Research article

Institute of Brewing & Distilling

Received: 3 September 2014 Revised: 30 September 2014 Accepted: 2 November 2014 Published online in Wiley Online Library: 19 March 2015

(wileyonlinelibrary.com) DOI 10.1002/jib.208

Enhancement of urea uptake in Chinese rice wine yeast strain N85 by the constitutive expression of *DUR3* for ethyl carbamate elimination

Xiaomin Li,[1,2,3] Chao Shen,[1,2,3] Dianhui Wu,[1,2,3] Jian Lu,[1,2,3,4]* Jian Chen[1,2,3] and Guangfa Xie[3,5]

Most of the fermented alcoholic beverages, particularly Chinese rice wine, contain the potentially human carcinogenic compound ethyl carbamate (EC). As a major EC precursor in Chinese rice wine, urea in fermentations can be transported into the yeast cell by urea permease and finally metabolized by urea carboxylase and allophanate hydrolase *in vivo*. To eliminate EC in Chinese rice wines, the present study constructed high urea uptake yeast strains N1-D, N2-D and N-D, by introducing a strong promoter (*PGK1p*) into the urea permease gene (*DUR3*) of the industrial Chinese rice wine yeast N85, and by the restoration of the *URA3* gene at the same time. With these self-cloned, high urea uptake strains, the urea and EC in the terminal Chinese rice wine samples were reduced to different extents. With two copies of overexpressed *DUR3*, the N-D strain could reduce the urea and the EC by 53.4 and 26.1%, respectively. No difference in fermentation characteristics was found between the engineered strains and the parental industrial yeast strain N85. These results could help to optimize the genetic manipulation strategy for EC elimination in Chinese rice wine production.

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords: Chinese rice wine; yeast; urea uptake; ethyl carbamate; *DUR3*

Introduction

Chinese rice wine is a national unique and traditional alcoholic beverage with more than 4000 years of history. However, a survey of Chinese rice wines has shown that they contain ethyl carbamate (EC) with an average concentration of 160 $\mu g\,kg^{-1}$ *(1)*, which is a threat to the health of consumers *(2)*. Previous studies have shown that EC can be formed from various precursors such as urea, citrulline and *N*-carbamyl compounds, by a spontaneous reaction with ethanol. The most common route of EC formation is from a reaction of ethanol with urea, during food and beverage processing or storage *(3,4)*.

Urea is mainly accumulated in yeast by the arginase-dependent degradation pathway, and it can be exported into the surrounding medium, where it reacts with ethanol to form EC *(3)*. At the same time, the extracellular urea can be imported into the cell and further metabolized into ammonia and CO_2 by urea carboxylase and allophanate hydrolase, a bi-functional enzyme Dur1,2p encoded by *DUR1,2* in *Saccharomyces cerevisiae (5)*. Urea can be imported into a *S. cerevisiae* cell in two ways. One route is active transport through the membrane using urea permease, which is encoded by the NCR sensitive *DUR3* gene, and the other route is via either passive or facilitated diffusion, when the extracellular urea concentration is 0.5 $mmol\,L^{-1}$ or above (6-8).

As summarized by Zhao *et al. (9)*, current available methods for EC elimination can be classified into physical, chemical, enzymatic and metabolic engineering routes. Among them, controlling temperature and adding acid urease has been used to reduce EC formation or concentration, but this could not eliminate it. Modifications of yeast strains by metabolic engineering methods have been proven to be effective strategies for reducing the EC concentration in alcohol beverages, by blocking the arginase expression or by the high expression of the urea metabolizing enzymes *(2,10,11)*. However, our recent work, using the former strategy, has reduced EC to 50% in Chinese rice wine fermentations using the engineered yeast strain N85-c2, suggesting that there are other genes with genetic manipulation potential for EC elimination, such as those encoding urea transportation proteins *(12)*.

* Correspondence to: Jian Lu, National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi, 214122, People's Republic of China.
E-mail: jlu@jiangnan.edu.cn

[1] The Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University, 1800 Lihu Road, Wuxi, 214122, People's Republic of China

[2] National Engineering Laboratory for Cereal Fermentation Technology, Jiangnan University, 1800 Lihu Road, Wuxi, 214122, People's Republic of China

[3] School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, 214122, People's Republic of China

[4] Industrial Technology Research Institute of Jiangnan University in Suqian, 888 Renmin Road, Suqian, 223800, China

[5] National Engineering Research Centre for Chinese Rice Wine, China Shaoxing Rice Wine Group Co., Ltd, Shaoxing, 312000, People's Republic of China

In the present study, a *DUR3* expression cassette containing the strong promoter *PKG1p* was constructed for generating the engineered Chinese rice wine yeast strain for reducing the urea and EC content. The capacity of high urea uptake and the negative influence on EC formation of the engineered strains were validated. In addition, the expression level of urea permease, growth behaviour and fermentation characteristics of the engineered strains were compared with the parental strain.

Materials and methods

Strains, plasmids and media

The plasmid pMD18-T was used for nucleotide fragment cloning and pMGKR for the *DUR3* expression cassette construction. *Escherichia coli* JM109 for vector propagation was cultivated aerobically at 37 °C in Luria–Bertani medium, supplemented with 2% (w/v) agar and 100 μg mL^{-1} ampicillin (Sangon, Shanghai, China) as required. All yeast strains (Table 1) were cultured at 30 °C in yeast peptone dextrose medium (YPD), which was solidified with 2% (w/v) agar as necessary. Minimum medium [MM; 0.17% (w/v) YNB (yeast nitrogen base without amino acids), 2% (w/v) glucose, 0.5% (w/v) ammonium sulphate, 2% (w/v) agar] was used for culturing and selecting prototrophic strains. SM-Ura medium with 0.005% (w/v) uracil supplemented to the MM was used for the Δ*ura3* mutant culture. The 5-FOA medium with 0.125% (w/v) 5-FOA (5-fluororotic acid) added to the SM-Ura medium was used for the Δ*ura3* mutant selection. Sporulation was performed on potassium acetate (KAc) medium [1% (w/v) KAc, 2% (w/v) agar] for 3 days at 26 °C to verify the diploid strains.

Nucleic acid manipulation

All primers were designed according to the sequence of the *S. cerevisiae S288C* genome (http://www.yeastgenome.org/; Table 2). Genome DNA used for PCR amplification was extracted from strain N85. PCR products were purified from agarose gel using a MiniBEST Agarose Gel DNA Extraction kit (Takara, Dalian, China). Plasmids were extracted using a MiniBEST Plasmid Purification Kit (Takara Dalian, China). Constructed plasmids and PCR products were verified by DNA sequencing (Sangon, Shanghai, China).

Construction of *DUR3* expression haploid strains

The *DUR3* expression cassette was constructed as described in Fig. S1 in the Supporting Information. In detail, the orf of *DUR3* was amplified with specific primers D3-1/D3-2 using the genomic DNA of N85 as the template, then inserted into the pMD18-T vector, which was then double-digested with *Bam* HI and *Sal* I for ligation with the similarly digested pMGKR. The resulting plasmid pMGKR-*DUR3* was used as the template to amplify the fragment *PGK1p-DUR3-PGK1t* with primers PDT-1/PDT-2. The upstream (*URA3*-L) and downstream (*URA3*-R) fragments of *URA3* were generated by PCR amplification using genomic DNA of N85 and sets of primers, U3-1p/U3-1 t and U3-2p/U3-2 t, respectively. PCR products of URA3-L, URA3-R and *PGK1p-DUR3-PGK1t* were pooled in equimolar amounts as templates according to the previously reported touchdown PCR method *(13)*, to generate the *DUR3* expression cassette, *URA3L-PGK1p-DUR3-PGK1t-URA3R*, which was verified by sequencing. The *DUR3* expression cassette was electro-transformed into the two mating type haploid strains Na (*MATa*) and Nα (*MATα*), plated onto 5-FOA plates, and then streak cultured onto MM and SM-Ura plates. Colonies that appeared on SM-Ura but not on MM were considered to be transformants. The transformants, designated Na-uD and Nα-uD, were confirmed by analytical PCR with primers U3-1p/U3-2 t and with their genomic DNA as templates.

Construction of prototroph diploid strains

The *URA3* restore cassette, *HOL-URA3-HOR*, was constructed as follows. First, the left and right arms of *HO*, HO-L and HO-R, as well as *URA3* ORF were amplified with genomic DNA of N85 as template and primers HO1/HO1-1 (for HO-L), HO2-1/HO2 (for HO-R) and U3-1/U3-2 (for *URA3*), respectively. Second, equimolar amounts of the resulting PCR fragments were mixed as templates for fusion PCR with primers HO1/HO2. The amplified product, *HOL-URA3-HOR*, was then purified and sequenced.

Haploids engineered strains, Na-uD and Nα-uD, were electro-transformed with the *HO-URA3-HO* cassette according to the above method and plated on MM plates. The colonies were identified by PCR with primers U3-1/U3-2, and confirmed by streak culture on SM-Ura and 5-FOA plates. Colonies that appeared on SM-Ura but not on 5-FOA were considered to be transformants. The transformants, with either mating type, named Na-D and Nα-D,

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

Strain	Parental	Genotype	Source
N85		*MATa/ MATα*	NERCCRW[a]
Na	N85	*MATa ho*	Wu *et al.* (13)
Nα	N85	*MATα ho*	Wu *et al.* (13)
Na-uD	Na	*MATa ho ura3::DUR3*	This study
Nα-uD	Nα	*MATα ho ura3::DUR3*	This study
Na-D	Na-uD	*MATa ho::URA3 ura3::DUR3*	This study
Nα-D	Nα-uD	*MATα ho::URA3 ura3::DUR3*	This study
N1-D	Na/Nα-D	*MATa/MATα ho/ho::URA3 URA3/ura3::DUR3*	This study
N2-D	Na-D/Nα	*MATa/MATα ho::URA3/ho ura3::DUR3/URA3*	This study
N-D	Na-D/Nα-D	*MATa/MATα ho::URA3/ ho::URA3 ura3::DUR3/ ura3::DUR3*	This study

[a]National Engineering Research Centre for Chinese Rice Wine.

Table 2. Oligonucleotide primers used in this study

Primer name	Primer sequence (5′–3′)[a]
D3-1	CGC**GGATCC**TGTCATTGTTATAATATGG[b]
D3-2	ACGC**GTCGAC**CTAAATTATTTCATCAAC[c]
U3-1p	GTAATCTCCGAGCAGAAG
U3-1 t	AGGAATCTAAAATAAATGTCTGCCCATTCTGC
U3-2p	ATTCTGCGTTCGTTGATTGGTTGATTATGACACC
U3-2 t	ATACTGTTACTTGGTTCTGGC
Pp-1	TATTTTAGATTCCTGACTTCAACTCAAGACGC
Pp-2	TGTTTTATATTTGTTGTAAAAAGTAG
PDT-1	AATGGGCAGACATTTATTTTAGATTCCTGACTTCAACTCAAGACGC
PDT-2	GTCATAATCAACCAATCAACGAACGCAGAATTTTC
HO1	ATCCTCATAAGCAGCAATC
HO1-1	CTTTATGGACCCTGCAAACTCACCTTCAACTGTC
U3-1	GACAGTTGAAGGTGAGTTTGCAGGGTCCATAAAGCTT
U3-2	CAGCAGCCTCGACATGATTTCAGATTAGAGTACAAACGCAT
HO2-2	TGTACTCTAATCTGAAATCATGTCGAGGCTGCTG
HO2	ACAAATCAGTGCCGGTAAC
MAT	AGTCACATCAAGATCGTTTATGG
MAT-a	ACTCCACTTCAAGTAAGAGTTTG
MAT-α	GCACGGAATATGGGACTACTTCG

[a]Overlapping sequences required for the fusion PCR are underlined.
[b]*Bam* H I site is indicated in bold.
[c]*Sal* I site is indicated in bold.

were confirmed by analytical PCR using primers HO-1/HO-2 and with their genome DNA as template. The haploid strain was then fused with the reverse-mating-type strain, which was either the wild type strain or the engineered strain, to generate a homozygous diploid strain as described previously *(12)*. Diploids were identified by PCR with primers MAT/MAT-a/MAT-α and verified on KAC plates.

Urea uptake analysis in the engineered strains

Yeast cells of each engineered strain, as well as the parental strain N85, were cultured on 0.17% YNB without amino acids and ammonium sulphate, 2% glucose and 0.2% urea. The urea uptake ability was determined by detecting the urea concentration in the supernatant after 8 h using an Agilent 1200 series HPLC system with fluorescence detector as described previously *(12)*. RNA isolation was performed using a CellAmp Direct RNA Prep kit for Real Time RT-PCR (Takara, Dalian, China) after mortar and pestle with liquid nitrogen. Reverse transcription and quantitative polymerasa chain reaction (qPCR) was conducted with a One Step SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China) using a CFX96 Touch RT-PCR detection system (BioRad, CA, USA). Samples were analysed by PCR in triplicate and normalized to internal *ACT1* mRNA levels. Primers for *ACT1* and *DUR3* genes were synthesized as per Zhao *et al*. *(14)*. Melting curve analysis was performed to ensure that a single product was produced in each reaction.

Small-scale Chinese rice wine fermentation and metabolite analysis

The engineered diploid yeast strains and the parental strain N85 were used as starter cultures for fermentation. Each strain was pre-cultured in YPD medium and then mixed with steamed rice and wheat Qu. Primary fermentation was carried out at 30 °C in 3000 mL conical flasks fitted with a fermentation lock for air-out. Weight loss was monitored every day, until the daily weight loss was <2 g (about 4–5 days). After that, the flasks were placed at 15 °C for 15 days for secondary fermentation. As fermentations ended, metabolites such as residual glucose, organic acid, amino nitrogen and ethanol in the fermented rice wine were determined using official methods according to the Chinese National Standard GB 13662-2008. Urea and EC in the final fermentations were detected with the methods described *(12)*.

Statistical analysis

All data were obtained from three replicate trials for each experiment. One-way analysis of variance (ANOVA) was employed to analyse data. Significant differences were considered at the 95% confidence level ($p < 0.05$). Statistical analysis was performed using SAS software.

Results and discussion

Haploid and diploid strain construction with the *DUR3* expression cassette

To constitutively express urea permease in the industrial Chinese rice wine yeast N85, the strong promoter *PGK1p* of *S. cerevisiae (15)* was used to construct the *DUR3* expression cassette of *URA3L-PGK1p-DUR3-PGK1t-URA3R*. The cassette was then electrotransformed into the haploid strains Na and Nα isolated in our previous work *(13)*, which resulted in the uracil auxotroph engineered strains, Na-uD and Nα-uD (Fig. S2). The growth ability of Na-uD and Nα-uD was poorer than that of the parental strain (data not shown), thus restricting industrial applications. Therefore, the *URA3* restore cassette was constructed for restoring it at the *HO* loci, obtaining Na-D and Nα-D. Diploid strains with three kinds of

genotypes, N1-D, N2-D and N-D, were obtained by haploid fusion, further identified by PCR with primers MAT/MAT-a/MAT-α and verified on KAc plates. All engineered strains and their genotypes obtained in this study are summarized in Table 1.

Transport of urea into engineered yeast cells by the overexpression of *DUR3*

Urea permease is a 735 amino acid integral membrane protein containing 16 transmembrane segments *(6)*. In addition to transporting urea into yeast cells, it also facilitates the uptake of polyamines and regulates intracellular boron concentrations *(16,17)*. However, the existence of preponderant nitrogen, such as polyamines, would repress the expression of urea permease *(6,17,18)*. When grown on YNB medium complemented with urea as the sole nitrogen source, the engineered yeast cells with either one or two copies of the *DUR3* expression cassette showed a 10 times greater urea uptake than the parental strain N85 (Fig. 1). In addition, two copies of *DUR3* expression cassette increased expression of *DUR3* by 40.83 ± 0.17-fold in the diploid strain N-D, which was much higher than in the previous study using the same strategy using promoter *PKG1p*, with only a 14.2-fold up-regulation *(8)*. These results indicate that, with the imported strong promoter *PKG1p*, *DUR3* was overexpressed and this helped to reduce the extracellular urea content.

Fermentation characteristics of the engineered diploid strains

In order to test the overexpression of *DUR3* on the fermentation performance of the engineered strains, small-scale Chinese rice wine fermentation trials were carried out with the engineered diploid strains, N1-D, N2-D and N-D, and with the parental strain N85, respectively. The weight loss curve (Fig. 2) was made according to the conical flask weight changes during the fermentation process. Results show that the growth curves of each strain followed the same trend. The concentrations of ethanol, residual sugar and amino acid nitrogen in the terminal fermentation samples were determined as shown in Table 3. No significant differences in fermentation behaviour between the parental strain and its *DUR3* high-expression derivatives were observed (differing by no more than 5%; $p < 0.05$).

More importantly, the effect of *DUR3* overexpression on urea uptake and EC formation during the rice wine fermentation was evaluated. As expected, the residual amounts of urea in the rice

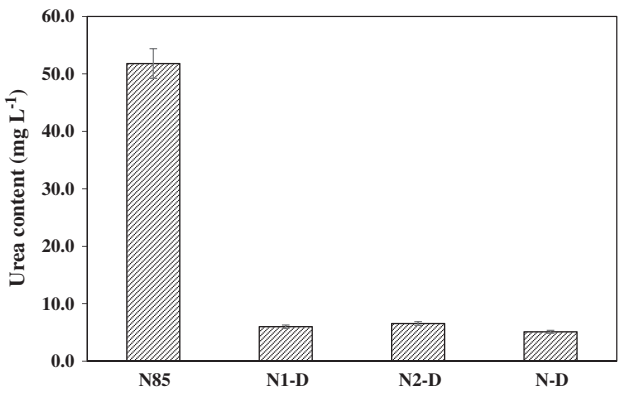

Figure 1. Urea transport capacity of N85 and the *DUR3* overexpressing diploid strains.

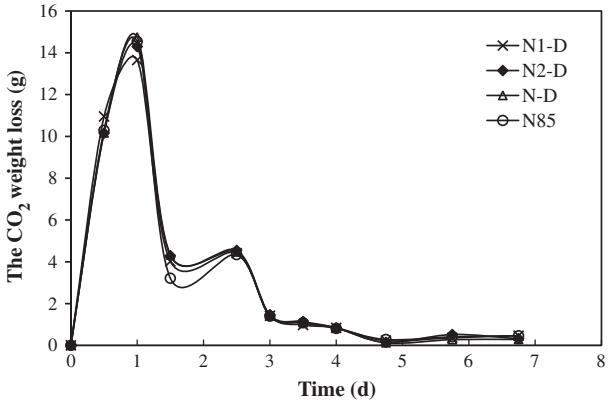

Figure 2. Fermentation performance of N85 and the diploid derivatives with high urea uptake feasibility.

wine samples, fermented with the engineered diploid strains, N1-D, N2-D and N-D, were 34.1, 35.2 and 20.1 mg L^{-1}, a reduction of 20.8, 18.3 and 53.4%, respectively, compared with the parental strain N85. The EC concentrations in the fermentation liquor brewed using the engineered strains, N1-D, N2-D and N-D were reduced by 11.5, 11.1 and 26.1%, respectively. Previous studies using sake yeast reduced the EC content by 15% using engineered strains manipulated in the same manner *(8)*. The reductions in the amount of urea and in the EC concentration by homogenous diploid strains with double copies of the *DUR3* expression cassette, were twice as high as in the diploids with only one copy of *PKG1p* integrated in *DUR3*. With more urea permease expressed in the yeast cell, more environmental urea was imported into the yeast cell. However, the concentrations of urea and EC were not eliminated as expected. More copies of *DUR3* may be needed for importing urea using the permease. Furthermore, utilization of urea as a nitrogen source requires the action of the bi-functional enzyme Dur1,2p, whose catalytic cycle involves the carboxylation of urea followed by hydrolysis of the resulting urea-1-carboxylate intermediate to yield 2 equivalents of carbon dioxide and ammonia *(19)*. It is therefore speculated that the expression level of Dur1,2p should be increased at the same time, or else more urea in yeast cells will block the import of extracellular urea in spite of the overexpression of urea permease.

In conclusion, constitutive expression of *DUR3* in a yeast strain was shown to have some positive effects on urea and EC reduction during Chinese rice wine fermentation. The constructed engineered strains N1-D, N2-D and N-D, with high urea removal feasibility, were substantially equivalent to the parental strain N85 in terms of fermentation characteristics, except for the residual urea and EC in the final fermented rice wines. Furthermore, it is hypothesized that more copies of *DUR3* combined with the *DUR1,2* expression cassette in the yeast cell could be even more effective in reducing the urea and EC concentration. Different from the previous study, which also integrated *PKG1p* in the upstream of *DUR3* gene, in the present study no antibiotic genes were introduced, but there was a higher exhibition of the *DUR3* expression level and the yeast was more effective in reducing the urea concentration and EC formation *(8)*. Thus, the genetically modified strains from this current study could have a good industrial application potential, since no exogenous genes or antibiotic genes were introduced during the genetic manipulation process. However, to achieve industrial Chinese rice wine strains with a higher

Table 3. Characteristics of Chinese rice wine brewed with different yeast strains

Strains	N85	N1-D	N2-D	N-D
Alcohol content (%)	14.9 ± 0.1	15.1 ± 0.3	14.6 ± 0.1	15.1 ± 0.1
Total sugar ($g\,L^{-1}$)	1.98 ± 0.11	2.07 ± 0.09	2.05 ± 0.13	2.01 ± 0.07
Total acid ($g\,L^{-1}$)	3.02 ± 0.10	3.05 ± 0.12	2.95 ± 0.08	2.98 ± 0.11
Amino acid nitrogen ($g\,L^{-1}$)	1.07 ± 0.03	0.99 ± 0.02	0.95 ± 0.03	1.11 ± 0.04
pH value	5.17 ± 0.30	5.16 ± 0.24	5.12 ± 0.19	5.19 ± 0.23
Urea ($mg\,L^{-1}$)	43.1 ± 1.3	34.1 ± 1.90	35.2 ± 1.50	20.1 ± 1.20
Ethyl carbamate ($\mu g\,L^{-1}$)	43.78 ± 2.02	38.74 ± 1.73	38.92 ± 1.36	32.36 ± 1.17

EC elimination rate, more work should carried out, such as examining the spontaneous overexpression of urea permease and an examination of the urea metabolizing enzymes, urea carboxylase and allophanate hydrolase.

Acknowledgements

This study was supported by the Major State Basic Research Development Program of China (973 Programs, 2012CB720802 and 2013CB733602), the Natural Science Foundation of Jiangsu Province, China (BK20140144), the Key Program of National Natural Science Foundation of China (31130043), the Fundamental Research Funds for the Central Universities (JUSRP51302A and JUSRP1015), the open project of the Key Laboratory of Industrial Biotechnology, Ministry of Education, Jiangnan University (KLIB-KF201308), the Program of Introducing Talents of Discipline to Universities (111 Project; 111-2-06) and the Program Development of Safe Food Deep Processing Technology Innovation Platform (2012B091400030).

References

1. Wu, P. G., Pan, X. D., Wang, L. Y., Shen, X. H., and Yang, D. J. (2012) A survey of ethyl carbamate in fermented foods and beverages from Zhejiang, China. *Food Control 23*, 286–288.
2. Zimmerli, B., and Schlatter, J. (1991) Ethyl carbamate: Analytical methodology, occurrence, formation, biological activity and risk assessment. *Mutat. Res. 259*, 325–350.
3. 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.
4. Ough, C. S., Crowell, E. A., and Gutlove, B. R. (1988) Carbamyl compound reactions with ethanol. *Am. J. Enol. Vitic. 39*, 239–242.
5. Weber, J. V., and Sharypov, V. I. (2008) Ethyl carbamate in foods and beverages: A review. *Environ. Chem. Lett. 7*, 233–247.
6. 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.
7. An, D., and Ough, C. S. (1993) Urea excretion and uptake by wine yeasts as affected by various factors. *Am. J. Enol. Vitic. 44*, 35–40.
8. Dahabieh, M. S., Husnik, J. I., and Van Vuuren, H. J. (2010) Functional enhancement of Sake yeast strains to minimize the production of ethyl carbamate in Sake wine. *J. Appl. Microbiol. 109*, 963–973.
9. Zhao, X. R., Du, G. C., Zou, H. J., Fu, J. W., Zhou, J. W., and Chen, J. (2013) Progress in preventing the accumulation of ethyl carbamate in alcoholic beverages. *Trends Food Sci. Technol. 32*, 97–107.
10. Park, H. D., Shin, M. C., and Woo, I. S. (2001) Antisense-mediated inhibition of arginase (*CAR1*) gene expression in *Saccharomyces cerevisiae*. *J. Biosci. Bioeng. 92*, 481–484.
11. 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*, 393a–393a.
12. Wu, D., Li, X., Shen, C., Lu, J., Chen, J., and Xie, G. (2014) Decreased ethyl carbamate generation during Chinese rice wine fermentation by disruption of *CAR1* in an industrial yeast strain. *Int. J. Food Microbiol. 180*, 19–23.
13. Wu, D. H., Li, X. M., Shen, C., Lu, J., Chen, J., and Xie, G. F. (2013) Isolation of a haploid from an industrial Chinese rice wine yeast for metabolic engineering manipulation. *J. Inst. Brew. 119*, 288–293.
14. Zhao, X. R., Zou, H. J., Fu, J. W., Chen, J., Zhou, J. W., and Du, G. C. (2013) Nitrogen regulation involved in the accumulation of urea in *Saccharomyces cerevisiae*. *Yeast 30*, 437–447.
15. Matsushika, A., Inoue, H., Kodaki, T., and Sawayama, S. (2009) Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: Current state and perspectives. *Appl. Microbiol. Biotechnol. 84*, 37–53.
16. Navarathna, D. H., Das, A., Morschhauser, J., Nickerson, K. W., and Roberts, D. D. (2011) Dur3 is the major urea transporter in *Candida albicans* and is co-regulated with the urea amidolyase Dur1,2. *Microbiol. 157*, 270–279.
17. Uemura, T., Kashiwagi, K., and Igarashi, K. (2007) Polyamine uptake by DUR3 and SAM3 in *Saccharomyces cerevisiae*. *J. Biol. Chem. 282*, 7733–7741.
18. Kashiwagi, K., and Igarashi, K. (2011) Identification and assays of polyamine transport systems in *Escherichia coli* and *Saccharomyces cerevisiae*. *Meth. Mol. Biol. 720*, 295–308.
19. ter Schure, E. G., van Riel, N. A., and Verrips, C. T. (2000) The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev. 24*, 67–83.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.