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Construction of Amylolytic Industrial Brewing Yeast Strain with High Glutathione Content for Manufacturing Beer with Improved Anti-Staling Capability and Flavor

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In beer, glutathione works as the main antioxidant compound, which also correlates with the stability of the beer flavor. In addition, high residual sugars in beer contribute to major nonvolatile components, which are reflected in a high caloric content. Therefore, in this study, the *Saccharomyces cerevisiae GSH1* gene encoding glutamylcysteine synthetase and the *Saccharomycopsis fibuligera ALP1* gene encoding α-amylase were coexpressed in industrial brewing yeast strain Y31 targeting the α-acetolactate synthase (AHAS) gene (*ILV2*) and alcohol dehydrogenase gene (*ADH2*), resulting in the new recombinant strain TY3. The glutathione content in the fermentation broth of TY3 increased to 43.83 mg/l as compared with 33.34 mg/l in the fermentation broth of Y31. The recombinant strain showed a high α-amylase activity and utilized more than 46% of the starch as the sole carbon source after 5 days. European Brewery Convention tube fermentation tests comparing the fermentation broths of TY3 and Y31 showed that the flavor stability index for TY3 was 1.3-fold higher, whereas its residual sugar concentration was 76.8% lower. Owing to the interruption of the *ILV2* gene and *ADH2* gene, the contents of diacetyl and acetaldehyde as off-flavor compounds were reduced by 56.93% and 31.25%, respectively, when compared with the contents in the Y31 fermentation broth. In addition, since no drug-resistant genes were introduced to the new recombinant strain, it should be more suitable for use in the beer industry, owing to its better flavor stability and other beneficial characteristics.

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Beer aging often occurs during storage, resulting in a strange smell. Among the many factors involved in beer aging, oxidation is the main cause, as it produces aldehyde compounds that directly contribute to the beer flavor. Glutathione (GSH: γ-L-glutamyl-L-cysteinylglycine) is the best-known example of a nonenzymatic defense system and is already widely used by the beer brewery industry because of its redox activity [22]. GSH plays an important role in DNA and protein syntheses, as well as protein transportation [7], and recent research has shown that increasing the content of GSH helps improve the flavor stability of beer [11, 29, 30, 35]. The *GSH1* gene encodes glutamylcysteine synthetase, which is required for the rate-limiting step of glutathione synthesis in *Saccharomyces cerevisiae* [1]. Therefore, overexpression of the *GSH1* gene improves the GSH content in yeast. Wang *et al*. [31] also reported that increasing the concentration of GSH in beer regulates several metabolisms involved in reducing the content of certain oxycarbonyl compounds (*i.e*., diacetyl, pentanedione).

Acetaldehyde is one of the main off-flavor compounds and is a natural by-product of fermentation. While creating a pungent aroma at a high concentration, it leaves a more pleasant green-apple aroma at a diluted concentration. Moreover, as the main aromatic aldehyde, acetaldehyde affects beer staling through an aldol condensation reaction during beer storage. Thus, decreasing the amount of acetaldehyde could improve the resistant staling value (RSV) of beer [28, 31]. Previous reports have shown that disrupting the *ADH2* gene encoding alcohol dehydrogenase can reduce the acetaldehyde content in the final beer [6, 28, 25, 31, 36].

Table 1. Microbial strains and plasmids used in this study.

Strains or plasmids	Relevant genotype	Source
Strains		
DH5α	*F recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ (*argF-Lac-ZYA*)	Stratagene
Saccharomyces cerevisiae Y31	Industrial yeast strain	Tsingtao Brewery Co., Ltd (Qingdao, China)
Saccharomycopsis fibuligera ZF02	Wild yeast strain	Stored in author's lab
Saccharomyces cerevisiae TY3	Recombined industrial yeast strain *ALP1 GSH1 CUP1*Δ(*ilv2-adh2*)	This study
Plasmids		
pICG	Ap^R*ADH2GSH1CUP1URA3*	[25]
pMPS	Cloning vector, *amp*	[20]
pADH	Cloning vector, *amp*	[27]

Residual saccharides, such as maltose and maltotriose, which result in a high caloric content and unusual flavor, are another problem for the brewery industry, as *S. cerevisiae* is hardly able to hydrolyze starchy materials due to a lack of extracellular depolymerizing enzymes. Thus, in conventional beer fermentation, large amounts of exogenous enzymes are added to liberate fermentable sugars from polysaccharides-rich substrates, but this is costly and sometimes causes allergenic-related symptoms [3, 19]. It should be noted that many oenological *S. cerevisiae* strains have already been genetically engineered for either aroma [10, 23] or other characteristics [26, 27, 34] for use in wine. Meanwhile, α-amylase, dextranase, and glucoamylase genes have been introduced to industrial brewing yeast strains to address the lack of amylolytic activity [8, 12, 15, 16, 28].

Accordingly, in this study, the *GSH1* gene from *S. cerevisiae* and the α-amylase gene (*ALP1*) from *Saccharomycopsis fibuligera* were coexpressed in an industrial brewing yeast strain by interrupting the *ILV2* and *ADH2* genes in order to promote anti-staling, a low caloric content, and better flavor for the beer.

Materials and Methods

Strains and Culture Condition

The sources and relevant genotypes of the strains and plasmids used in this research are listed in Table 1.

The yeasts were cultured in a YPD medium (1% yeast extract, 2% peptone, 2% glucose), whereas the recombinant strains were cultured and selected on YNBS media (0.67% yeast nitrogen base w/o amino acids with 1% soluble starch as sole carbon source) with copper sulfate ($CuSO_4$). All the solid media contained 1.5% agar. YPD or 12°P wort was used for the assay of growth capability, fermentation ability, α-amylase activity, alcohol dehydrogenase activity, α-acetolactate synthase activity, and glutathione production.

Construction of Recombinant Strains

Plasmid pICG containing the *GSH1* and *CUP1* genes was constructed in a previous study [29]. The *ALP1* gene encoding α-amylase was amplified from the genomic DNA of ZF02 *via* PCR using primers ALP1-F/ALP1-R (Table 2) (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, China). The strong promoter in *S. cerevisiae*, PGK1 promoter ($PGK1_p$) from pMPS [28], was introduced to promote the expression of the *ALP1* gene, whereas the α signal factor was introduced for the secretion of the *ALP1* gene. All these fragments were then ligated into the *Sac*I and *Sph*I sites of pADH [31], generating pAPM (Fig. 1A).

The cassettes GC (*ilv2*Δ::*GSH1*) and PM (*adh2*Δ::*ALP1*) (Fig.1B) were prepared by PCR using primers ILV2-F/ILV2-R and ADH2-F/ADH2-R. The two fragments were then cotransformed to Y31 using the lithium acetate (LiAc) method, as previously described [24]. Thereafter, the recombinant strains were selected on YNBS plates with 8 mM $CuSO_4$.

Assay of Enzyme Activities of Concern

The α-amylase activity was measured using the dinitrosalicylic acid (DNS) method [18] with some modification. One unit (U) of α-amylase activity was defined as the amount of enzyme catalyzing the production of 1nmole of reducing sugar at 37°C per minute.

The alcohol dehydrogenase activity was measured spectrophotometrically using a modified version of Bergmeyer's method [4], as described by Blandino *et al*. [5]. One unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μmole of NADH per minute under the specified conditions.

The α-acetolactate synthase activity was detected using the method described by Zhang *et al*. [34].

Table 2. Primers used in this research.

Primers	Sequences 5' to 3'
ALP1-F	GGTCTAGACATCTTTCAATACCCGCC (*Xba*I)
ALP1-R	AAAGAGCTCTGTTTCCCGTGATGACC (*Sac*I)
ILV2-F	CCCGACAATAAAGTAAATAG
ILV2-R	AGAAAGAAGCGTAAGATC
ADH2-F	GCTGTTATGTTCAAGGTC
ADH2-R	TTCAGAGGAGCAGGACAA
CUP-F	CGCTATACGTGCATATGTTC

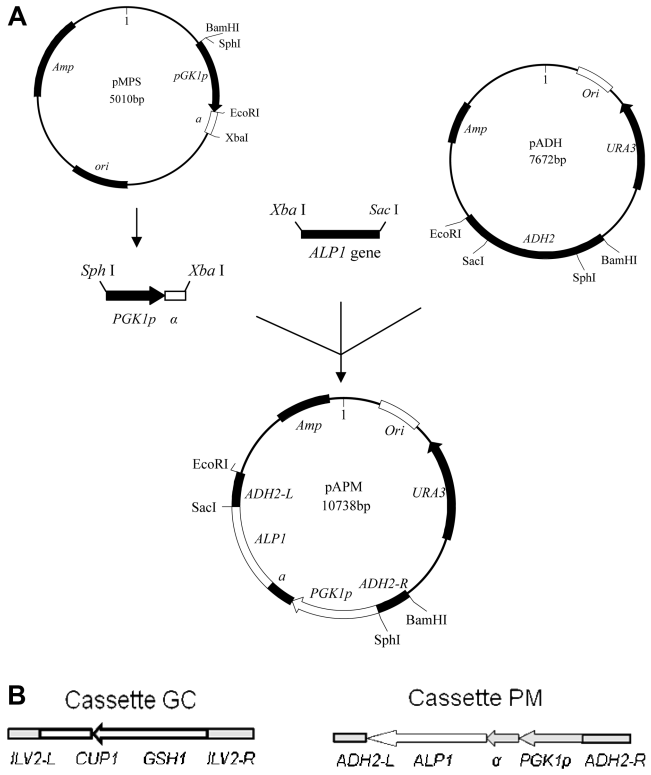

Fig. 1. Construction of related plasmids.

PCR Verification of Recombinant Strain

PCR was performed to confirm the recombinant strains in a 20 µl volume with 0.08 µl of *Taq* DNA Polymerase, 1× ThermoPol reaction buffer, 200 µM dNTPs, 100 ng of template DNA, and 0.1 µM primers (refer to Table 2). The cycle conditions were 94°C for 5 min followed by 30 cycles at 94°C for 30 s, 50°C for 40 s, 72°C for 3 min 10 s, and finally 72°C for 15 min.

Genetic Stability Test of Recombinant Strains

The recombinant strains were cultured on YPD medium at 28°C for 24 h for each generation, and plate streaking was performed for the 50th generation strains. Then 100 single-grown colonies were chosen randomly and transferred to sterilized water for 4 h of starvation at room temperature. Thereafter, a Greiner Inoculation Loop of the starved yeast suspension was inoculated onto YNBS medium with 8 mM $CuSO_4$ and cultured at 28°C for 2 days.

The alcohol dehydrogenase, α-amylase, and α-acetolactate synthase activities of the 1st and 50th generation strains were analyzed as described above.

Assay of Starch Utilization Rate

The rate of starch utilization was determined by measuring the residual glucose in the culture supernatants as previously described by Liu *et al*. [16].

Assay of Biomass, Glutathione Content, and Preservative Qualities

The biomass was determined by the method described by Wang *et al*. [29], whereas the glutathione content was determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [9] and expressed as the milligrams of glutathione per gram of dried yeast cells or milligrams of glutathione per 100 ml of fermented liquor.

The preservative qualities of the fermentative broths and corresponding anti-aging characters of the various strains were determined by measuring the thiobarbituric acid (TAB) value and resistant staling value (RSV) [21], both of which are significant indexes reflecting beer freshness. The RSV was taken as a reference of the beer flavor freshness period: RSV=1/4(12/ΔTBA12+24/ΔTBA24+ 36/ΔTBA36+48/ΔTBA48). A stability index (SI), counted as 100× diphenylpicrylhydrazyl (DPPH) scavenging activity/TBA value, was also introduced to measure the flavor stability of the beer [32, 35].

Fermentation Test and Pilot-Scale Brewing

The fermentation test was performed as described by Wang *et al*. [31]. The fermentation was first carried out at 12°C for 10 days in conical flasks. The yeast pellets harvested from the conical flasks were then inoculated to a 6-L European Brewery Convention (EBC) tube with 5 L 12 °P wort for pilot-scale brewing at 12°C for 16 days.

The real extract and attenuation degree in the fermentation broth were measured using an Alcolyzer Plus Beer machine, WBA-505b (Kyoto Electronics Manufacturing Co., Ltd, Tokyo, Japan). The acetaldehyde, diacetyl, and main residual sugar contents were measured by GC/MS, as described by Landaud *et al*. [13]. The GSH content was determined using the DTNB method, the TBA value and RSV were determined as described by Parson *et al*. [21], and the SI was calculated to reflect the preservative qualities of the beer. The data were finally analyzed using a one-way analysis of variance (ANOVA).

Beer fermented by various strains was stored at 4°C for 10 days. Comparative and qualitative tests to evaluate the sensorial characteristics of the final beer were conducted on-site by six tasting experts from the Tsingtao Brewery Co., according to company quality control procedures.

RESULTS AND DISCUSSION

Construction of Recombinant Strains

Industrial brewing yeast strain Y31 was used as the host strain. Cassette GC (*ilv2*Δ::*GSH1*) and cassette PM (*adh2*Δ::*ALP1*) were prepared using a PCR and co-transformed into Y31 by homologous recombination. As Y31 cannot grow on YNBS medium and can only grow on YPD medium with less than 5 mM $CuSO_4$, 42 recombinant strains were selected from an YNBS plate with 8 mM $CuSO_4$ and named using the nomenclature TY1, TY2, up to TY42.

Assay of Enzyme Activities and Starch Utilization

All 42 recombinant strains, the host strain Y31, and donor strain ZF02 were cultured for different enzyme activities and GSH content measurements. All the recombinant strains showed α-amylase activities (5.54–6.72 U/ml) similar to the donor strain ZF02 (6.99 U/ml), except for the host strain Y31. Moreover, the recombinant strains exhibited a higher GSH content (7.85–10.01 mg/g in dried cells) than

Y31 (3.76 mg/g in dried cells). In contrast, the alcohol dehydrogenase activity and α-acetolactate synthase activity of the recombinant strains were 44.48–58.03% (2.85–3.77 U/mg) and 42.91–56.75% (0.38–0.49 U/mg protein) lower, respectively, than that of Y31.

A starch utilization test was also applied to the recombinant strains, donor strain, and host strain. When analyzing the residual glucose in the culture medium after 5 days, the recombinant strains were found to have utilized more than 46% of the starch, whereas almost no starch was consumed by the host strain Y31 (data not shown). After considering the different enzyme activity measurements and starch utilization results, TY3 was chosen for further study because of its better performance.

PCR Verification

The flanking *ILV2* and *ADH2* sequences in the fragments were designed for homologous recombination to the *ILV2* and *ADH2* alleles of the Y31 chromosome. The integration of the *GSH1* gene and *CUP1* gene into the *ILV2* locus and the integration of the *ALP1* gene into the *ADH2* locus of the genomic DNA were confirmed by a PCR (Fig. 2) using different primer pairs (Table 2) in the TY3 genomic DNA: CUP-F/ILV2-R, ILV2-F/ILV2-R, ALP1-F/ALP1-R, and ADH2-F/ADH2-R, where primer CUP-F was the 5' end sequence of the *CUP1* gene, primers ILV2-L and ILV2-R were the 5' and 3' end sequences of the *ILV2* gene, primers ALP1-F and ALP1-R were the 5' and 3' end sequences of the *ALP1* gene, and primers ADH2-F and ADH2-R were the 5' and 3' end sequences of the *ADH2* gene. When using primers ADH2-F/ADH2-R, the sizes of the PCR products were 5.60 and 2.50 kb, which was consistent with our previous study, where one copy of the *ADH2* gene was more difficult than the other to disrupt. The *ADH2* gene is considered essential for yeast growth, as it would appear that knocking out the *ADH2* gene totally affects the yeast biomass (not published). In this study, since only one of the two copies of the *ADH2* gene was interrupted, the alcohol dehydrogenase activity in the recombinant strain was reduced to approximately half of that observed in Y31, which had both copies of the *ADH2* gene intact.

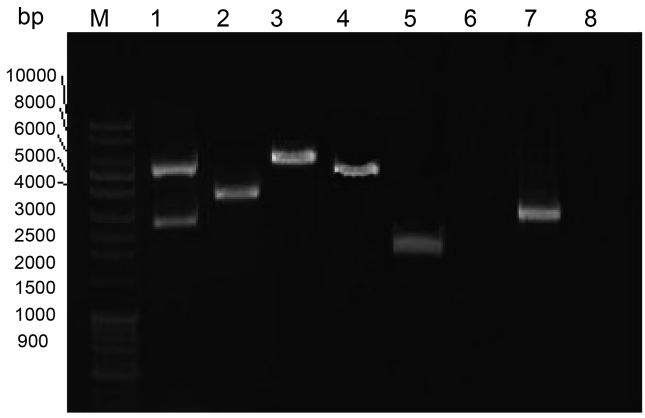

Fig. 2. PCR analysis of recombinant strains.
Lane M: Marker D10000; lane 1: TY3(ADH2-F/ADH2-R); lane 2: Y31(ILV2-F/ILV2-R); lane 3: TY3(ILV2-F/ILV2-R); lane 4: TY3(CUP-F/ILV2-R); lane 5: TY3(ALP1-F/ALP1-R); lane 6: Y31(ALP1-F/ALP1-R); lane 7: Y31(ADH2-F/ADH2-R); lane 8: Y31(CUP-F/ILV2-R).

When taking the genomic DNA of host strain Y31 as the template, the sizes of the PCR products agreed well with the predicted sizes. Thus, all the fragments were verified as being successfully integrated into the genomic DNA of Y31.

Genetic Stability Analysis

All 100 strains from the 50^{th} generation of TY3 were able to grow stably on a YNBS plate with 8 mM $CuSO_4$, whereas Y31 could not, indicating that the *CUP1* gene was stably expressed in the recombinant strains and the genetic stability was 100%. Moreover, the long-term stability of the *ALP1* gene expression ensured long-term stable growth in the YNBS medium. Similar results for different enzyme activities between the 1^{st} and 50^{th} generations of recombinant strain TY3 (data not shown) also indicated the genetic stability of TY3.

Fermentation Test and Pilot-Scale Brewing

The recombinant strain TY3 and host strain Y31 were tested with regards their fermentation ability and flavor generation in a pilot-scale brewing.

The CO_2 reduction and attenuation degree (data not shown) of TY3 in the conical flask fermentation were both higher than those of Y31, indicating that the genetic modifications to Y31 had not made any difference, but rather increased the fermentation ability of the recombinant strain owing to the expression of the *ALP1* gene. When compared with the fermentation broth of Y31, the diacetyl content in the fermentation broth of TY3 was 48.76% (126.50±0.50 ppb, $p<0.01$) lower, the acetaldehyde content was about 41% (8.50±0.02 ppm, $p<0.01$) lower, and the extracellular GSH content was 63.43% (8.58±0.07 mg/l, $p<0.01$) higher.

For the EBC tube fermentation, the recombinant strain TY3 and host strain Y31 initially showed similar diacetyl, acetaldehyde, and GSH contents in the fermentation broth; however, as the fermentation proceeded, TY3 began to exhibit lower diacetyl and acetaldehyde contents and a higher GSH content. By the end of the EBC tube fermentation, the diacetyl content in the TY3 fermentation broth was 56.93% lower, the acetaldehyde content was 31.25% lower, and the extracellular GSH content was 31.46% higher when compared with that in the Y31 fermentation broth (Fig. 3). Therefore, these results indicate that the disruption of the *ILV2* and *ADH2* genes certainly reduced the diacetyl and acetaldehyde contents, which are the two major off-flavor compounds in beer. Meanwhile,

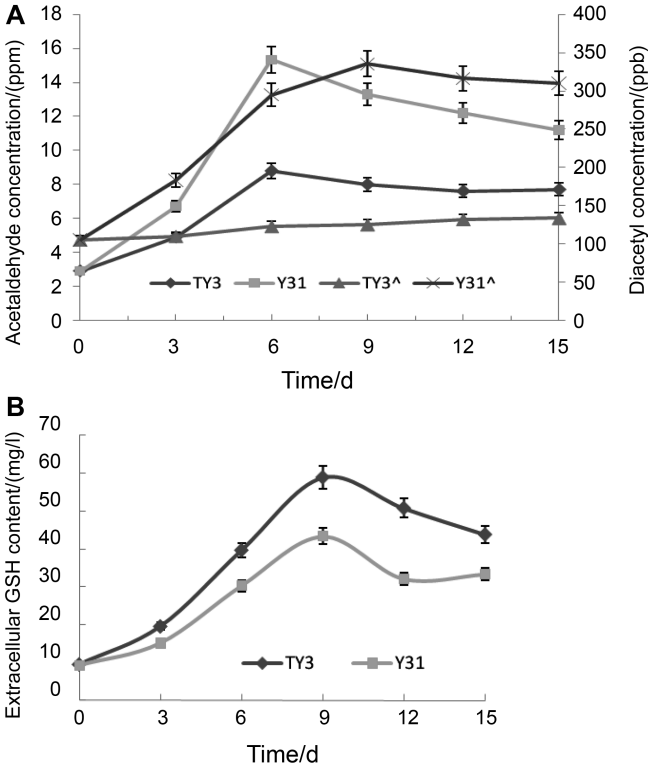

Fig. 3. Determination of acetaldehyde (**A**), diacetyl (**A**), and extracellular GSH (**B**) concentrations in Y31 and TY3 during EBC tube fermentation.
^ Data for diacetyl concentration.

the overexpression of the *GSH1* gene ensured an improved GSH content in the fermentation broth of the recombinant strain.

The TBA value and RSV reflect the beer freshness, where a smaller TBA value indicates a better antioxidizability, whereas a higher RSV indicates a longer flavor freshness period. Thus, as shown by the results in Table 3, the decreased TBA value and increased RSV measured for the TY3 fermentation broth would seem to suggest that beer brewed using TY3 may have a better antioxidizability and longer freshness period. Moreover, when calculating the stability index (SI) for the fermentation broths from different strains, which represents the antioxidative potential of beer and correlates with the sensory tests on beer flavor stability [2], the higher SI for TY3 (1.3 times the SI for the host strain) further confirmed that beer brewed using TY3 would be more antioxidative and have a more stable flavor than beer brewed using Y31. In a previous study [31], the present authors suggested that GSH in beer works as a major antioxidant and crucial redox mediator that can help reduce the content of certain oxycarbonyl compounds. Importantly, the acetaldehyde content in the TY3 fermentation broth was 31.25% lower than that in the Y31 fermentation broth. As the main off-flavor and oxycarbonyl compound in beer, the concentration of acetaldehyde not only affects the flavor of beer, but also has a negative influence on the TBA value [14]. Therefore, the present results further proved the significant effect of GSH and acetaldehyde on beer staling. Six experts from Tsingtao Brewery Co., Ltd evaluated the beers brewed using the recombinant strain TY3 and host strain Y31, and all considered the beer brewed using TY3 better tasting than that brewed using Y31.

To investigate the impact of the *ALP1* gene expression on the fermentation performance, the α-amylase activity in the fermentation broths was measured during the EBC tube fermentation. As shown in Fig. 4, TY3 consistently exhibited a high α-amylase activity during the fermentation, whereas Y31 did not. When measuring the concentrations of the key residual saccharides after the EBC tube fermentation, maltose and maltotriose were both detected in the Y31 broth, whereas there was no maltose left in the TY3 broth and an obvious reduction in the concentration

Table 3. Parameters of fermented liquor. (mean ± SD, n=3)

Parameter	TY3	Y31	F value	P value
Real attenuation (%)	71.44±0.13	66.14±0.09	392.38	$3.83e^{-5}$, S
Real extract (%)	4.07±0.01	4.53±0.02	23.88	0.008, S
Maltose (mg/l)	--	0.85±0.001	21675	$1.28e^{-8}$, S
Maltotriose (mg/l)	0.87±0.007	2.90±0.006	1,741.23	$1.97e^{-6}$, S
Starch residual (%)	64.27±0.55	100±0.00	6,918.96	$1.25e^{-7}$, S
Acetaldehyde (ppm)	7.70±0.07	11.20±0.16	159.78	$2.26e^{-4}$, S
Diacetyl (ppb)	133.67±12.33	310.34±46.33	1,596.02	$2.35e^{-6}$, S
Pentidione (ppb)	61.67±0.33	184.00±16.00	2,748.76	$7.92e^{-7}$, S
Extracellular GSH (mg/l)	43.83±0.28	33.34±1.79	159.09	$2.27e^{-4}$, S
Intracellular GSH (mg/g)	11.02±0.03	7.77±0.01	878.76	$7.71e^{-6}$, S
TBA	0.541±0.001	0.703±0.00	51.42	0.002, S
RSV	733.0±145.0	400.3±100.3	1,342.32	$3.31e^{-6}$, S
SI	137.0±29.0	105.3±26.3	35.16	$3.63e^{-4}$, S

S, significant (P<0.01).

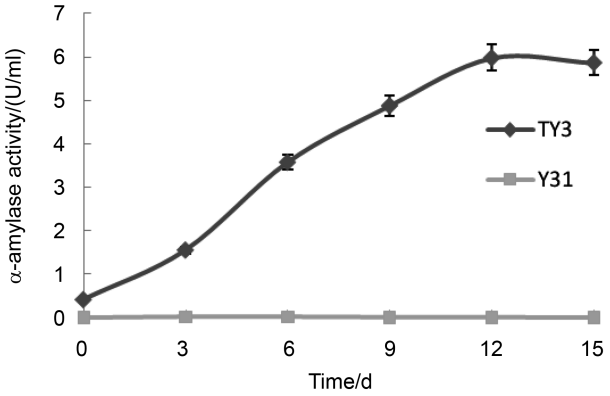

Fig. 4. Assay of α-amylase activities of Y31 and TY3 during fermentation.
Y31 (■), TY3 (◆).

of maltotriose when compared with that in the Y31 broth (from 2.90 to 0.87 mg/l). Moreover, the fermentation degree for the TY3 broth was higher at 71.44% when compared with 66.14% for the Y31 broth. Previous reports have suggested that lower concentrations of residual saccharides in the fermentation broth may be related to a reduced caloric content in the final beer [15, 28]. Thus, beer brewed using the recombinant strain TY3 could also have a lower caloric content than beer brewed using the host strain Y31, thereby helping to address consumer health problems associated with obesity and tooth decay [17, 20].

In conclusion, the genetically modified strain TY3 produced a better fermentation performance than the host strain Y31 in terms of a higher GSH production, enhanced flavor stability, and lower diacetyl and acetaldehyde production. Moreover, no heterologous DNA fragments were introduced to the host strain, making strain TY3 relatively safe and more acceptable for application in the beer industry.

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References

1. Adamis, P. D. B., A. D. Panek, S. G. F. Leite, and E. C. A. Eleutherio. 2003. Factors involved with cadmium absorption by a wild-type strain of *Saccharomyces cerevisiae*. *Braz. J. Microbiol* **34:** 55–60.
2. Back, W., O. Franz, and T. Nakamura. 2001. Das antioxidative potenzial von Bier. *Brauwelt* **141:** 209–215.
3. Baur, X., Z. Chen, and I. Sander. 1994. Isolation and denomination of an important allergen in baking additives: α-Amylase from *Aspergillus orizae* (Asp O II). *Clin. Exp. Allergy* **24:** 1465–1470.
4. Bergmeyer, H. V., K. Gacoehm, and M. Grassl. 1974. *In* H. V. Bergmeyer (ed.). *Methods of Enzymatic Analysis*, Vol. 2, pp. 428–429. Academic Press, NY.
5. Blandino, A., I. Caro, and D. Cantero. 1997. Comparative study of alcohol dehydrogenase activity of flor yeast extracts. *Biotech. Lett.* **19:** 651–654.
6. Cai, Y., Q. Mu, Z. Y. Wang, B. R. Zhang, and B. J. Yan. 2008. Construction of self-cloning industrial brewing yeast with high-glutathione production and low ADH II enzyme activity. *Microbiolgy* **35:** 1171–1175 [*In Chinese*]
7. Douglas, K. T. 1987. Mechanisms of action of glutathione-dependent enzymes, pp. 103–167. *In* A. Meister (ed.). *Advances in Enzymology*. John Wiley & Sons, NY.
8. Eksteen, J. M., P. van Rensburg, R. R. C. Otero, and I. S. Pretorius. 2003. Starch fermentation by recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. *Biotechnol. Bioeng.* **84:** 639–646.
9. Fan, X., X. He, X. Guo, N. Qu, Ch. Wang, and B. Zhang. 2004. Increasing glutathione formation by functional expression of γ-glutamylcysteine synthetase gene in *Saccharomyces cerevisiae*. *Biotech. Lett.* **26:** 415–417.
10. Gange, M. A., F. Pinãga, S. Valleâs, D. Ramoân, and A. Querol. 1999. Aroma improving in microvinification processes by the use of a recombinant wine yeast strain expressing the *Aspergillus nidulans xlnA* gene. *Int. J. Food Microbiol.* **47:** 171–178.
11. Jiang, K., Q. Li, and G. X. Gu. 2007. Improvement of beer anti-staling capability by genetically modifying industrial brewing yeast with high glutathione content. *Chinese J. Biotechnol.* **23:** 1071–1076. [*In Chinese*]
12. Kang, N. Y., J. N. Park, J. E. Chin, H. B. Lee, S. Y. Im, and S. Bai. 2003. Construction of an amylolytic industrial strain of *Saccharomyces cerevisiae* containing the *Schwanniomyces occidentalis* α-amylase gene. *Biotech. Lett.* **25:** 1847–1851.
13. Landaud, S., P. Lieben, and D. Picque. 1998. Quantitative analysis of diacetyl, pentanedione and their precursors during beer fermentation by an accurate GC/MS method. *J. Inst. Brew.* **104:** 93–99.
14. Li, H., C. X. Song, Y. Y. Wu, and W. J. Zhang. 2005. Correlation studies of beer resistant indexes and beer staling value. *Liquor-making SciTechnol.* **1:** 57–60. [*In Chinese*]
15. Liu, X. F., Z. Y. Wang, J. J. Wang, Y. Lu, X. P. He, and B. R. Zhang. 2009. Expression of *GAI* gene and disruption of *PEP4* gene in an industrial brewer's yeast strain. *Lett. Appl. Microbiol.* **49:** 117–123.
16. Liu, Z. R., G. Y. Zhang, Z. F. Long, and S. G. Liu. 2005. Heterologous expression of amylase gene from *Saccharo-mycopsis fibuligera* in an industrial strain of *Saccharomyces Cerevisiae*. *Wuhan Univ. J. Nat. Sci*. **10:** 1041–1046.
17. McTigue, K. M., R. Harris, and B. Hemphill. 2003. Screening and interventions for obesity in adults: Summary of the evidence for the U.S. Preventive Services Task Force. *Ann. Int. Med.* **139:** 933–949.
18. Miller, J. L., W. E. Glennon, and A. L. Burton. 1960. Measurement of carboxymethylcellulase activity. *Anal. Biochem.* **2:** 127–132.
19. Nieto, A., J. A. Prieto, and P. Sanz. 1999. Stable high-copy number integration of *Aspergillus orizae* α-amylase cDNA in an industrial baker's yeast strain. *Biotech. Progr.* **15:** 459–466.

20. Nogueira, F. N., D. N. Souza, and J. Nicolau. 2000. *In vitro* approach to evaluate potential harmful effects of beer on health. *J. Dent. Res.* **28:** 271–276.
21. Parsons, R. and R. Cope. 1983. The assessment and prediction of beer flavor stability. *Proceedings of the Congress of the European Brewery Convention*. Information Press, Oxford
22. Penninckx, M. J. 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res.* **2:** 295–305.
23. Pézer-Gonzalez, J. A., R. Gonzalez, A. Querol, J. Sendra, and D. Ramoón. 1993. Construction of a recombinant wine yeast strain expression β-(1,4)-endoglucanase and its use in micro-vinification processes. *Appl. Environ. Microbiol.* **59:** 2801–2806.
24. Schiestl, R. H. and R. D. Gietz. 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.* **16:** 339–346.
25. Sun, J. S., W. J. Zhang, F. C. Jia , Y. Yang, Z. P. Lin, J. Z. Feng, P. Martin, and D. L. Wang. 2006. Disruption of brewer's yeast alcohol dehydrogenase II gene and reduction of acetaldehyde content during brewery fermentation. *J. Am. Soc. Brew. Chem.* **64:** 195–201.
26. Van Rensburg, P., M. L. A. Strauss, M. G. Lambrechts, R. R. C. Otero, and I. S. Pretorius. 2007. The heterologous expression of polysaccharidase-encoding genes with oenological relevance in *Saccharomyces cerevisiae*. *J. Appl. Microbiol.* **103:** 2248–2257.
27. Vilanova, M., P. Blanco, S. Cortéz, M. Castro, T. G. Villal, and C. Sieiro. 2000. Use of PGU1 recombinant *Saccharomyces cerevisiae* strain in oenological fermentation. *J. Appl. Microbiol.* **89:** 876–883.
28. Wang, J. J., Z. Y. Wang, X. F. Liu, X. N. Guo, X. P. He, P. C. Wensel, and B. R. Zhang. 2010. Construction of an industrial brewing yeast strain to manufacture beer with low caloric content and improved flavor. *J. Microbiol. Biotechnol*. doi: 10.4014/jmb.0910.10013
29. Wang, Z. Y., X. P. He, W. H. Li, N. Liu, and B. R. Zhang. 2008. Construction of self-cloning brewing yeast with high-glutathione and low-diacetyl production. *Int. J. Food Sci. Tech.* **43:** 989–994.
30. Wang, Z. Y., X. P. He, and B. R. Zhang. 2007. Over-expression of *GSH1* gene and disruption of *PEP4* gene in self-cloning industrial brewer's yeast. *Int. J. Food Microbiol.* **119:** 192–199.
31. Wang, Z. Y., J. J. Wang, X. F. Liu, X. P. He, and B. R. Zhang. 2009. Recombinant industrial brewing yeast strains with *ADH2* interruption using self-cloning *GSH1+CUP1* cassette. *FEMS Yeast Res.* **9:** 574–581.
32. Yan, M., Q. Li, and G. X. Gu. 2005. The estimation of endogenesis antioxidative activity of beer by DPPH radical scavenging capacity. *Sci. Technol. Food Ind.* **26:** 82–83, 87. [*In Chinese*]
33. Zhang, J. N., X. P. He, X. N. Guo, N. Liu, and B. R. Zhang. 2005. Genetically modified industrial brewing yeast with high-glutathione and low-diacetyl production. *Chinese J. Biotechnol.* **21:** 942–946. [*In Chinese*]
34. Zhang, Y., Z. Y. Wang, X. P. He, N. Liu, and B. R. Zhang. 2008. New industrial brewing yeast strains with *ILV2* disruption and *LSD1* expression. *Int. J. Food Microbiol.* **123:** 18–24.
35. Zhang, Y. X., Q. Li, W. Shen, Y. Xie, and G. X. Gu. 2008. Effects of knockout ECM25/YJL210W gene in brewing yeast on beer flavor stability. *Chinese J. Biotechnol.* **24:** 1420–1427.
36. Zhao, L. J., D. L. Wang, Y. L. Cheng, J. Z. Zhou, and B. G. Ge. 2006. Study on the control of aldehyde content in beer by molecular biological measures. *Liquor-making Sci. Technol.* **1:** 45–47. [*In Chinese*]