutive expression of *DUR3* in $K7^{D3}$ induced expression of *DUR1,2* by 3.61-fold.

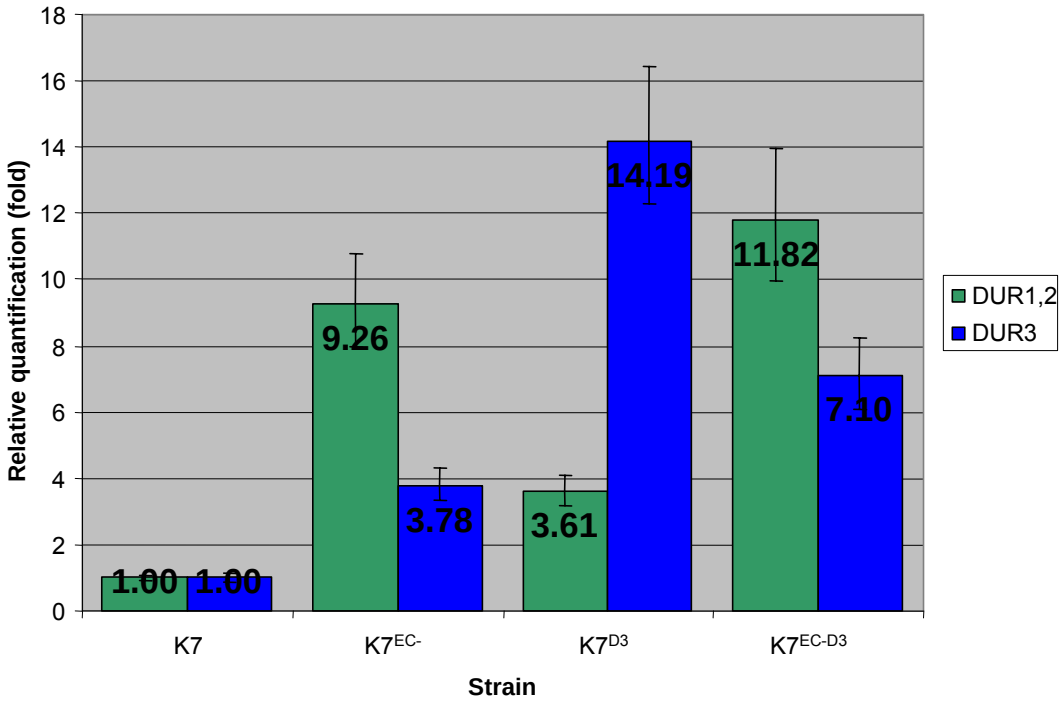


Figure 30. Analyses of gene expression (qRT-PCR) of K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ confirmed functionality of the *DUR3* cassette and constitutive expression of *DUR1,2* and *DUR3* in non-inducing (NCR) conditions. Total RNA was extracted from cells harvested after 24 hour fermentation (20°C) in filter sterilized Calona Chardonnay must that was inoculated to a final OD_{600} = 0.1. Total RNA was subsequently reverse transcribed, and the resultant cDNA was amplified in the presence of SYBR green dye. *DUR1,2* and *DUR3* gene expression was standardized to *ACT1* expression and data for $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$ was calibrated to the parental strain K7. Fermentations were conducted in triplicate and the data averaged; error bars represent 95% confidence intervals.

3.2.3.4 Effect of the integrated *DUR3* cassette on the transcriptome of $K7^{D3}$. Total RNA from yeast cells isolated from 24 hour fermentations in Chardonnay must was used analyze the impact of the integrated *DUR3* cassette on global gene expression in $K7^{D3}$. Reported changes in gene expression were cut off at a minimum 4-fold change in expression ($SLR_{Avg} \geq 2$ or $SLR_{Avg} \leq -2$) to ensure elimination of experimental noise inherent in microarray analysis; this cut-off is supported by the previously published statistical examination of a wine yeast's transcriptome during fermentation (Marks, et al. 2008).

Integration of the *DUR3* cassette into the *TRP1* locus of $K7^{D3}$ had a minimal effect on the yeast's transcriptome. *DUR3* was upregulated by 36.95-fold in $K7^{D3}$; *TRP1* was downregulated by 1.25-fold but was not included in Table 17 as it fell below the 4-fold cut off. Besides *DUR3*, three genes were upregulated greater than 4-fold in $K7^{D3}$ (Table 17); one gene (*HAC1*) was common to both of the engineered strains $K7^{EC-}$ and $K7^{D3}$ (Tables 10 and 17). Four genes were downregulated more than 4-fold in $K7^{D3}$. Despite falling below the 4-fold cut off, one gene (*RUD3*) was included in Table 17 because it was common to the list of genes downregulated in both $K7^{EC-}$ and $K7^{D3}$ (Tables 10 and 17) and because it is very close to the 4-fold cut off. No metabolic pathways were affected by the presence of the integrated *DUR3* cassette; however, integration of the *DUR3* cassette upregulated one gene (*FIG1*) and downregulated one gene (*TID3*) involved in meiosis/sporulation indicating a possible effect on meiosis/sporulation efficiency (Table 17).

Table 17. Effect of the integrated *DUR3* cassette in the genome of K7 on global gene expression patterns in *S. cerevisiae* $K7^{D3}$ (≥ 4-fold change). Reported changes are relative to the parental strain K7. Total RNA from K7 and $K7^{D3}$, harvested at 24 hours into fermentation of filter sterilized Chardonnay must, were used for hybridization to microarrays. Fermentations were conducted in duplicate and the data were averaged (p≤0.005).

Genes expressed at higher levels in $K7^{D3}$		
Fold Change	**Gene Symbol**	**Biological Process**
36.95	*DUR3*	Urea permease
5.67	*HAC1*	bZIP (basic-leucine zipper) 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 $K7^{D3}$		
Fold Change	**Gene Symbol**	**Biological Process**
-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
-3.98	*RUD3*	Protein involved in organization of Golgi

3.2.4 Recombinant strains $K7^{D3}$ and $K7^{EC-D3}$ exhibit highly enhanced urea uptake ability in 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.

The parental Sake strain K7, and the recombinant yeast $K7^{EC-}$ 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 sulphate (Figure 31). This is likely due to the NCR of the *DUR* genes in the presence of the preferred nitrogen source (ammonium sulphate). In contrast, $K7^{D3}$ and $K7^{EC-D3}$ were highly efficient at urea uptake (Figure 31), confirming that integration of the *DUR3* cassette results in the production of a functional protein that 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 significant difference in the urea uptake rates of $K7^{D3}$ and $K7^{EC-D3}$ was observed (Figure 31), despite integration of identical *DUR3* cassettes in both strains. The observed difference in urea uptake between $K7^{D3}$ and $K7^{EC-D3}$ can be explained by the need for urea degradation (DUR1,2p) after its import by the cell. While *DUR1,2* must be induced and then synthesized in $K7^{D3}$, DUR1,2p is constitutively expressed in $K7^{EC-D3}$ leading to rapid degradation of urea to which might mask an increased uptake; the radiolabel would be quickly lost as $^{14}CO_2$.

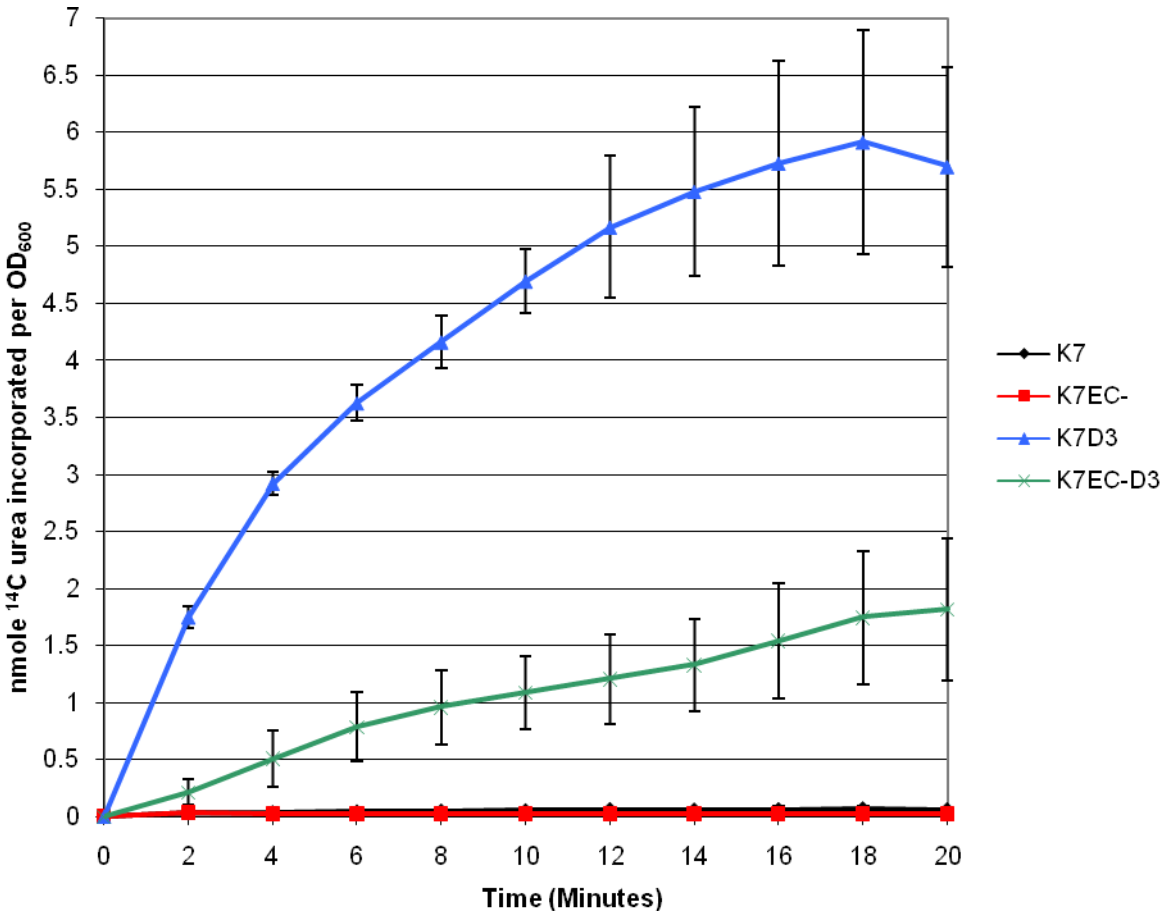


Figure 31. Uptake of ^{14}C-urea by K7, $K7^{EC-}$, $K7^{D3}$ and $K7^{EC-D3}$ under conditions of NCR. Strains were cultured (final OD_{600} = 1) in a 1% (w/v) ammonium sulphate minimal medium prior to exposure to 0.27 mM ^{14}C-urea (6.8 mCi/mmol). Assays were conducted in triplicate and the data averaged; error bars represent one standard deviation.

When compared to either $K7^{D3}$ or $K7^{EC-D3}$, both K7 and $K7^{EC-}$ exhibited relatively poor urea uptake under the conditions tested (Figure 31). In order to differentiate between K7 and $K7^{EC-}$, Figure 31 was modified by significantly reducing the scale of the Y-axis; the maximum value of the Y-axis was reduced from 7.0 nmole (Figure 31) to 0.1 nmole (Figure 32). K7 was capable of accumulating significantly more urea than $K7^{EC-}$ (Figure 32), probably due to the constitutive expression of *DUR1,2* in $K7^{EC-}$; ^{14}C-urea might be actively degraded as it is imported into the cell thus giving the illusion that transport is less efficient. This phenomenon was also observed when the abilities of $K7^{D3}$ and $K7^{EC-D3}$ to transport urea into the cell were compared (Figure 31).

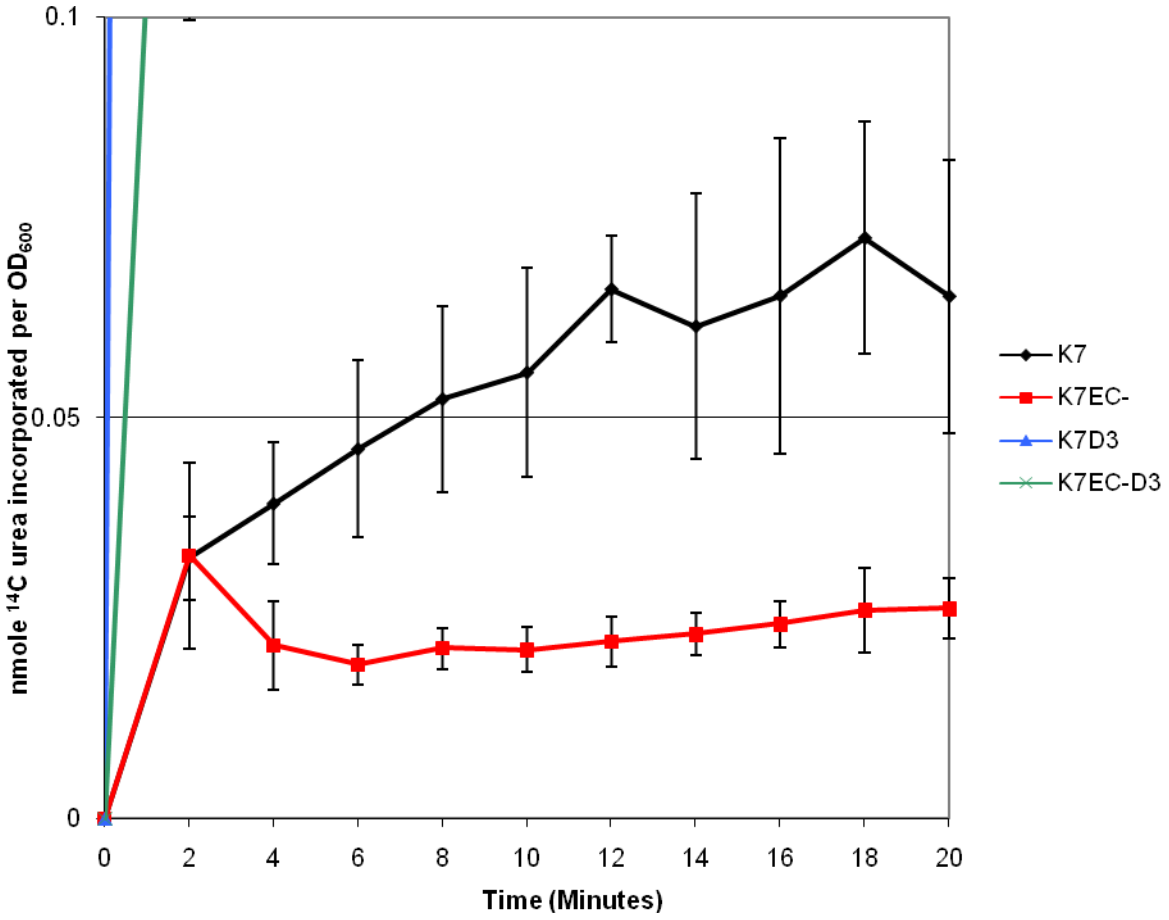


Figure 32. Uptake of ^{14}C-urea by K7 and K7^{EC-} under conditions of NCR. Strains were cultured (final OD_{600} = 1) in a 1% (w/v) ammonium sulphate minimal medium prior to exposure to 0.27 mM ^{14}C urea (6.8 mCi/mmol). Assays were conducted in triplicate and the data averaged; error bars represent one standard deviation.

3.2.5 Recombinant strains K7^{D3} and K7^{EC-D3} exhibit highly enhanced urea uptake ability in conditions of NCR de-repression

Radiolabelled ^{14}C-urea uptake assays were performed in order to assess the ability of engineered strains containing the integrated *DUR3* cassette to uptake urea under non-repressive conditions.

Both the parental Sake strain K7, and the recombinant yeast $K7^{EC-}$ containing the *DUR1,2* cassette integrated into the *URA3* locus, did not incorporate any significant amount of ^{14}C-urea in a minimal medium containing 1% (w/v) L-proline (Figure 33). This result, which parallels the inability of K7 and $K7^{EC-}$ to incorporate ^{14}C-urea in an ammonium sulphate minimal medium (Figure 31), is likely due to the slow induction and membrane trafficking of functional DUR3p. In contrast, $K7^{D3}$ and $K7^{EC-D3}$ were highly efficient at urea uptake (Figure 33), confirming that integration of the *DUR3* cassette results in the production of a functional protein, and that transcription of *DUR3* from the *PGK1* promoter and terminator signals is strong regardless of NCR state.

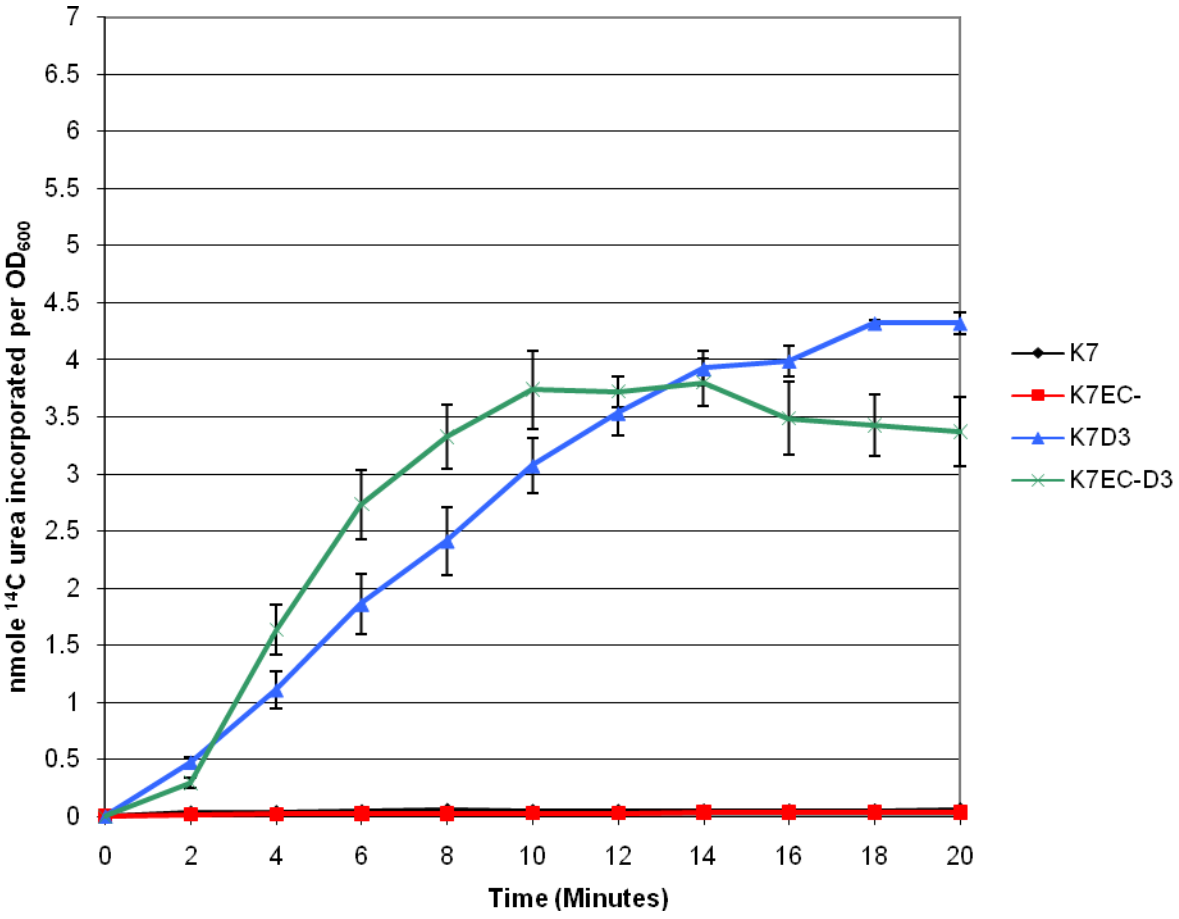


Figure 33. Uptake of ^{14}C-urea by K7, $K7^{EC-}$, $K7^{D3}$ and $K7^{EC-D3}$ under conditions of NCR de-repression. Strains were cultured (final OD_{600} = 1) in a 1% (w/v) L-proline minimal medium prior to exposure to 0.27 mM ^{14}C urea (6.8 mCi/mmol). Assays were conducted in triplicate and the data averaged; error bars represent one standard deviation.

When compared to either $K7^{D3}$ or $K7^{EC\text{-}D3}$, both K7 and $K7^{EC-}$ exhibited relatively poor urea uptake under conditions of NCR de-repression (Figure 33), K7 was capable of accumulating significantly more urea than $K7^{EC-}$ (Figure 34). To differentiate between K7 and $K7^{EC-}$, Figure 33 was modified by reducing the scale of the Y-axis; the maximum value of the Y-axis was reduced from 7.0 nmole (Figure 33) to 0.1 nmole (Figure 34). K7 was capable of accumulating significantly more urea than $K7^{EC-}$ (Figure 34); ^{14}C-urea might be actively degraded as it is imported into $K7^{EC-}$ where *DUR1,2* is constitutively expressed, thus masking the strain's true ability to import urea.

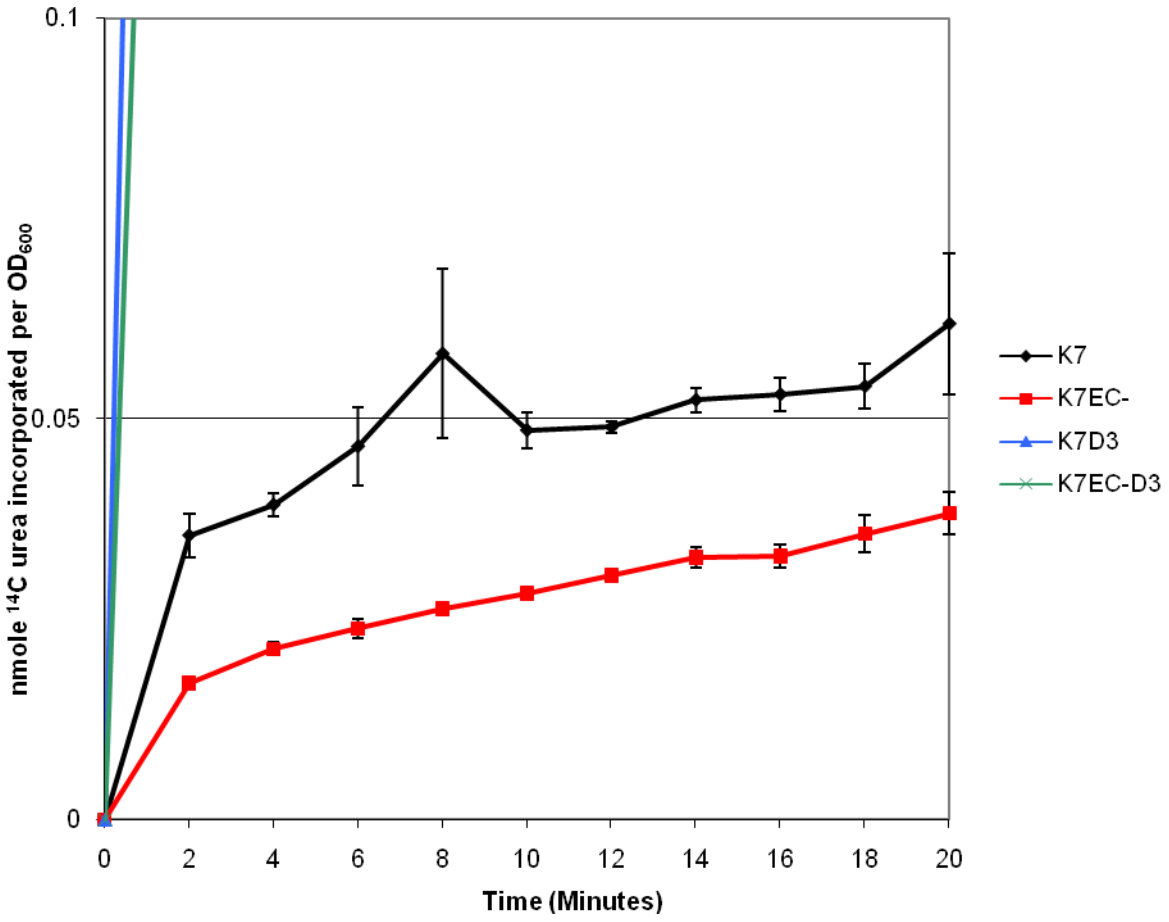
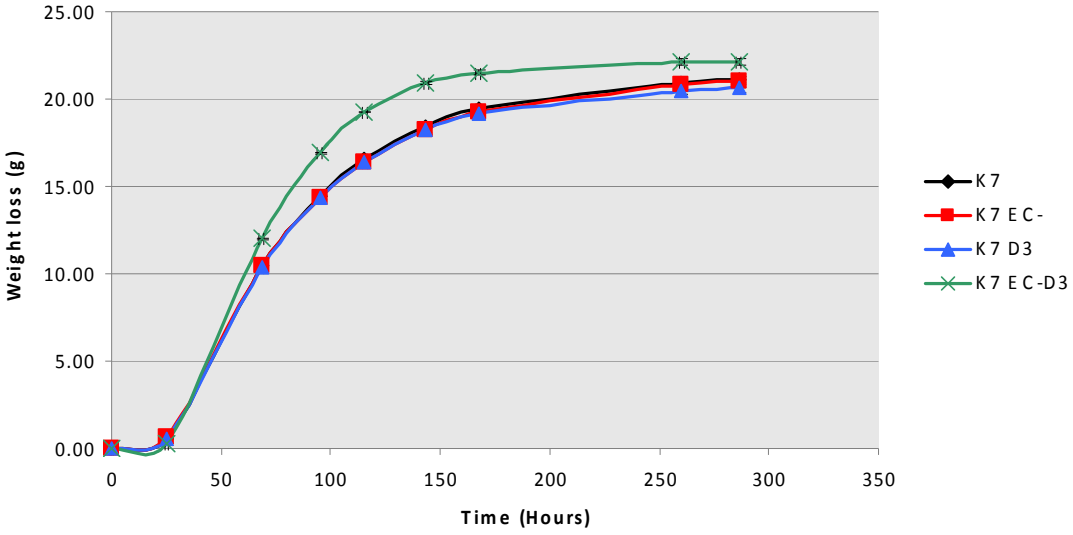


Figure 34. Uptake of ^{14}C-urea by K7 and $K7^{EC-}$ under conditions of NCR de-repression. Strains were cultured (final OD_{600} = 1) in a 1% (w/v) L-proline minimal medium prior to exposure to 0.27 mM ^{14}C urea (6.8 mCi/mmol). Assays were conducted in triplicate and the data averaged; error bars represent one standard deviation.

3.2.6 Phenotypic characterization of $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$

3.2.6.1 Fermentation rate of $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ in Chardonnay wine. The fermentation profiles of the parental and metabolically engineered strains are shown in Figures 35a,b. The robust fermentations were completed within 300 hours, and the fermentation rates of all but one engineered strain closely matched those of their respective parental strains, thus indicating substantial equivalence. The engineered strain $K7^{EC\text{-}D3}$ completed the fermentation by approximately 200-250 hours while the strains $K7^{EC\text{-}}$, and $K7^{D3}$ required 300 hours to finish; the parental strain K7 also required 300 hours to complete the fermentation.

a)



b)

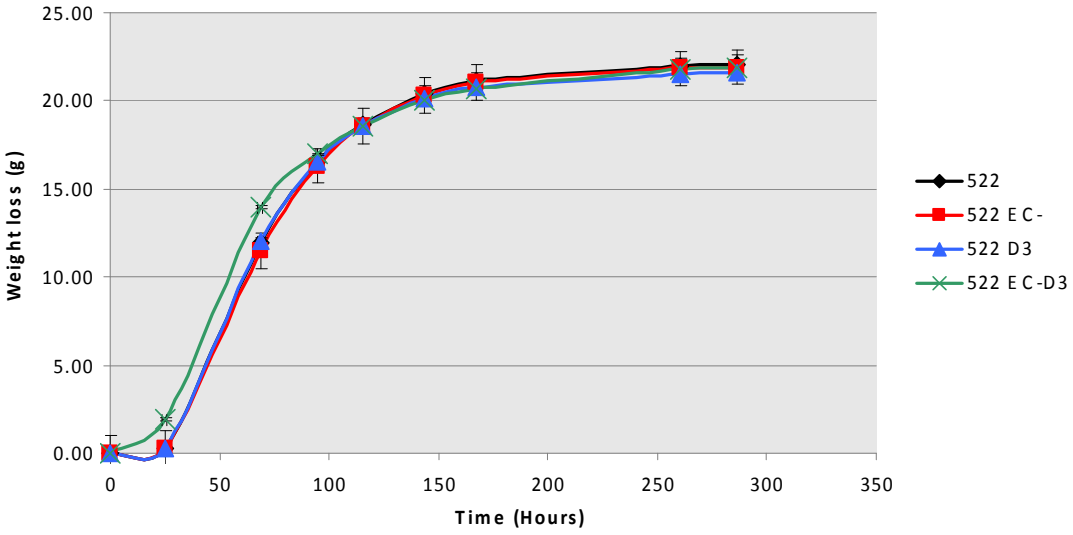


Figure 35. Fermentation profiles (weight loss) of (a) Sake yeast strains K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ and (b) wine yeast strains 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} in Chardonnay wine. Chardonnay wine was produced from unfiltered Calona Chardonnay must inoculated to a final OD_{600} = 0.1 and incubated to completion (~300 hours) at 20°C. Fermentations were conducted in triplicate and data were averaged; error bars indicate one standard deviation.

3.2.6.2 Ethanol production by $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ in Chardonnay wine. The effect of the *DUR3* cassette on ethanol production in Chardonnay wine by strains $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ was investigated by LC analysis at the end of fermentation. Compared to their respective parental strains, $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ produced Chardonnay wine with substantially equivalent ethanol content (Table 18).

Table 18. Ethanol produced by Sake yeast strains (K7, $K7^{EC\text{-}}$, $K7^{D3}$, and $K7^{EC\text{-}D3}$) and wine yeast strains (522, $522^{EC\text{-}}$, 522^{D3}, and $522^{EC\text{-}D3}$) in Chardonnay wine. Ethanol content (%v/v) was measured by LC at the end of fermentation. Fermentation profiles are given in Figures 35a,b. Data were analyzed for statistical significance ($p \leq 0.05$) using two factor ANOVA analysis.

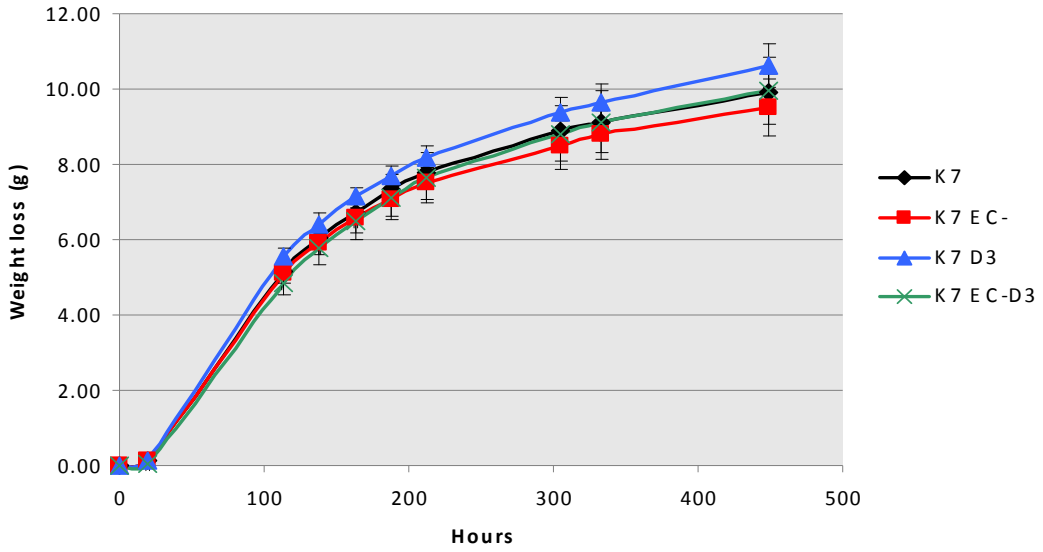
	K7	**$K7^{EC\text{-}}$**	**$K7^{D3}$**	**$K7^{EC\text{-}D3}$**	***p*** *
Replicate 1	12.89	12.84	12.89	12.91	--
Replicate 2	12.90	12.75	13.01	12.95	--
Replicate 3	12.96	12.97	12.97	12.89	--
Ethanol average (n=3)	**12.92**	**12.85**	**12.96**	**12.92**	**ns**
STDEV	0.04	0.11	0.06	0.03	--

	522	**$522^{EC\text{-}}$**	**522^{D3}**	**$522^{EC\text{-}D3}$**	***p*** *
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**	**ns**
STDEV	0.06	0.03	0.10	0.04	--

* si, ns: significant at $p \leq 0.05$, or non-significant

3.2.6.3 Fermentation rate of $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ in Sake wine. The fermentation profiles of the parental and metabolically engineered strains are shown in Figures 36a,b. In all cases, the fermentations proceeded at a slower rate than those in grape must; fermentations were completed within 450 hours compared to 300 hours in Chardonnay grape must (Figure 35). The fermentation rates of engineered strains closely matched those of the respective parental strains, thus indicating substantial equivalence in fermentation rate.

a)



b)

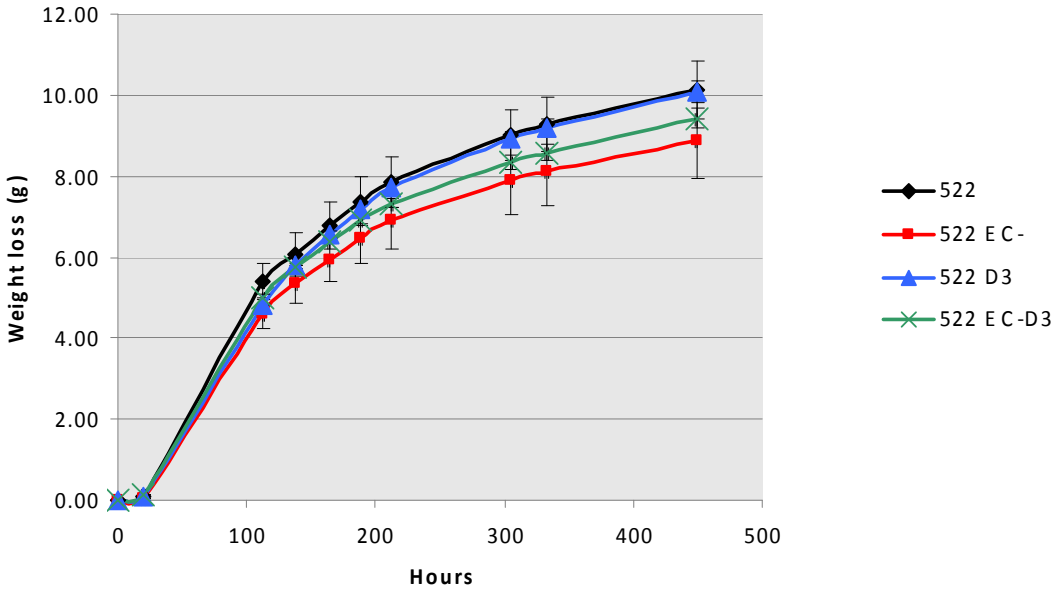


Figure 36. Fermentation profiles (weight loss) of (a) Sake yeast strains K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ and (b) wine yeast strains 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} in Sake. Sake wine was produced from white rice (Kokako Rose) and koji mash (Vision Brewing) inoculated to a final OD_{600} = 0.1 and incubated to completion (~450 hours) at 18°C. Fermentations were conducted in triplicate and data were averaged; error bars indicate one standard deviation.

3.2.6.4 Ethanol production by $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ in Sake wine. The effect of the *DUR3* cassette on ethanol production in Sake wine by strains $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ was investigated by LC analysis at the end of fermentation. Compared to their respective parental strains, $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ produced Sake wine with substantially equivalent ethanol content (Table 19).

Table 19. Ethanol produced by Sake yeast strains (K7, $K7^{EC\text{-}}$, $K7^{D3}$, and $K7^{EC\text{-}D3}$) and wine yeast strains (522, $522^{EC\text{-}}$, 522^{D3}, and $522^{EC\text{-}D3}$) in Sake wine. Ethanol content (% v/v) was measured by LC at the end of fermentation. Fermentation profiles are given in Figures 36a,b. Data were analyzed for statistical significance ($p \leq 0.05$) using two factor ANOVA analysis.

	K7	**$K7^{EC\text{-}}$**	**$K7^{D3}$**	**$K7^{EC\text{-}D3}$**	***p*** *
Replicate 1	9.33	9.22	8.90	8.63	--
Replicate 2	9.20	8.14	9.58	8.96	--
Replicate 3	10.01	8.14	9.64	9.01	--
Ethanol average (n=3)	**9.51**	**8.50**	**9.37**	**8.87**	**ns**
STDEV	0.44	0.62	0.41	0.21	--

	522	**$522^{EC\text{-}}$**	**522^{D3}**	**$522^{EC\text{-}D3}$**	***p*** *
Replicate 1	8.78	7.46	8.77	8.99	--
Replicate 2	8.80	7.81	8.99	8.86	--
Replicate 3	8.61	9.22	8.98	8.37	--
Ethanol average (n=3)	**8.73**	**8.16**	**8.91**	**8.74**	**ns**
STDEV	0.10	0.93	0.12	0.33	--

* si, ns: significant at $p \leq 0.05$, or non-significant

3.2.7 Constitutive expression of *DUR3* in yeast strains $K7^{D3}$ and 522^{D3} reduces EC in Chardonnay wine by 24.97% and 81.38%, respectively.

In order to assess the reduction of EC by functionally enhanced yeast strains, Chardonnay wine was made with the parental strains K7 and 522 and the recombinant strains $K7^{EC\text{-}}$, $K7^{D3}$, $K7^{EC\text{-}D3}$, $522^{EC\text{-}}$, 522^{D3}, and $522^{EC\text{-}D3}$ and the EC content quantified by GC/MS at the end of fermentation. The fermentation profiles are given in Figures 35a,b.

During wine making, $K7^{D3}$ and 522^{D3} reduced EC as efficiently as $K7^{EC\text{-}}$ and $522^{EC\text{-}}$, respectively (Table 20). In Chardonnay wine, $K7^{D3}$ reduced EC by 12.64% while $K7^{EC\text{-}}$ reduced EC by 6.85%; 522^{D3}

reduced EC by 82.96% while 522^{EC-} reduced EC by 81.48%. Constitutive co-expression of *DUR1,2* and *DUR3* did not result in synergistic EC reduction in $K7^{EC-D3}$ or 522^{EC-D3} (Table 20).

Table 20. Reduction of EC by functionally enhanced yeast strains during wine making. The concentration of EC (μg/L) in Chardonnay wine produced by Sake yeast strains K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ and wine yeast strains 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} from unfiltered Calona Chardonnay must was quantified by GC/MS. Triplicate fermentations were incubated to completion (~300 hours) at 20°C and fermentation profiles are given in Figures 35a,b.

Yeast strain	**K7**	**$K7^{EC-}$**	**$K7^{D3}$**	**$K7^{EC-D3}$**
Replicate 1	34.19	34.25	29.39	29.74
Replicate 2	40.31	30.98	29.98	31.29
Replicate 3	28.69	30.89	30.78	30.06
Average (n=3)	**34.40**	**32.04**	**30.05**	**30.36**
STDEV	5.81	1.91	0.70	0.82
RSD (%)	16.89	5.96	2.33	2.70
Reduction (%)	**--**	**6.85**	**12.64**	**11.73**

Yeast strain	**522**	**522^{EC-}**	**522^{D3}**	**522^{EC-D3}**
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**
STDEV	15.22	3.15	3.07	3.78
RSD (%)	8.34	9.33	9.88	10.64
Reduction (%)	**--**	**81.48**	**82.96**	**80.52**

3.2.8 Constitutive expression of *DUR3* in yeast strains $K7^{D3}$ and 522^{D3} reduces EC in Sake wine by 18.40% and 10.45%, respectively.

To assay the EC reduction of functionally enhanced yeast strains during Sake making, Sake wine was brewed with the parental strains K7 and 522 and the recombinant strains $K7^{EC-}$, $K7^{D3}$, $K7^{EC-D3}$, 522^{EC-}, 522^{D3}, and 522^{EC-D3} and the EC content quantified by GC/MS at the end of fermentation. The fermentation profiles are given in Figures 36a,b.

As observed before (Section 3.1.5), engineered Sake yeast strains reduce EC more effectively when assessed by brewing Sake; the Sake yeasts $K7^{EC-}$ and $K7^{EC-D3}$ both reduced EC content by ~84%

(Table 21). During the wine making trial described in Section 3.2.7, $K7^{EC-}$ and $K7^{EC-D3}$ both reduced EC by only ~15% (Table 20).

In contrast to the results described in Section 3.2.7 for Chardonnay wine, constitutive expression of *DUR3* did not reduce EC as effectively as constitutive expression of *DUR1,2* (Tables 20 and 21). In Sake wine, $K7^{D3}$ reduced EC by 14.97% while $K7^{EC-}$ reduced EC by 87.07%; 522^{D3} reduced EC by 11.49% while 522^{EC-} reduced EC by 84.30% (Table 21). Constitutive co-expression of *DUR1,2* and *DUR3* had no synergistic effect on EC reduction during Sake production (Table 21); there was no appreciable difference in EC reduction between the *DUR1,2* expressing strains and those which constitutively expressed both *DUR1,2* and *DUR3.*

Table 21. Reduction of EC by functionally enhanced yeast strains during Sake making. The concentration of EC (μg/L) in Sake wine produced by Sake yeast strains K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ and wine yeast strains 522, 522^{EC-}, 522^{D3}, and 522^{EC-D3} from white rice (Kokako Rose) and koji mash (Vision Brewing) was quantified by GC/MS. Triplicate fermentations were incubated to completion (~450 hours) at 18°C and the fermentation profiles are given in Figures 36a,b.

Yeast strain	**K7**	**$K7^{EC-}$**	**$K7^{D3}$**	**$K7^{EC-D3}$**
Replicate 1	109.83	13.37	93.42	15.72
Replicate 2	80.68	12.3	79.86	18.6
Replicate 3	108.26	12.96	80.77	17.79
Average (n=3)	**99.59**	**12.88**	**84.68**	**17.37**
STDEV	16.40	0.54	7.58	1.49
RSD (%)	16.47	4.19	8.95	8.58
Reduction (%)	**--**	**87.07**	**14.97**	**82.56**

Yeast strain	**522**	**522^{EC-}**	**522^{D3}**	**522^{EC-D3}**
Replicate 1	77.05	11.6	85.85	16.12
Replicate 2	104.01	12.17	82.17	13.07
Replicate 3	85.11	18.03	67.57	13.51
Average (n=3)	**88.72**	**13.93**	**78.53**	**14.23**
STDEV	13.84	3.56	9.67	1.65
RSD (%)	15.60	25.56	12.31	11.60
Reduction (%)	**--**	**84.30**	**11.49**	**83.96**

4 DISCUSSION

4.1 Constitutive expression of the *DUR1,2* cassette reduces EC production in wine and Sake

A common theme throughout the history of mankind has been the lack of effective solutions to problems until the advent of certain technologies. Such has been the case for the problem of EC in fermented foods and beverages, more specifically EC in grape and Sake wine. Since its discovery and subsequent characterization as a carcinogen in the mid 20th century (Nettleship, Henshaw and Meyer 1943), EC has been found ubiquitously in almost all wines and spirits (Canas, et al. 1989; Coulon, et al. 2006). Although the actual health implications of EC consumption by humans has yet to be definitively established, the Canadian government has imposed a legal limit (30 μg/L) and the US FDA has imposed a voluntary limit (15 μg/L) on the EC content of wines (Butzke and Bisson 1998). Furthermore, several agencies, including the National Institute of Health's National Toxicology Programme and the University of California Davis Department of Enology and Viticulture, have published preventative action manuals for limiting the EC content of wines and spirits (Butzke and Bisson 1998).

Despite the mandatory and voluntary limits imposed on the EC content in food and beverages, its presence continues to be a pervasive problem. A recent survey by our group found that of 20 randomly chosen, commercially available wines from six wine producing countries, 14 and 17 exceeded the Canadian and US limits, respectively (Coulon, et al. 2006). It is therefore obvious that current methods for 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 (Kodama, et al. 1994; Ough and Trioli 1988), and, to a lesser extent, the metabolic engineering of *CAR1* deficient yeast strains was exploited to limit EC in Sake (Kitamoto, et al. 1991; Park, Shin and Woo 2001; Yoshiuchi, Watanabe and Nishimura 2000).

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 by 89% (Coulon, et al. 2006). This result, which is a consequence of the constitutive expression of an otherwise inactive urea amidolyase (*DUR1,2*) gene, far exceeds any other method of EC reduction and does not lengthen production time or

incur any additional costs to winemakers/consumers. The metabolically enhanced 522 strain contains a constitutively expressed *DUR1,2* gene, no antibiotic resistance marker genes or foreign DNA and is thus non-transgenic and 'self-cloned'. This yeast has received approval from the FDA, Health Canada, and Environment Canada for commercial wine production.

4.2 Integration of the *DUR1,2* cassette into the genomes of Sake yeast strains K7 and K9 yielded the functional urea degrading Sake yeasts $K7^{EC-}$ and $K9^{EC-}$

S. cerevisiae produces wine with varying amounts of residual urea, mainly due to NCR of its urea amidolyase encoding gene (*DUR1,2* - YBR208C); synthesis of the urea degrading urea amidolyase enzyme is thus prevented and urea is excreted in to the wine (Kodama, et al. 1994; Monteiro and Bisson 1991; Monteiro, Trousdale and Bisson 1989; Ough, et al. 1990; Ough, et al. 1991; Stevens and Ough 1993). The successful integration of the *DUR1,2* cassette (Figure 15) (Coulon, et al. 2006) into the *URA3* locus of the popular Sake yeast strains K7 and K9 yielded the urea degrading strains $K7^{EC-}$ and $K9^{EC-}$. In addition to the *URA3* flanking sequences required for homologous recombination, the *DUR1,2* cassette contains the *S. cerevisiae DUR1,2* gene under the control of the constitutive *S. cerevisiae PGK1* promoter and terminator signals.

4.2.1 Genetic characterization of $K7^{EC-}$ and $K9^{EC-}$

The targeted integration of the *DUR1,2* cassette into the *URA3* locus (YEL021W) was confirmed on Southern blots hybridized with both *DUR1,2* and *URA3* (Figures 16 and 17). These blots revealed that $K7^{EC-}$ and $K9^{EC-}$ both contain a single copy of the ~9.1 kb linear *DUR1,2* cassette integrated into one of their *URA3* loci. Genomic hybridization also confirmed that both the diploid strains $K7^{EC-}$ and $K9^{EC-}$ retained a non-disrupted *URA3* locus, thus maintaining their uracil prototrophy. Nutritional prototrophy is important for industrial yeasts as they are required to ferment substrates with highly variable nutrient contents.

As the *DUR1,2* cassette does not contain any antibiotic resistance markers, no positive selection method was available to identify transformants that carried the integrated cassette; as a result, urea degrading yeasts were initially identified by colony PCR. Screening was completed with the assistance of

the co-transforming plasmid pUT332 which contains both the *bla* and Tn5*ble* resistance markers (Ampicillin and phleomycin, respectively); pUT332 was used in order to reduce the number of transformants that had to be screened by colony PCR. By successive subculturing on non-selective media, the plasmid was lost and this was confirmed on Southern blots hybridized with the *bla* and Tn5*ble* genes (Figure 18). Sake strains $K7^{EC-}$ and $K9^{EC-}$ are the first metabolically engineered Sake yeast strains to be constructed without the integration of antibiotic resistance markers or *E. coli* vector sequences, thus making them suitable for commercialization; these strains will be more likely to be accepted by consumers than strains containing antibiotic resistance marker genes or foreign DNA.

One strand of the integrated *DUR1,2* cassettes in $K7^{EC-}$ and $K9^{EC-}$ was sequenced. Upon comparison to previously published sequences (Coulon, et al. 2006), a single nucleotide difference was observed in the sequence of $K9^{EC-}$ (Table 8). This single nucleotide difference was localized to the 5' *URA3* flanking region of the cassette and is likely due to a genetic polymorphism between the Sake strain K9 and the wine strain 522. No differences were observed in the *DUR1,2* coding region or the *PGK1* promoter and terminator signals.

Analysis of *DUR1,2* expression during wine fermentation revealed that integration of the *DUR1,2* cassette indeed relieves repression of *DUR1,2* by NCR, as the *PGK1* promoter is much stronger than the inducible/repressible, NCR sensitive *DUR1,2* promoter. *DUR1,2* mRNA was approximately 10-fold more abundant in $K7^{EC-}$ and $K9^{EC-}$ than in their respective parent strains (Figure 20).

The global gene expression pattern of the engineered strain $K7^{EC-}$ as compared to the pattern of the parent strain K7 was studied at 24 hours into Chardonnay must fermentation. Besides *DUR1,2* (6.35-fold overexpression), 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 5795 ORFs (4692 verified and 1103 uncharacterized, SGD, March, 2008) in the yeast cell. One gene of interest that was upregulated ≥ 4-fold in $K7^{EC-}$ (Table 10) was *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 upregulated (5.67-fold) in the engineered strain $K7^{D3}$ which contains the *DUR3* gene under the control of the *PGK1* promoter and

terminator signals (Table 17). The data also suggests that no metabolic pathways were affected by the presence of the *DUR1,2* cassette integrated into $K7^{EC-}$; however, three (*RME1*, *SSP1*, *SDS3*) of the seven genes downregulated in $K7^{EC-}$ are involved in different aspects of meiosis/sporulation (Table 10). 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.

4.2.2 The Sake yeasts $K7^{EC-}$ and $K9^{EC-}$ conduct efficient alcoholic fermentations

As measures of substantial equivalence, fermentation rates, glucose/fructose utilization and ethanol production was evaluated in the metabolically engineered strains $K7^{EC-}$ and $K9^{EC-}$. Prior to commercialization, engineered yeasts must obtain approval from regulatory agencies such as the FDA, Health Canada, and Environment Canada. In North America, approval is granted on the basis of substantial equivalence, which means that should a foodstuff from a genetically modified organism (GMO) be proven to be 'as safe as' the food produced by traditional means, then both should be treated equally (Kessler 1992). Proof of substantial equivalence and safety for a genetically engineered organism generally requires detailed data regarding the organism's genotype, phenotype, transcriptome, proteome, and metabolome.

In both Chardonnay must and Sake mash, $K7^{EC-}$ and $K9^{EC-}$ conducted efficient alcoholic fermentations (Figures 21 and 22), comparable to those of the parental strains K7 and K9; fermentations were completed in ~300 hours in Chardonnay wine and ~500-600 hours in Sake wine. Furthermore, residual glucose and fructose in Sake wine produced with $K7^{EC-}$ and $K9^{EC-}$ (0.05, 0.010 g/L glucose and 0.72, 0.30 g/L fructose, respectively) and the parental strains K7 and K9 were comparable (0.06, 0.08 g/L glucose and 0.65, 0.31 g/L fructose, respectively). All four yeasts produced similar amounts of ethanol in Sake wine (Table 11).

4.2.3 The Sake yeasts $K7^{EC-}$ and $K9^{EC-}$ reduce EC poorly in Chardonnay wine, yet efficiently in Sake wine

The two functionally enhanced Sake yeasts $K7^{EC-}$ and $K9^{EC-}$ were evaluated for their ability to reduce EC content in both Chardonnay wine and Sake wine. In Chardonnay wine, $K7^{EC-}$ and $K9^{EC-}$ were

ineffective in reducing EC; $K7^{EC-}$ and $K9^{EC-}$ reduced EC by 30% and 0%, respectively (Table 12). In contrast, $K7^{EC-}$ and $K9^{EC-}$ both effectively reduced EC by 68% in Sake wine (Table 13).

During the course of Sake fermentations, yeast cells are exposed to a substantially different set of environmental conditions and stresses than those experienced during wine fermentation. Specialized Sake yeast strains have been selected for the production of Sake wine (Shobayashi, et al. 2007) and fermentation conditions undoubtedly play a role in Sake yeast metabolism. While several environmental parameters may play a role in explaining the ineffective EC reduction of Sake strains $K7^{EC-}$ and $K9^{EC-}$ during Chardonnay wine making (Tables 12 and 13), the effect of yeast available nitrogen on native NCR controlled genes is likely the predominant factor.

The type and quantity of yeast assimilable nitrogen (YAN) in Sake mash is substantially different from grape must. Grape must of almost all varietals is usually high in free arginine and proline (Kliewer 1970). In contrast, Sake mash is rich in polypeptides that form structures known as protein bodies (PB) (Kizaki, et al. 1991). These protein bodies, which fall into two major categories (PB-I and PB-II), are primarily composed of prolamins and glutelins, respectively (Hashizume, et al. 2006). During the course of the fermentation yeast draw on the pool of free amino acids which is replenished by degradation of PB by koji-provided acid carboxypeptidases and acid proteases (Hashizume, et al. 2006; Iemura, et al. 1999a; Iemura, et al. 1999b). This controlled release process, which may limit the large scale induction of certain amino acid catabolic enzymes, functions to prevent nitrogen exhaustion (Wu, et al. 2006) and subsequent TOR mediated transcriptional reprogramming leading to the de-repression of NCR sensitive genes (Cooper 2002; Hofman-Bang 1999).

In contrast to the nitrogen available in Sake mash, yeasts draw on the large pool of free amino acids in grape must to create biomass during fermentation. However, at approximately 2-4 days into the fermentation, cells stop dividing and enter a stationary growth phase in which they ferment actively. While there is new evidence to suggest that ethanol stress may play a role transitioning into stationary phase (Marks, et al. 2003; Marks, et al. 2008), the general consensus in the literature, however, is that nitrogen exhaustion is the primary reason why yeast cells enter into stationary phase (Hauser, et al. 2001; Rossignol, et al. 2003; Shobayashi, et al. 2007; Wu, et al. 2006). Nitrogen exhaustion, which is detected by the TOR pathway, is accompanied by large scale transcriptional changes leading to the de-

repression of genes involved in the catabolism of poor nitrogen sources, including urea (Cooper 2002; Hofman-Bang 1999). Interestingly, the second most abundant amino acid in grape must (Kliewer 1970), proline, is not metabolized during fermentation. Proline catabolism requires molecular oxygen and, as fermentations are anaerobic, proline cannot be utilized (Ingledew, Magnus and Sosulski 1987). Thus, after nitrogen exhaustion shifts cells into stationary phase, the high concentration of proline in the ferment likely reinforces, sustains and strengthens the transcriptional reprogramming associated with NCR de-repression.

Of particular interest to EC reduction is the de-repression of the NCR sensitive urea amidolyase encoding gene *DUR1,2*. Induction of *DUR1,2* allows yeast to degrade urea in an increasingly nitrogen scarce environment. Thus, during the later stages of wine fermentation, *DUR1,2* can be induced and maintained throughout the fermentation as long as yeast are starved for nitrogen. As a result, parental strains produce less EC overall since they tend to induce *DUR1,2* at the middle/end of the fermentation, thereby reducing the effectiveness of functionally enhanced clones (Table 12). During Sake brewing however, no nitrogen exhaustion is experienced by yeast cells and *DUR1,2*, along with the other NCR sensitive genes, remains largely repressed, resulting in higher absolute EC values and an increase in the effectiveness of the metabolically enhanced yeasts containing the constitutive *DUR1,2* cassette (Table 13). Data obtained from global gene expression studies during grape wine and Sake wine fermentations confirm that, during grape wine fermentation, the native *DUR1,2* and *DUR3* genes are both induced at the transition into stationary phase (Rossignol, et al. 2003); this is consistent with nitrogen exhaustion and subsequent transcriptional reprogramming inducing stationary phase. In Sake wine making, *DUR1,2* and *DUR3* are transcriptionally inactive throughout the course of fermentation (Wu, et al. 2006), which is consistent with an adequate nitrogen supply.

Another difference between grape must and rice mash concerns osmotic stress. As a result of the continuous saccharification process during Sake brewing, (Figure 4), yeasts are subjected to substantially less osmotic stress during Sake fermentation than during wine fermentation where all of the sugars are present at inoculation (Takagi, et al. 2005; Wu, et al. 2006). This reduced stress during Sake fermentations is thought to play a role in the ability of Sake yeasts to produce up to 20% (w/v) ethanol in Sake wine (Shobayashi, et al. 2007). During osmotic stress, yeast cells activate the 'high osmolarity growth' (HOG) pathway which allows cells to survive osmotic stress via the biosynthesis and

intracellular retention of small molecules such as trehalose and glycerol (Westfall, Ballon and Thorner 2004). These molecules help to offset the osmotic pressure created by the high concentration of extracellular sugars (often 20-30% w/v, equimolar amounts of glucose and fructose in grape must), which would otherwise crenate the cells. Although *S. cerevisiae* has evolved to tolerate substantial osmotic stress, induction and maintenance of tolerance mechanisms requires significant energy expenditure that could otherwise be devoted to biomass creation and other metabolic processes (Wu, et al. 2006). For Sake yeast, which has evolved in a niche of markedly reduced osmotic stress, the osmotic shock in grape must may be a factor in explaining the poor EC reduction of engineered Sake strains during wine making. Specifically, the yeast stress response tends to down-regulate translation globally, which in turn could decrease the levels of *DUR1,2*p (Cooper 2002; Hauser, et al. 2001; Rossignol, et al. 2003).

Finally, the addition of koji to Sake fermentations plays an important role in the availability of some vital auxiliary factors. One such factor is ergosterol, a cholesterol derivative compound present in yeast cell membranes that is vital for ethanol tolerance (Inoue 2000). Ethanol causes rigidity of the cell membrane and induces fatal cracks; ergosterol, like cholesterol, functions to decrease the packing density of membrane phospholipids and increase membrane fluidity, thus counteracting the effects of ethanol (Inoue 2000). Yeast cells require oxygen to synthesize ergosterol (Jahnke 1983), and during anaerobic fermentations this dependency on ergosterol may be a limiting factor on cell viability and ethanol production. In fact, wine fermentations are often oxygenated briefly prior to or during fermentation in order to facilitate the production of ergosterol (Rossignol, et al. 2003). In Sake fermentations however, ergosterol is supplied primarily from koji which are cultured aerobically (Wu, et al. 2006), and as such Sake yeast may have evolved to be dependent on this external source of ergosterol. In fact, Sake yeast strains were shown to induce all of the ergosterol biosynthetic genes during the course of Sake fermentation thus indicating an unexpected shortage of ergosterol despite the presence of koji derived ergosterol (Wu, et al. 2006). Thus, when functionally enhanced Sake yeasts are used to ferment grape must which is not oxygenated, the lack of ergosterol contribution from koji may play a role in a reduction in cell health, viability and EC reduction.

Given the observed differences in the EC reduction of the functionally enhanced yeasts $K7^{EC-}$ and $K9^{EC-}$ in grape must and Sake mash, it is imperative that, in order to gather the most relevant data, the

functionality of engineered yeasts be tested in their niche environments; this should prevent the identification of false negatives and will expedite commercialization of new strains.

4.3 Constitutive expression of the urea permease, *DUR3*, in yeast cells is a viable alternative method to reduce EC in fermented alcoholic beverages

The most efficient metabolically enhanced wine yeast 522^{EC-}, which contains the *DUR1,2* cassette integrated into the *URA3* locus, reduced EC by 89% in Chardonnay wine (Coulon, et al. 2006). In order to further improve EC reduction, we created yeast strains that acted as 'urea sponges', reabsorbing any urea secreted during fermentation or urea native to the must. This goal was completed through the constitutive expression of the urea permease *DUR3* (YHL016C), a NCR sensitive gene native to *S. cerevisiae* (Cooper and Sumrada 1975; ElBerry, et al. 1993; Sumrada, Gorski and Cooper 1976). Due to the abundance of other high quality nitrogen sources during primary fermentation, there is no need for yeast cells to actively import urea, thus the urea transporter is transcriptionally silenced. Given the success of the constitutively expressed *DUR1,2* cassette previously characterized by our group (Coulon, et al. 2006), we chose to utilize similar methodology in order to transcriptionally activate and constitutively express *DUR3* in wine and Sake yeasts.

4.3.1 Construction of a linear *PGK1p-DUR3-PGK1t-kanMX* cassette for integration into the *TRP1* locus of wine and Sake yeasts

Although constitutive expression of *DUR3* could have been more easily achieved through an episomal plasmid expression system, creation of a linear cassette for integration into the yeast genome was a more attractive option. The main problem with a plasmid borne system is that without a positive selection pressure, such as the addition of antibiotics to the must, plasmid loss in *S. cerevisiae* generally occurs within 3-5 generations (Jones, Pringle and Broach 1992) thereby reverting engineered strains to wild type. Coupled with the nutrient prototrophy of industrial yeasts, positive selection during winemaking is extremely impractical as grape must/rice mash is essentially a 'rich' medium for yeast growth. Furthermore, addition of antibiotics to the fermentation substrate is undesirable and negates the goal of creating 'self-cloned' yeast strains. Additionally, *S. cerevisiae* is highly amenable to gene

uptake and integration via homologous recombination (Ausubel, et al. 1995; Griffiths et al., 2005), making precise manipulation, replacement, and deletion of genes in the genome possible.

In order to create a linear cassette for the constitutive expression of *DUR3* (Figure 25), the *DUR3* ORF was placed under the control of the *S. cerevisiae PGK1* promoter and terminator signals (Figure 10). *PGK1* (YCR012W) encodes 3-phosphoglycerate kinase (EC 2.7.2.3) which is a key enzyme in the glycolytic pathway responsible for transferring the acyl phosphate from 1,3-bisphosphoglycerate to ADP thereby creating one molecule of energy rich ATP (Blake 1981; Hitzeman 1980; Lam 1977). Being a key enzyme in the ubiquitous process of glycolysis, *PGK1*, while technically inducible, is constitutively expressed so long as yeast are grown in the presence of glucose (Lam 1977). During both wine and Sake fermentation glucose is abundant and cells never experience carbon exhaustion (Marks, et al. 2008; Wu, et al. 2006). Additionally, as glycolysis is essential during fermentation (Lam 1977), placing *DUR3* under the control of *PGK1* ensured constitutive expression throughout the course of fermentation.

As this section of the research was to be a proof of concept study only, the antibiotic resistance marker *kanMX* was used in order to simplify the selection of positive clones (Figure 11). A positive selection marker allowed us to distinguish *DUR3* clones from untransformed cells thus eliminating the need for costly and time consuming colony PCR selection. While this approach was suitable for this study, it will be necessary to construct an antibiotic resistance free cassette similar to that of *DUR1,2* when *DUR3* strains are developed for commercial purposes.

In order to target the linear *DUR3* expression cassette to a specific locus in the genome the *PGK1p-DUR3-PGK1t-kanMX* cassette was flanked on either side with 300 bp of *TRP1* homology and successfully integrated into the *TRP1* locus (Figure 13). While homologous recombination in *S. cerevisiae* is possible in laboratory strains with as few as 40 nucleotides of flanking homology (Baudin 1993; Manivasakam 1995), we used 300 homologous nucleotides on either end of the *DUR3* cassette to ensure efficient and specific integration into the *TRP1* locus. Shorter regions of homology increases the likelihood that strain sequence polymorphisms will reduce integration efficiency. Efficiencies increase 30- to 50-fold when flanking sequences of several hundred base pairs in length are used (Wach 1996).

While the *DUR3* cassette could have been integrated into any locus, the *TRP1* locus was chosen for a number of reasons. Firstly, *TRP1* is a well characterized and common auxotrophic marker (Hampsey 1997; Mortimer 1966; Stolz 1998). Secondly, *TRP1* is closely located (1 cM) to the centromere of chromosome four (Mortimer 1966) and is thus highly stable, as genetic recombination during meiosis is proportional to a locus' distance from the centromere (Griffiths, et al., 2005). As a result of this genetic stability, engineered strains would be suitable for active dry yeast production and industrial use.

Prior to integration into test strains, the *DUR3* cassette in the plasmid pUCMD was sequenced (single strand) to confirm its structure; analysis revealed that the plasmid contained all the desired DNA fragments in the correct order and orientation (Figure 14). Furthermore, sequencing data revealed nine single nucleotide changes along the length of cassette (Table 14 and Figure 23); however, all four mismatches in the *DUR3* coding region were silent and the deduced DUR3p was identical in amino acid sequence and length to that published for DUR3p on SGD. Five of the nine base pair mismatches localized to the *PGK1* promoter; four were C to T conversions. Given that the *in silico* sequence was assembled from single pass, single strand sequencing, the most logical explanation for the mismatches is sequencing error. This conclusion is supported by the fact that none of the identified mismatches in the *PGK1* promoter were previously reported in the utilization of the *PGK1* promoter for constitutive expression (Coulon, et al. 2006; Husnik, et al. 2006; Volschenk, et al. 1997). Furthermore, during characterization of the wine yeast ML01 (Husnik, et al. 2006), two mismatches that were found in one copy of the *PGK1* promoter were absent in the other copy (Husnik, et al. 2006), indicating that the sequence of the *PGK1* promoter may be prone to sequencing errors. Given that the integrated *DUR3* cassette was fully functional, further sequencing of the cassette to identify bona fide mismatches was deemed non-essential.

4.3.2 Integration of the *DUR3* cassette into the genomes of K7, $K7^{EC-}$, 522, and 522^{EC-} yielded the functional urea transporting yeasts $K7^{D3}$, $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3}

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, thus leading to high EC. In order to constitutively

express *DUR3* throughout fermentation, the *DUR3* cassette (Figure 25) was integrated into the *TRP1* locus of K7, $K7^{EC-}$, 522, and 522^{EC-}, which yielded the functionally enhanced strains $K7^{D3,}$ $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3} (Table 16). The enhanced strains were genetically, phenotypically, and functionally characterized.

4.3.2.1 Integration of the *DUR3* cassette into the genomes of $K7^{D3}$, $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3} results in constitutive expression of *DUR3*. Correct integration of the *DUR3* cassette into the *TRP1* locus was confirmed by Southern blots probed with both *DUR3* and *TRP1* fragments. *S. cerevisiae* strains $K7^{D3}$, $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3} were all shown to contain a single copy of the ~6.5 kb linear *DUR3* cassette integrated in their *TRP1* loci (Figure 26). Blotting also confirmed that the diploid strains $K7^{D3}$, $K7^{EC-D3}$, 522^{D3}, and 522^{EC-D3} retained a non-disrupted *TRP1* locus, thus maintaining their tryptophan prototrophy and wild type phenotype.

In the *DUR3* cassette, expression of *DUR3* is controlled by the *PGK1* promoter and terminator signals. As was previously observed (Section 4.2.1) during the characterization of *DUR1,2* cassette clones, *PGK1* is a strong promoter capable of driving constitutive expression under NCR conditions. To confirm the constitutive expression of *DUR3* from the integrated *DUR3* cassette, a northern blot was hybridized with a *DUR3* probe; blots of K7, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ total RNA revealed strong expression of *DUR3* in $K7^{D3}$ and $K7^{EC-D3}$ (Figure 29). RNA for the northern blot was isolated from cells 24 hours into a fermentation of Chardonnay must; at 24 hours, quality nitrogen sources are still abundant and thus NCR is still strong. Thus, under the control of the *PGK1* promoter and terminator signals, *DUR3* expression is high in cells containing the integrated *DUR3* cassette despite non-inducing conditions. Quantification of constitutive expression by qRT-PCR revealed a 14- and 7-fold increase in *DUR3* mRNA at 24 hours into Chardonnay must fermentations in $K7^{D3}$ and $K7^{EC-D3}$, respectively (Figure 30). Furthermore, high level expression of both *DUR1,2* and *DUR3* was maintained in $K7^{EC-D3}$ (12- and 7-fold, respectively), which had both the *DUR1,2* and *DUR3* cassettes integrated into its genome. These data suggest that K7, and presumably other strains of *S. cerevisiae*, is tolerant to altered, high level expression of multiple proteins. Coupled with data confirming the functionality of both DUR1,2p (Table 21) and DUR3p (Figure 31), this result validates the metabolic engineering of *S. cerevisiae* for polygenic traits. In strains in which only one cassette (*DUR1,2* or *DUR3*) was integrated, constitutive expression of that gene induced expression of the other gene (Figure 30). These results indicate 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 (constitutive expression of *DUR1,2*), the urea degradation intermediate, allophanate, causes induction of *DUR3*. Allophanate is a known inducer of all *DUR* genes (Cooper 1982; Cooper 2002; Cooper and Sumrada 1975; ElBerry, et al. 1993; Hofman-Bang 1999; Uemura, Kashiwagi and Igarashi 2007; Whitney and Cooper 1972; Whitney, Cooper and Magasanik 1973) and exerts its effect through an upstream induction sequence that binds the transcriptional activators *DAL81* and *DAL82*, and through an upstream activation sequence that binds the NCR GATA factor *GLN3* (Hofman-Bang 1999). Similarly, when cells are actively importing urea (constitutive expression of *DUR3*), the increased intracellular urea concentration induces *DUR1,2* expression such that the intracellular concentration of urea can be lowered before it becomes toxic.

The global gene expression patterns of the metabolically engineered strain $K7^{D3}$ and the parent strain K7 were studied at 24 hours into Chardonnay must fermentation. Besides *DUR3* (36.95-fold overexpression), seven genes were affected ≥ 4-fold; thus, 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 5795 ORFs (4692 verified and 1103 uncharacterized, SGD, March, 2008) in the yeast cell. One gene that was upregulated ≥ 4-fold in $K7^{D3}$ (Table 17) was common to those upregulated in the *DUR1,2* cassette containing engineered strain $K7^{EC-}$ (Table 10); *HAC1* (5.67-fold) encodes a transcription factor involved in the unfolded protein response and is likely needed for increased translation and folding of *DUR3* constitutively expressed from the strong *PGK1* promoter. The data also suggests that no metabolic pathways were affected by the presence of the *DUR3* cassette integrated into $K7^{EC-}$. However, integration of the *DUR3* cassette into $K7^{D3}$ appears to have had some effect on the regulation of meiosis/sporulation, processes controlled by nutrient deficiency (Malone 1990). One gene (*FIG1*) was upregulated and one gene (*TID3*) was downregulated in $K7^{D3}$ (Table 17); both are involved in meiosis/sporulation. As constitutive expression of *DUR3* results in increased nitrogen availability it is reasonable that the *DUR3* cassette would exert some effect on sporulation gene regulation.

4.3.2.2 The integrated *DUR3* cassette results in enhanced urea uptake. In order 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, $K7^{EC-}$, $K7^{D3}$, and $K7^{EC-D3}$ was studied. Under the conditions tested, the strains $K7^{D3}$ and $K7^{EC-D3}$ were both highly

capable of importing ^{14}C-urea while K7 and K7^{EC-} were unable to incorporate any appreciable amounts of ^{14}C-urea (Figure 31). These data indicate that integration of the *DUR3* cassette is responsible for the constitutive expression of an active urea permease (DUR3p) under NCR conditions.

In the ^{14}C-urea uptake assay, the strains K7, K7^{EC-}, K7^{D3}, and K7^{EC-D3} were cultured in an ammonium sulphate minimal medium that results in repression of all NCR sensitive genes (Cooper 1982; Hofman-Bang 1999). In all of the strains (K7, K7^{EC-}, K7^{D3} and K7^{EC-D3}) transcription of *DUR3* from its native promoter was repressed due the presence of ammonium sulphate; however in strains K7^{D3} and K7^{EC-D3}, *DUR3* was expressed despite the presence of ammonium sulphate in the medium due to the presence of the recombinant *DUR3* cassette. Given that the conditions of fermentation are largely repressive to NCR sensitive genes, the efficient uptake of ^{14}C-urea by K7^{D3} and K7^{EC-D3} in conditions of strong NCR is indicative of efficient EC reduction.

Despite integration of the same *DUR3* cassette, K7^{D3} and K7^{EC-D3} exhibited different rates of ^{14}C-urea uptake; K7^{D3} was able to incorporate approximately 3-fold more ^{14}C-urea than K7^{EC-D3} (Figure 31). Since there is no data or rationale to explain why K7^{D3} would be more efficient at urea uptake than K7^{EC-D3}, the most logical explanation is that the constitutive expression of *DUR1,2* in K7^{EC-D3} masks the accumulation of ^{14}C-urea. In order to counteract the influx of toxic urea, K7^{D3} must induce transcription of *DUR1,2* and, in turn, produce functional *DUR1,2*p. *DUR1,2* is known to be controlled at the level of transcription (Genbauffe and Cooper 1986; Genbauffe and Cooper 1991); enzyme activity correlates well with transcript abundance and the half-life of *DUR1,2* transcripts is the same in both the presence and absence of an inducer (Jacobs, Dubois and Wiame 1985). In the case of K7^{EC-D3}, *DUR1,2* is constitutively expressed and the functional enzyme is capable of degrading urea as it is incorporated into the cell. Thus, both K7^{D3} and K7^{EC-D3} likely incorporate urea with equivalent efficiencies, however, this conclusion was not confirmed by experimental data. The same masking phenomenon was observed when comparing the uptake of ^{14}C-urea by K7 and K7^{EC-} (Figure 32).

4.3.2.3 The metabolically engineered yeasts K7^{D3}, K7^{EC-D3}, 522^{D3}, and 522^{EC-D3} ferment at similar rates and produce similar amounts of ethanol in Chardonnay and Sake wine. As measures of substantial equivalence, fermentation rate and ethanol production was evaluated in the metabolically engineered strains K7^{D3}, K7^{EC-D3}, 522^{D3}, and 522^{EC-D3}. In both Chardonnay must and Sake mash, K7^{D3}, K7^{EC-D3}, 522^{D3},

and $522^{EC\text{-}D3}$ conducted substantially equivalent alcoholic fermentations (Figures 35 and 36). Furthermore, the amount of ethanol produced in Chardonnay and Sake wine by $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ was shown to be similar (Tables 18 and 19). These results indicate that the $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ strains are suitable for commercialization from a fermentation standpoint.

4.3.2.4 Variability of metabolically engineered yeasts to effectively reduce EC in Chardonnay wine and in Sake wine. In order to assess the EC reduction potential of the engineered strains $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$, fermentations of both Chardonnay must and Sake rice mash were conducted after which the EC content of the resultant wines was quantified. Consistent with prior observations of $K7^{EC\text{-}}$ (Section 4.2.2), EC reduction by $K7^{EC\text{-}}$ in Chardonnay wine was inefficient (6.85% - Table 20) due to low EC production by the parental strain K7. As expected, production of Sake wine with $K7^{EC\text{-}}$ yielded highly efficient EC reduction (87% - Table 21). Furthermore, $522^{EC\text{-}}$ was effective at reducing EC in both Chardonnay wine and Sake wine (81% and 84%, respectively - Tables 20 and 21).

In Chardonnay wine, $K7^{D3}$ and 522^{D3} reduced EC as efficiently as $K7^{EC\text{-}}$ and $522^{EC\text{-}}$, respectively (Table 20); both $K7^{D3}$ and $K7^{EC\text{-}}$ reduced EC by ~10% while 522^{D3} and $522^{EC\text{-}}$ both reduced EC by ~81%. The observed equivalency in EC reduction is likely a function of the need for cells to degrade urea once it is internalized, as urea is toxic to cells at high concentrations. Presumably, the 3.6-fold induction of *DUR1,2* observed in $K7^{D3}$ (Figure 30), coupled with constitutive urea import, is responsible for the strain's ability to reduce EC as efficiently as $K7^{EC\text{-}}$. Despite the lack of gene expression data, the same rationale is likely responsible for the reduction of EC in Chardonnay wine by 522^{D3}. These data suggest that K7 and 522 are highly sensitive to changes in the expression of genes involved in urea metabolism and that engineering yeasts to induce large scale expression changes (greater than 5-fold) may be unnecessary. Furthermore, these data are important because they validate the application of *DUR3* constitutive expression for the reduction of EC in grape wine, specifically in wine derived from musts with high endogenous urea. In such cases, EC reduction would likely be greatest in wine fermented by urea importing yeasts (*DUR3* cassette) rather than urea degrading yeasts (*DUR1,2* cassette).

In Sake wine, $K7^{D3}$ and 522^{D3} were much less effective in reducing EC than their *DUR1,2* counterparts $K7^{EC\text{-}}$ and $522^{EC\text{-}}$ (Table 21). These results can be explained by the requirements for *DUR1,2* expression in *S. cerevisiae* (Figure 37). In a system analogous to the well characterized *lac* operon in *E.*

coli, NCR in *S. cerevisiae* functions to force cells to metabolize the most energetically favourable nutrients. As such, expression of NCR regulated genes is highly dependent on two conditions being met: the presence of an inducer and the presence of an NCR associated transcriptional activator like *GLN3*. Indeed, expression of *DUR1,2* was shown to be highly dependent on *GLN3* under induced conditions (Cooper, et al. 1990). Likewise, *DUR1,2* expression on a proline medium (no NCR – *GLN3* active) was very low without an inducer present (Cooper, et al. 1990). As discussed previously (Section 4.2.3), fermentations of grape must can be subject to nitrogen exhaustion and thus yeasts may undergo transcriptional de-repression of NCR sensitive genes i.e. *GLN3* becomes activated. During Chardonnay must fermentation $K7^{D3}$ and 522^{D3} constitutively imported urea thus fulfilling the inducer requirement for *DUR1,2* expression. Upon nitrogen limitation, transcriptional reprogramming likely activated *GLN3* and, because of the constitutive presence of urea, *DUR1,2* was induced enabling $K7^{D3}$ and 522^{D3} to degrade urea and reduce EC. In contrast, fermentations of Sake rice mash are not subject to nitrogen exhaustion due to the presence of koji enzymes and rice protein bodies. Although $K7^{D3}$ and 522^{D3} still constitutively imported urea during Sake fermentations, *GLN3* remained inactive due to a plentiful supply of good nitrogen. Thus, without satisfying both conditions for transcriptional activation, the native *DUR1,2* genes remained repressed in $K7^{D3}$ and 522^{D3} and constitutively absorbed urea likely diffused back out of the cell, where it could form EC in the resultant Sake wine. Indeed, the facilitated diffusion system for urea (*DUR4*), which allows leakage from the cell, is energy independent, insensitive to NCR, and present in cells growing in the absence of any inducer (Cooper and Sumrada 1975).

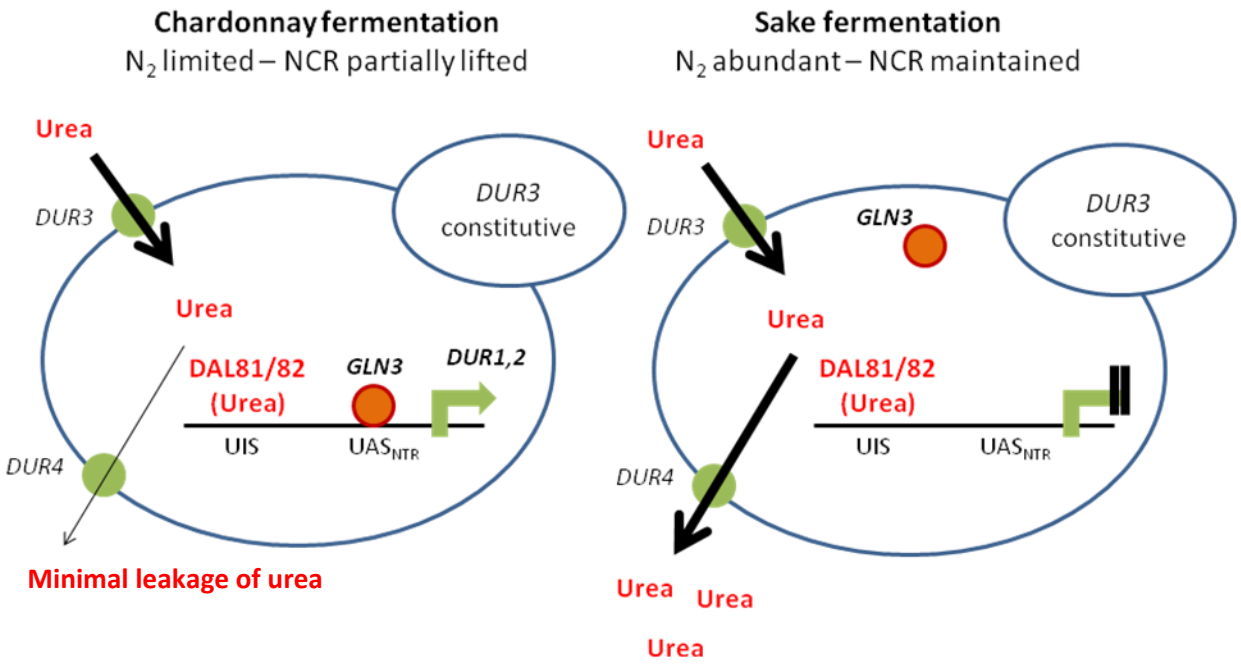


Figure 37. Schematic representation of inducible *DUR1,2* expression during Chardonnay and Sake wine fermentation by a urea importing yeast strain. During Chardonnay fermentation partial activation of *GLN3* coupled with the constitutive presence of the inducer allows for expression of *DUR1,2* and reduction of EC. Despite the constitutive presence of the inducer, constant NCR maintains repression of *DUR1,2* during Sake fermentations and constitutively imported urea is allowed to leak from the cell and form EC.

While Figure 37 explains the most likely reason for the inability of $K7^{D3}$ and 522^{D3} to reduce EC efficiently in Sake wine, there may be other minor issues. Firstly, yeast growth in rice mash may influence the degree of protein mistargeting, a common problem for membrane protein over expression. Unlike soluble proteins, which are translated and kept in the cytosol, membrane proteins must navigate the protein trafficking system which, consequently, is subject to a number of rate limiting steps (Reviewed in Wagner, et al. 2006). In eukaryotes, membrane protein production begins on the rough endoplasmic reticulum (ER) where ribosomes bound to ER translate proteins which are immediately translocated into the ER lumen (Wagner, et al. 2006). From there, proteins can begin to undergo a variety of post-translational modifications (most commonly glycosylation) and quality control checks (Wagner, et al. 2006). Proteins leave the ER by means of COPII coated vesicles that travel along microtubules through the cis-, mid- and trans- Golgi apparatus before being excreted into the plasma membrane via additional vesicles (Wagner, et al. 2006). Since protein trafficking machinery is limited in its speed and efficiency, and must be shared with other essential membrane proteins, excess

overexpressed protein often backs up (Wagner, et al. 2006). Such backups are often dealt with via ubiquitin-proteasome mediated degradation (Wagner, et al. 2006), or, as more often the case in *S. cerevisiae*, excess protein is diverted to the vacuole where it is either stored or degraded (Wagner, et al. 2006). Although expressed from a high copy plasmid rather than an integrated cassette, a recent study of the polyamine transport function of DUR3p reported significant amounts of over expressed DUR3p localizing to the vacuole suggesting a high degree of degradation (Uemura, Kashiwagi and Igarashi 2007). This result was subsequently confirmed by another study looking at optimizing *S. cerevisiae* membrane protein over expression (Newstead, et al. 2007). Thus, if cell growth in rice mash increases DUR3p mistargeting relative to growth in grape must, or, more likely, if cell survival in Sake wine (perhaps due to high ethanol stress and low osmotic stress) requires the expression of a greater number of membrane proteins, the amount of functional DUR3p would be diminished, thus lowering the EC reduction ability.

Finally, Sake brewing may change 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, Kashiwagi and Igarashi 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 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.

4.3.2.5 The metabolically engineered yeasts $K7^{EC\text{-}D3}$ and $522^{EC\text{-}D3}$ do not reduce EC more effectively than $K7^{EC\text{-}}$ and $522^{EC\text{-}}$ or $K7^{D3}$ and 522^{D3} in either Chardonnay or Sake wine. The effect of both the *DUR1,2* and *DUR3* cassettes on EC reduction was evaluated in Chardonnay and Sake wine. Strains $K7^{EC\text{-}D3}$ and $522^{EC\text{-}D3}$ were not capable of producing Chardonnay or Sake wine with less EC than $K7^{EC\text{-}}$ and $522^{EC\text{-}}$ or $K7^{D3}$ and 522^{D3} (Tables 20 and 21). Thus, it seems that constitutive expression of both *DUR1,2* and *DUR3* offers no synergistic advantage over the constitutive expression of either gene alone and that, in the case of metabolic engineering of *S. cerevisiae* to reduce EC in wine, it is prudent to limit engineering to

one gene. Yeast strains constitutively expressing only a single gene are logistically and functionally simpler, more cost effective, and can more expediently receive regulatory approval.

Data from this study suggested that in $K7^{EC\text{-}D3}$, which has both cassettes integrated, high level expression of both *DUR1,2* and *DUR3* was maintained (Figure 30), and that both cassettes produce functional enzymes (Figure 31). Moreover, urea degrading and urea importing yeast strains (integration of the *DUR1,2* and *DUR3* cassettes, respectively) each reduce EC by up to 90% (Tables 20 and 21); however, the lack of synergistic EC reduction in strains constitutively co-expressing *DUR1,2* and *DUR3* suggests the presence of confounding factors which must still be investigated. One such factor may be the induction of the native copies of *DUR3* in the engineered strains at or near the end of fermentation. If and when, at the end of fermentation, yeasts are subject to nitrogen exhaustion, the de-repression of NCR may result in the expression of *DUR3*; if the level of *DUR3* expression from the native promoter is equal or near equal to that of expression from the *PGK1* promoter then it follows that integration of the *DUR3* cassette should not confer any advantage in terms of urea uptake and EC reduction. To test this hypothesis, the ability for yeasts to uptake urea under conditions of NCR de-repression was examined (Figures 33 and 34). These ^{14}C-uptake assays were completed in an L-proline minimal medium to simulate conditions of NCR de-repression. Proline has long been recognized as a poor quality nitrogen source for yeast (Hofman-Bang 1999; Ingledew, Magnus and Sosulski 1987). Under de-repressive conditions, uptake of urea by the parental strain K7 and the engineered strains $K7^{EC\text{-}}$, $K7^{D3}$, and $K7^{EC\text{-}D3}$ largely mimicked uptake under repressive conditions (Figures 31 and 33). Both $K7^{D3}$ and $K7^{EC\text{-}D3}$, which contained the integrated *DUR3* cassette, were highly capable of incorporating ^{14}C-urea while the strains that did not contain the *DUR3* cassette, K7 and $K7^{EC\text{-}}$, were unable to incorporate any appreciable amounts of urea (Figure 33) indicating that constitutive expression of *DUR3* from the *PGK1* promoter and terminator signals does indeed confer an advantage to engineered yeast cells in terms of urea uptake. Furthermore, data obtained from global gene expression studies during grape wine and Sake wine fermentations confirm that during grape wine fermentation *DUR3* is only induced during nitrogen exhaustion (Rossignol, et al. 2003). Moreover, during Sake wine making, *DUR3* is transcriptionally inactive throughout the course of fermentation (Wu, et al. 2006). Taken together, these data indicate that expression of *DUR3* from its native promoter is not likely to be a significant reason for the lack of synergistic EC reduction in engineered strains that constitutively import and degrade urea. Although the de-repressive conditions of the assay permitted full induction of native *DUR3*, the reason for poor urea

uptake in K7 and $K7^{EC-}$ may be the use of stationary phase cells for the assay. In order to express *DUR3* from its native promoter, both an inducer must be present (urea) and NCR must be de-repressed (Hofman-Bang 1999). Despite the de-repression of NCR, the cells were grown in the absence of an inducer thus maintaining low-level *DUR3* expression; only when cells were exposed to ^{14}C-urea were both conditions for *DUR3* expression met.

In grape wine and Sake wine, the major contributor to EC is urea along with ethanol. Post-fermentation treatment of wine and Sake with an acid urease resulted in significant reduction of EC (Ough and Trioli 1988); moreover, fermentation of Sake rice mash with *CAR1* deficient yeast strains has been shown to produce Sake wine with no detectable urea or EC (Kitamoto, et al. 1991; Yoshiuchi, Watanabe and Nishimura 2000). These studies provide strong evidence that the most important precursor for EC is urea derived from *CAR1* mediated arginine degradation; however, in certain circumstances, such as wine that has undergone a MLF, a certain percentage of EC could be derived from alternate sources. While this rationale would explain a lack of synergistic EC reduction in a wine that had undergone MLF, it fails to explain the results of this study.

Besides urea, a number of other compounds have been implicated in the formation of EC. Carbamyl phosphate and citrulline are known to react with ethanol and form EC (Matsudo 1993; Ough 1976b); however, while they have been detected, carbamyl phosphate and citrulline are not usually found in grape or Sake wine (Ough, Crowell and Gutlove 1988). Citrulline and carbamyl phosphate are by-products of arginine metabolism in lactic acid bacteria and are only found in wines that have undergone MLF; in lactic acid bacteria arginine is metabolized via the arginine deiminase pathway in which arginine is degraded to citrulline which is then degraded to ornithine and carbamyl phosphate (Liu, et al. 1994). Utilization of the metabolically engineered yeast strain ML01, which conducts parallel alcoholic and malolactic fermentations without the addition of lactic acid bacteria (Husnik, et al. 2006), could help reduce EC in certain applications. In stone fruit spirits such as fruit brandies, amygdalin from the stone has been shown to degrade into cyanide during fermentation, the oxidized form (cyannate) of which reacts with ethanol to form EC (Schehl 2007). Finally, a number of other compounds, such as N-carbamyl α-amino acids, N-carbamyl β-amino acids, and allantoin (by-product of purine degradation) have been shown to react with ethanol and form EC (Ough, Crowell and Gutlove 1988); however, the contribution of these compounds to EC in alcoholic beverages has yet to be substantiated.

5 CONCLUSIONS

Amongst other reasons, increased awareness surrounding the health benefits of wine consumption has caused the popularity of wine to grow worldwide. Wine, and in particular red wine, has been shown to help prevent cardiovascular disease, dietary cancers, diabetes, hypertension, peptic users, and macular degeneration, amongst others (Bisson 2002). Along with a growing appreciation for the benefits of alcoholic beverage consumption, consumers have become increasingly conscious of consumption risks and therefore have begun to demand increasingly safe products. This mindset echoes beyond alcoholic beverages and is highlighted by the prevalence of recent food safety issues including mad cow disease, pesticide usage, heavy metal contamination, *E. coli* O157:H7, GMO corn, and other GMO crops. Of particular concern to this study is the increasing recognition of EC, a naturally occurring and well characterized carcinogen found in wine, Sake, and many other yeast fermented foods and beverages. Indeed, winemakers and governments have become progressively more cognisant of the widespread prevalence of the EC problem. Spearheaded by the FDA and the University of California at Davis' Department of Enology and Viticulture (Butzke and Bisson 1998), action manuals and data have been complied concerning a wide range of EC reduction techniques. Until recently, none of the techniques employed have been highly effective due to low efficacy, high cost, or lengthened production time. However, in 2006 our group developed a substantially equivalent, self-cloned, FDA approved, industrial wine yeast (522^{EC-}) capable of reducing EC in Chardonnay wine by 89%. The amount of EC in wines is proportional to residual urea, thus making EC reduction a matter of eliminating residual urea at the end of alcoholic fermentation. *S. cerevisiae* strain 522^{EC-} is capable of reducing EC by 89% due to constitutive expression of urea amidolyase (*DUR1,2*) which degrades urea to ammonia and carbon dioxide; the native copy of this gene is normally transcriptionally silent in fermentations with rich nitrogen supplies thus resulting in wines with high residual urea and thus high EC .

Numerous industrial strains of *S. cerevisiae* exist around the world, each filling an environmental niche. Arising from growth on different nutrient sources, strains have developed different fermentative properties and, as such, are uniquely suited to fermentation of different substrates. However, EC is a pervasive problem in all types of alcoholic fermentation and, as a result, EC reduction is important in all yeast strains. The self-cloned Sake yeast strains $K7^{EC-}$ and $K9^{EC-}$ containing the constitutively expressed *DUR1,2* cassette, were ineffective when tested in wine making trials; however, $K7^{EC-}$ and $K9^{EC-}$ were

highly capable of EC reduction during Sake brewing trials. Thus, it is clear that certain yeast strains are uniquely adapted to specific niches and that they are not optimally functional in other niches. The functionality of enhanced yeast strains must therefore be assessed in their native environment in order to most accurately gauge performance and avoid the identification of false negatives.

In an effort to further reduce EC levels beyond the capability of *DUR1,2* technology, this study was also focused on the creation of substantially equivalent, self-cloned, industrial wine yeasts capable of constitutive urea import. In order to create the industrial strains $K7^{D3}$, $K7^{EC\text{-}D3}$, 522^{D3}, and $522^{EC\text{-}D3}$ the *S. cerevisiae* urea permease (*DUR3*) was expressed under the control of the *S. cerevisiae PGK1* promoter and terminator signals and integrated into the *TRP1* locus of the relevant parental strains. DNA sequencing confirmed that the recombinant cassette contained no deleterious mutations or amino acid substitutions. Furthermore, Southern blotting confirmed proper cassette integration into the target *TRP1* locus. Constitutive expression of *DUR3* was confirmed by northern blotting and quantified by qRT-PCR. Global gene expression analysis indicated that the integration of the *DUR3* cassette had a minimal impact on the transcriptome of *S. cerevisiae* and that no major metabolic pathways were affected by the presence of the *DUR3* cassette. Urea import studies using ^{14}C-urea indicated that *DUR3* cassette containing strains indeed express functional DUR3p and that this functionality is preserved when *DUR1,2* and *DUR3* are constitutively co-expressed. Comparisons of small scale Chardonnay and Sake wine making demonstrated that, although constitutive co-expression of *DUR1,2* and *DUR3* does not yield synergistic EC reduction in Chardonnay or Sake wine, constitutive expression of *DUR3* alone is capable of reducing EC as efficiently as constitutive expression of *DUR1,2* in Chardonnay wine yet not in Sake wine. Thus, in grape wine making applications, and specifically in musts which contain high endogenous urea, *DUR3* constitutive expression is an important, valuable, and alternative strategy for EC reduction. A provisional patent has been filed concerning the constitutive expression of *DUR3* in *S. cerevisiae* for the reduction of EC in grape wine. Furthermore, all of the *DUR3* expressing strains can be constructed by self-cloned means, and thus would be more acceptable both worldwide due to their non-transgenic nature, and in countries such as Germany and Japan where self-cloned organisms are not considered as GMO's.

5.1 Future Directions

As a result of this study and previous work by our group (Coulon, et al. 2006), winemakers now have highly effective methods for EC reduction at their fingertips; however, several topics still require investigation. Firstly, in order to facilitate the commercialization of yeast strains constitutively expressing *DUR3*, a cassette must be developed which does not contain any antibiotic resistance markers. Secondly, experiments should be done in order to confirm which of the potential causes discussed in Section 4.3.2.4 are responsible for the constitutive expression of *DUR3* not reducing EC efficiently in Sake wine as it does in Chardonnay wine; answering this question may allow for the subsequent adaptation of *DUR3* technology to Sake making. Thirdly, the development of alternative methods of EC reduction would prove to be useful in further reducing EC beyond 90% thus making wines and spirits safer for consumers. Moreover, as urea and EC production by different strains is highly variable, studies into the molecular mechanisms behind these variations will aid scientists and winemakers in engineering new strains or selecting natural strains which produce little or no EC. Finally, it is imperative that, before the yeasts can be commercialized, the proteome and metabolome of the metabolically enhanced yeast strains described here be characterized as to establish substantial equivalence.

REFERENCES

Ashby, J. (1991) Genotoxicity data supporting the proposed metabolic activation of ethyl carbamate (urethane) to a carcinogen: the problem now posed by methyl carbamate. Mutat. Res. 260, 307-308.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1995) Short Protocols in Molecular Biology, 3rd ed., John Wiley & Sons, Inc., Boston, USA.

Baudin, A. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. Nucl. Acids Res. 21, 3329-3330.

Bisson, L.F. (2002) The present and future of the international wine industry. Nature 418, 696-699.

Blake, C. C. (1981) Phosphoglycerate kinase. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 293, 93-104.

Bond, U., Neal, C., Donnelly, D. and James, T. (2004) Aneuploidy and copy number breakpoints in the genome of lager yeasts mapped by microarray hybridisation. Curr. Genet. 45, 360-370.

Bruckner, R. and Titgemeyer, F. (2002) Carbon catabolite repression in bacteria: choice of the carbon source and autoregulatory limitation of sugar utilization. FEMS Microbiol. Lett. 209, 141-148.

Butzke, C.E. and Bisson, L.F. (1998) Ethyl Carbamate Preventative Action Manual. 2008. (http://www.cfsan.fda.gov/~frf/ecaction.html).

Bysani, N., Daugherty, J. R. and Cooper, T. G. (1991) Saturation mutagenesis of the UASNTR (GATAA) responsible for nitrogen catabolite repression-sensitive transcriptional activation of the allantoin pathway genes in *Saccharomyces cerevisiae*. J. Bacteriol. 173, 4977-4982.

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. J.N. Strathern, E.W. Jones (ed.), pp. 39-99, Cold Spring Harbor Press, New York.

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., Ferguson, D., Rai, R. and Bysani, N. (1990) The *GLN3* gene product is required for transcriptional activation of allantoin system gene expression in *Saccharomyces cerevisiae*. J. Bacteriol. 172, 1014-1018.

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.

Cox, K. H., Rai, R., Distler, M., Daugherty, J. R., Coffman, J. A. and Cooper, T. G. (2000) *Saccharomyces cerevisiae* GATA sequences function as TATA elements during nitrogen catabolite repression and when Gln3p is excluded from the nucleus by overproduction of Ure2p. J. Biol. Chem. 275, 17611-17618.

Cox, K. H., Kulkarni, A., Tate, J. J. and Cooper, T. G. (2004) *Gln3* Phosphorylation and Intracellular Localization in Nutrient Limitation and Starvation Differ from Those Generated by Rapamycin Inhibition of *Tor1/2* in *Saccharomyces cerevisiae*. J. Biol. Chem. 279, 10270-10278.

Dahl, G., Miller, J. and Miller, E. (1978) Vinyl carbamate as a promutagen and a more carcinogenic analog of ethyl carbamate. Cancer Res. 38, 3793-3804.

Dann, S. G. and Thomas, G. (2006) The amino acid sensitive TOR pathway from yeast to mammals. FEBS Letters 580, 2821-2829.

De Virgilio, C. and Loewith, R. (2006) The TOR signalling network from yeast to man. Int. J. Biochem. Cell Bio. 38, 1476-1481.

Dohmen, R. J. and Varshavsky, A. (2005) Heat-inducible degron and the making of conditional mutants. Methods Enzymol. 399, 799-822.

Dunn, B., Levine, R. P. and Sherlock, G. (2005) Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. BMC Genomics 6, 53-74.

Eckhardt, T. (1978) A rapid method for the identification of plasmid desoxyribonucleic acid in bacteria. Plasmid 1, 584-588.

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.

Farrell A., E., Plevin R., J., Turner B., T., Jones A., D., O'Hare, M. and Kammen D., M. (2006) Ethanol Can Contribute to Energy and Environmental Goals. Science 311, 506-508.

Gancedo , J. M. (1992) Carbon catabolite repression in yeast. Eur. J. Biochem. 206, 297-313.

Gatignol, A. (1987) Phleomycin resistance encoded by the *ble* gene from transposon Tn 5 as a dominant selectable marker in *Saccharomyces cerevisiae*. Mol. Gen. Genetics 207, 342-348.

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

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.

Gietz, R. D. and Woods, R. A. (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. 350, 87-96.

Goffeau, A., Barrell, B. G., Bussey, H., Davis, R. W., Dujon, B., Feldmann, H., Galibert, F., Hoheisel, J. D., Jacq, C., Johnston, M., Louis, E. J., Mewes, H. W., Murakami, Y., Philippsen, P., Tettelin, H. and Oliver, S. G. (1996) Life with 6000 Genes. Science 274, 546-567.

Goode, J. (2005) The Science of Wine: From Vine to Glass, University of California Press, Berkley, California.

Greig, D. and Travisano, M. (2003) Evolution: Haploid Superiority. Science 299, 524-525.

Griffiths, A. J. F., Miller, J. H., Suzuki, D. T., Lewontin, R. C., and Gelbart, W. M. (2005) An Introduction to Genetic Analysis. Eight ed., W. H. Freeman, New York, USA.

Guengerich, F. P. and Kim, D. H. (1991) Enzymatic oxidation of ethyl carbamate to vinyl carbamate and its role as an intermediate in the formation of 1,N^6-ethenoadenosine. Chem. Res. Toxicol. 4, 413-421.

Guldener, U., Heck, S., Fielder, T., Beinhauer, J. and Hegemann, J. (1996) A new efficient gene disruption cassette for repeated use in budding yeast. Nucl. Acids Res. 24, 2519-2524.

Hampsey, M. (1997) A review of phenotypes in *Saccharomyces cerevisiae*. Yeast 13, 1099-1133.

Han, J., Lee, J., Bibbs, L. and Ulevitch, R. (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265, 808-811.

Hashizume, K., Okuda, M., Sakurao, S., Numata, M., Koseki, T., Aramaki, I., Kumamaru, T. and Sato, H. (2006) Rice protein digestion by sake koji enzymes: comparison between steamed rice grains and isolated protein bodies from rice endosperm. J. Biosci. Bioeng. 102, 340-345.

Hauser, N., Fellenberg, R., Gil, R., Bastuk, S., Hoheisel, J. and Perez-Ortin, J. (2001) Whole genome analysis of a wine yeast strain. Comp. Funct. Genomics 2, 69-79.

Herskowitz, I. (1988) Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. 52, 536-553.

Hitzeman, R. A. (1980) Isolation and characterization of the yeast 3-phosphoglycerokinase gene (*PGK*) by an immunological screening technique. J. Biol. Chem. 255, 12073-12080.

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

Hughes, T., Roberts, C., Dai, H., Jones, A., Meyer, M., Slade, D., Burchard, J., Dow, S., Ward, T., Kidd, M., Friend, S. and Marton, M. (2000) Widespread aneuploidy revealed by DNA microarray expression profiling. Nat. Genet. 25, 333-337.

Husnik, J. I., Volschenk, H., Bauer, J., Colavizza, D., Luo, Z. and van Vuuren, H. J. J. (2006) Metabolic engineering of malolactic wine yeast. Metabol. Eng. 8, 315-323.

Iemura, Y., Takahashi, T., Yamada, T., Furukawa, K. and Hara, S. (1999a) Properties of TCA-Insoluble peptides in Kimoto (traditional seed mash for sake brewing) and conditions for liberation of the peptides from rice protein. J. Biosci. Bioeng. 88, 531-535.

Iemura, Y., Yamada, T., Takahashi, T., Furukawa, K. and Hara, S. (1999b) Properties of the peptides liberated from rice protein in Sokujo-moto. J. Biosci. Bioeng. 88, 276-280.

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.

Ingledew, W. M., Magnus, C. A. and Sosulski, F. W. (1987) Influence of Oxygen on Proline Utilization During the Wine Fermentation. Am. J. Enol. Vitic. 38, 246-248.

Inoue,T . (2000) Cloning and Characterization of a Gene Complementing the Mutation of an Ethanol-sensitive Mutant of Sake Yeast. Biosci. Biotech. Biochem. 64, 229-236.

Jacobs, E., Dubois, E. and Wiame, J. M. (1985) Regulation of urea amidolyase synthesis in *Saccharomyces cerevisiae*, RNA analysis, and cloning of the positive regulatory gene *DURM*. Curr. Genet. 9, 333-339.

Jahnke, L. (1983) Oxygen requirements for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. J. Bacteriol 155, 488-492.

Jones E. W., Pringle J. R., Broach J. R. (1992) The Molecular and Cellular Biology of the Yeast *Saccharomyces*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA.

Kessler, D. A. (1992) The safety of foods developed by biotechnology. Science 256, 1747-1749.

Kinzy, T. G. (1994) Multiple genes encode the translation elongation factor EF-1 gamma in *Saccharomyces cerevisiae*. Nucl. Acids Res. 22, 2703-2707.

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. (1970) Free amino acids and other nitrogenous fractions in wine grapes. J. Food Sci. 35, 17-21.

Kodama, K. (1993) Sake-brewing yeast; in The Yeasts, Rose, A. H. and Harrison, J. S. (eds.), pp. 129-168, Academic Press, London, United Kingdom.

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.

Lam, K. B. (1977) Isolation and characterization of *Saccharomyces cerevisiae* glycolytic pathway mutants. J. Bacteriol 130, 746-749.

Leithauser, M. T., Liem, A., Stewart, B. C., Miller, E. C. and Miller, J. A. (1990) 1,N^6-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.

Liu, S., Pritchard, G. G., Hardman, M. J. and Pilone, G. J. (1994) Citrulline Production and Ethyl Carbamate (Urethane) Precursor Formation From Arginine Degradation by Wine Lactic Acid Bacteria *Leuconostoc oenos* and *Lactobacillus buchneri*. Am. J. Enol. Vitic. 45, 235-242.

Lohr, D., Venkov, P. and Zlatanova, J. (1995) Transcriptional regulation in the yeast *GAL* gene family: a complex genetic network. FASEB J. 9, 777-787.

Maftahi, M., Gaillardin, C. and Nicaud, J. M. (1998) Generation of *Saccharomyces cerevisiae* deletants and basic phenotypic analysis of eight novel genes from the left arm of chromosome XIV. Yeast 14, 271-280.

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

Manivasakam, P. (1995) Micro-homology mediated PCR targeting in *Saccharomyces cerevisiae*. Nuc. Acids Res. 23, 2799-2800.

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

Marks, V. D., van der Merwe, G., van Vuuren, H. J. J. (2003) Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Research 3, 269-287.

Matsudo, T. (1993) Determination of ethyl carbamate in soy sauce and its possible precursor. J. Agricult. Food Chem. 41, 352-356.

Miklos, G. G. and Rubin, G. (1996) The Role of the Genome Project in Determining Gene Function: Insights from Model Organisms. Cell, 86, 521-529.

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 to Cells 1, 803-817.

Mortimer, R. K. (1966) Genetic mapping in *Saccharomyces*. Genetics 53, 165-173.

Nasmyth, K. (1993) Regulating the HO endonuclease in yeast. Curr. Opin. Genet. Dev. 3, 286-294.

National Institutes of Health National Toxicology Program. (2004) NTP Technical Report on the Toxicology and Carcinogenesis Studies of Urethane, Ethanol, and Urethane/Ethanol in B6C3F_1 Mice (Drinking Water Studies). TR 510, 1-351.

Newstead, S., Kim, H., von Heijne, G., Iwata, S. and Drew, D. (2007) High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 104, 13936-13941.

Nettleship, A., Henshaw, PS. and Meyer, HL. (1943) Induction of pulmonary tumors in mice with ethyl carbamate (urethane). J. Natl. Cancer Inst. 4, 309-319.

Nikawa, J., Akiyoshi, M., Hirata, S. and Fukuda, T. (1996) *Saccharomyces cerevisiae IRE2/HAC1* is involved in *IRE1*-mediated *KAR2* expression. Nucl. 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.

Ough, C. S. (1976a) Ethyl carbamate in fermented beverages and foods. I. Naturally occurring ethyl carbamate. J. Agric. Food Chem. 24, 323-328.

Ough, C. S. (1976b) Ethyl carbamate in fermented beverages and foods. II. Possible formation of ethyl carbamate from diethyl dicarbonate addition to wine. J. Agric. Food Chem. 24, 328-331.

Ough, C. S., Crowell, E. A. and Gutlove, B. R. (1988) Carbamyl Compound Reactions with Ethanol. Am. J. Enol. Vitic. 39, 239-242.

Ough, C. S., Crowell, E. A. and Mooney, L. A. (1988) 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., Huang, Z., An, D. and Stevens, D. (1991) Amino Acid Uptake by Four Commercial Yeasts at Two Different Temperatures of Growth and Fermentation: Effects on Urea Excretion and Reabsorption. Am. J. Enol. Vitic. 42, 26-40.

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.

Ough, C. S. and Trioli, G. (1988) Urea Removal from Wine by an Acid Urease. Am. J. Enol. Vitic. 39, 303-307.

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, K., Liem, A., Stewart, B. C. and Miller, J. A. (1993) Vinyl carbamate epoxide, a major strong electrophilic, mutagenic and carcinogenic metabolite of vinyl carbamate and ethyl carbamate (urethane). Carcinogenesis 14, 441-450.

Pena-Castillo, L. and Hughes, T. (2007) Why Are There Still Over 1000 Uncharacterized Yeast Genes? Genetics 176, 7-14.

Perez-Ortin, J., Garcia-Martinez, J. and Alberola, T. (2002) DNA chips for yeast biotechnology. The case of wine yeasts. J. Biotechnol. 98, 227-241.

Rossignol, T., Dulau, L., Julien, A. and Blondin, B. (2003) Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast 20, 1369-1385.

Salmon, J. and Barre, P. (1998) Improvement of Nitrogen Assimilation and Fermentation Kinetics under Enological Conditions by Derepression of Alternative Nitrogen-Assimilatory Pathways in an Industrial *Saccharomyces cerevisiae* Strain. Appl. Environ. Microbiol. 64, 3831-3837.

Schehl, B. (2007) Contribution of the fermenting yeast strain to ethyl carbamate generation in stone fruit spirits. Appl. Microbiol. Biotech. 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.

Shobayashi, M., Ukena, E., Fujii, T. and Iefuji, H. (2007) Genome-wide expression profile of sake brewing yeast under shaking and static conditions. Biosci. Biotechnol. Biochem. 71, 323-335.

Smart, W. C., Coffman, J. A. and Cooper, T. G. (1996) Combinatorial regulation of the *Saccharomyces cerevisiae CAR1* (arginase) promoter in response to multiple environmental signals. Mol. Cell. Biol. 16, 5876-5887.

Stevens, D. F. and Ough, C. S. (1993) Ethyl Carbamate Formation: Reaction of Urea and Citrulline with Ethanol in Wine Under Low to Normal Temperature Conditions. Am. J. Enol. Vitic. 44, 309-312.

Stolz, L. E. (1998) *INP51*, a yeast inositol polyphosphate 5-phosphatase required for phosphatidylinositol 4,5-bisphosphate homeostasis and whose absence confers a cold-resistant phenotype. J. Biol. Chem. 273, 11852-11861.

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. Analytical Biochem. 274, 150. Oxford University Press, New York, USA.

Takagi, H., Takaoka, M., Kawaguchi, A. and Kubo, Y. (2005) Effect of L-Proline on Sake Brewing and Ethanol Stress in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. 71, 8656-8662.

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

van Vuuren, H. J. J., Daugherty, J. R., Rai, R. and Cooper, T. G. (1991) Upstream induction sequence, the cis-acting element required for response to the allantoin pathway inducer and enhancement of operation of the nitrogen-regulated upstream activation sequence in *Saccharomyces cerevisiae*. J. Bacteriol. 173, 7186-7195.

Vine, R. P., Harkness, E. M. and Linton, S. J. (2002) Winemaking: From Grape Growing to Marketplace, Second ed., Kluwer Academic/Plenum, New York.

Volschenk, H., Viljoen, M., Grobler, J., Bauer, F., Lonvaud-Funel, A., Denayrolles, M., Subden, R. E. and Van Vuuren, H. J. J. (1997) Malolactic Fermentation in Grape Musts by a Genetically Engineered Strain of *Saccharomyces cerevisiae*. Am. J. Enol. Vitic. 48, 193-197.

Wach, A. (1996) PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast 12, 259-265.

Ward, A. (1992) Rapid analysis of yeast transformants using colony-PCR. Biotechniques, 13, 350.

Wagner, S., Bader, M. L., Drew, D. and de Gier, J. (2006) Rationalizing membrane protein overexpression. Trends in Biotech. 24, 364-371.

Westfall, P. J., Ballon, D. R. and Thorner, J. (2004) When the stress of your environment makes you go HOG wild. Science 306, 1511-1512.

Whitney, P. A. and Cooper, T. G. (1972) Urea Carboxylase and Allophanate Hydrolase. Two components of adenosine triphosphate: urea amidolyase 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.

Wu, H., Zheng, X., Araki, Y., Sahara, H., Takagi, H. and Shimoi, H. (2006) Global gene expression analysis of yeast cells during sake brewing. Appl. Environ. Microbiol. 72, 7353-7358.

Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33, 103-119.

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. Indust. Microbiol. Biotech. 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.