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# Construction of an Industrial Brewing Yeast Strain to Manufacture Beer with Low Caloric Content and Improved Flavor

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**In this study, the problems of high caloric content, increased maturation time, and off-flavors in commercial beer manufacture arising from residual sugar, diacetyl, and acetaldehyde levels were addressed. A recombinant industrial brewing yeast strain (TQ1) was generated from T1 [*Lipomyces starkeyi* dextranase gene (*LSD1*) introduced, α-acetohydroxyacid synthase gene (*ILV2*) disrupted] by introducing *Saccharomyces cerevisiae* glucoamylase (*SGA1*) and a strong promoter (*PGK1*), while disrupting the gene coding alcohol dehydrogenase (*ADH2*). The highest glucoamylase activity for TQ1 was 93.26 U/ml compared with host strain T1 (12.36 U/ml) and wild-type industrial yeast strain YSF5 (10.39 U/ml), respectively. European Brewery Convention (EBC) tube fermentation tests comparing the fermentation broths of TQ1 with T1 and YSF5 showed that the real extracts were reduced by 15.79% and 22.47%; the main residual maltotriose concentrations were reduced by 13.75% and 18.82%; the caloric contents were reduced by 27.18 and 35.39 calories per 12 oz. Owing to the disruption of the *ADH2* gene in TQ1, the off-flavor acetaldehyde concentrations in the fermentation broth were 9.43% and 13.28%, respectively, lower than that of T1 and YSF5. No heterologous DNA sequences or drug resistance genes were introduced into TQ1. Hence, the gene manipulations in this work properly solved the addressed problems in commercial beer manufacture.**

**Keywords:** Brewing, low calorie, glucoamylase, dextranase, yeast

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Beer is a beverage of immense commercial interest and complexity. It is composed of volatile compounds, like alcohol, acetaldehyde, pentanedione, and diacetyl, as well as nonvolatile compounds like carbohydrates [29]. Currently the consumption of alcoholic beer beverage is growing annually worldwide [27]. However, high caloric content in beer is attributed to consumers' health problems associated with obesity and tooth decay [15, 19]. Both volatile and nonvolatile components of beer contribute to its overall caloric content. Apart from alcohol, carbohydrates provide the most part of caloric content. During the brewing process, some carbohydrates remain as non-fermentable, highly caloric residual saccharides because of the absence of amylolytic activity in brewing yeast. Among these residual saccharides is maltotriose. Maltotriose, like maltose, is the second most abundant sugar in wort, which is normally hydrolyzed into glucose molecules by yeast; however, this metabolite cannot be completely consumed. The slow and incomplete consumption of maltotriose may result in beer having a high caloric content and unusual flavor. Therefore, the reduction of excessive residual saccharides during the brewing process is a key objective.

Traditionally, large amounts of exogenous enzymes (*i.e.*, α-amylase, dextranase, and glucoamylase) [20] are added prior to fermentation to liberate fermentable sugars from polysaccharide-rich substrates. However, this has been prohibitively expensive and responsible for consumer allergenic-related symptoms [2, 18]. A genetically manipulated *Saccharomyces cerevisiae* fermentation strain with amylolytic properties could address these problems associated with residual saccharides. Some researchers have introduced genes encoding α-amylase, dextranase, or glucoamylase to laboratory yeast strains [4, 9, 13, 25]. However, laboratory-generated recombinant yeast strains differ markedly from industrial strains because they are manipulated with shuttle

vectors containing both antibiotic-resistance and yeast-resistance markers deemed as a risk to beverage consumers [9, 28]. Safer industrial recombinant strains have recently been constructed [8, 12, 14, 31, 32]. However, these strains often show low levels of enzyme expression and could not improve the beer quality comprehensively since only one or two genes were modified to target-specified beer indexes. Alternatively, the complexity of beer warrants a more rigorous multigene regulation approach for comprehensive beer characteristic improvement.

In addition to the issue of residual saccharides, other compounds such as acetaldehydes, diacetyls, and pentanedione, which impart off-flavors and odors when excessive levels persist, affect the quality of beer as well. Acetaldehyde is a natural fermentation by-product and is the direct precursor of ethanol, and it leaves a pungent and irritating green-apple aroma at high concentrations but a more pleasant fruity aroma at dilute concentrations. It also affects beer freshness [32]. The higher acetaldehyde concentration in Chinese beer (3–8 mg/l) relative to overseas fine beer (<2 mg/l) [30] has been the focus of many beer researches in China. Reports have shown that disruption of the *ADH2* gene encoding alcohol dehydrogenase reduces the acetaldehyde content in beer [7, 26, 33]. The diacetyl in beer leaves an undesirable off-flavor at high concentrations too. It also increases beer maturation time, which is a rate-limiting bottleneck step in production. Decreasing the diacetyl content in beer by interrupting its metabolism is an effective way to reduce beer maturation time [17]. Reports have shown that disruption of the gene *ILV2* encoding diacetyl's precursor α-acetolactate synthase reduces the diacetyl content in beer [8, 31].

In a previous work, recombinant strain T1 [8] was constructed by disrupting the *S. cerevisiae* α-acetohydroxyacid synthase gene (*ILV2*) and introducing the *Lipomyces starkeyi* dextranase gene (*LSD1*) as a selective marker.

In this work, it is the first time to modifying the industrial yeast strain with the following characteristics: (1) Simultaneously overexpressing the amylolytic genes encoding *S. cerevisiae* glucoamylase (*SGA1*) and *L. starkeyi* dextranase (*LSD1*) to reduce the caloric content of beer. (2) Disrupting the genes encoding alcohol dehydrogenase (*ADH2*) and α-acetolactate synthase (*ILV2*) to limit the synthesis of the off-flavor components acetaldehyde and diacetyl.

Through the alternative multigene approach, we succeeded in reducing the residual concentrations of saccharides, diacetyl, and acetaldehyde in the final fermentation broth. Since only yeast-derived genes were introduced into the new *S. cerevisiae* strain TQ1, its use in commercial beer production should be relatively safe to the public and therefore suitable for improved industrial application.

## Materials and Methods

### Strains, Plasmids, and Cultivation Conditions

The sources and relevant genotypes of the strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α cells were used for general DNA manipulation. T1, modified from the industrial brewing yeast strain *S. cerevisiae* YSF5 (Tsingtao Brewery Company, Qingdao, China) in a previous study *via* the introduction of *LSD1* as a selective marker and simultaneous disruption of *ILV2* [8], was used as the host strain. The YSF5 strain was used as the DNA donor of the *SGA1* gene.

*E. coli* was cultured at 37°C in Luria–Bertani (LB) medium [22] containing ampicillin (100 μg/ml) when required. Yeast strains for transformation were grown in YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose] at 28°C. Recombinant

**Table 1.** Strains and plasmids used in this study.

| Strains or plasmids | Genotype | Source |
|---|---|---|
| Strains | | |
| *Escherichia coli* DH5α | *sup*E44 Δ*lac*U169 (φ80*lac*ZΔM15) *hsd*R17 *rec*Al *end*Al *gyr*A96 *thi*-1 *rel*Al | Stratagene |
| Brewing yeast strains | | |
| *Saccharomyces cerevisiae* YSF5 | Wild-type | Tsingtao Brewery Company (Qingdao, China) |
| T1 | Self-cloning yeast strain | [8] |
| TQ1 | Self-cloning yeast strain | This work |
| Plasmids | | |
| YEp352 | Cloning vector, *URA3 amp* | [12] |
| pYCUP | Recombinant plasmid, *URA3 amp* | [8] |
| pMP1 | Cloning vector, *amp* | [8] |
| pMPS | Cloning vector, *amp* | This work |
| pYA | Recombinant plasmid, *URA3 amp* | This work |
| pQD1 | Recombinant plasmid, *URA3 amp* | This work |
| pTQ | Recombinant plasmid, *URA3 amp* | This work |

**Table 2.** Oligonucleotide primers used in the PCR amplification reactions.

| Primer | Sequences (5' to 3') |
|---|---|
| S1 | TA TCTAGACCAAACGATGAGATTTCCTTC (*Xba*I) |
| S2 | CA GAATTC TACGTAAGCTTCAGCCTC (*Eco*RI) |
| SGA1-L | GTCTCTAGACTCGAGAACATTACTATAT (*Xba*I) |
| SGA1-R | TCCGAGCTCTACAATCCTGGCAACAAG (*Sac*I) |
| ADH2-L | ACGAATTC GCTGTTATGTTCAAGGTC (*Eco*RI) |
| ADH2-R | TCGGATCC TTCAGAGGAGCAGGACAA (*Bam*HI) |
| PGK1-L | CTTTCGACGGTATCGATAAGCT |
| PGK1-R | TTTTACATCGTCAACCTGGGCT |
| LSD1-L | GGAACGTTGTTGATTGTTTT |
| LSD1-R | TTCGACATGAACTTCCTTGGC |
| CUP1-L | CGCTATACGTGCATATGTTC |
| ILV2-L | CCCGACAATAAAGTAAATAG |
| ILV2-R | AGAAAGAAGCGTAAGATC |

strains were selected from the YPD plate (1.5% agar) using 9 mM copper sulfate ($CuSO_4$) as the selection marker.

**DNA Manipulation, Plasmids Construction, and Yeast Transformation**

Genomic DNA of yeast was prepared as described by Burke *et al*. [6]. The *ADH2* gene, the sequence of which was used for homologous recombination, was amplified from YSF5 genomic DNA using PCR with the primers ADH2-L/ADH2-R (Table 2) (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, Shanghai, China). The PCR product was subsequently subcloned into YEp352 to generate the plasmid pYA. The DNA corresponding to the *SGA1* gene was amplified from YSF5 genomic DNA using PCR with the primers SGA1-L/SGA1-R (Table 2). Although the glucoamylase gene already existed in the genome of YSF5 and its derivative T1, the expression of this gene was extremely weak. The strong promoter in *S. cerevisiae*, the *PGK1* promoter ($PGK1_p$) from pMP1 [8], was therefore introduced to ensure higher expression of this *SGA1* gene. α Signal factor was employed for the purpose of secreting expression of *SGA1*. The fragment containing the *CUP1* gene from pYCUP was introduced as a selective marker. All these fragments were then ligated into the *Sac*I and *Sph*I sites of pYA by T4 DNA ligase to generate plasmid pTQ (Fig. 1A) [22].

Plasmid pTQ was digested with *Pvu*II, and the fragment containing the 5.0-kb expression cassette TQ (Fig. 1B) was purified using a DNA Gel Extraction Kit (Beijing Probe Bioscience Technology Co., Ltd, Beijing, China) and transformed into T1 using the lithium acetate (LiAc) method [23]. Since T1 has no nutritional marker, the *CUP1* gene that encodes a metallothionein, which binds copper, was adopted for the selection of the recombinant strains. Recombinant strains were selected on the YPD plate using 9 mM $CuSO_4$ as the selection marker.

**Enzymes Activities Assays**

Alcohol dehydrogenase activity was assayed using a modified Bergmeyer method [3]. The extraction technique was modified from the original approach [24] as described by Blandino *et al*. [5].

The activity of glucoamylase was measured using the DNS method [16]. One unit (U) of glucoamylase activity was defined as the amount of enzyme catalyzing the production of 1 nmole of glucose in 30 min at 37°C.

Dextranase activity was determined from the rate of increase in reducing sugars as measured by the DNS method. One unit (U) of dextranase was defined as the amount of enzyme required to liberate 1 μmole of glucose equivalent from dextran T-70 in 40 min at 50°C and pH 5.5 under the previously described conditions [21].

α-Acetolactate synthase activity was detected using the method as described by Zhang *et al*. [8].

**Genetic Stability Test of Recombinant Strain**

The recombinant strain was transferred onto the YPD slant for 15 generations. Each generation was cultivated for 36 h at 28°C. Plate streaking of the 1st, 4th, 8th, 12th, and 15th generation strains was performed. After 48 h of cultivation at 28°C, 100 single-grown colonies were chosen randomly and transferred to 0.5 ml of sterilized water and starved for 4 h at room temperature. A Greiner inoculation loop of the starved yeast suspension was used to inoculate YPD plates containing 9 mM $CuSO_4$ and stored at 28°C for 2 days.

Alcohol dehydrogenase, glucoamylase, dextranase, and α-acetolactate synthase activities of the 1st, 4th, 8th, 12th, and 15th generation strains were analyzed as described above.

**PCR Verification of Recombinant Strain**

PCR analysis was performed using the primers listed in Table 2. PCR was performed in a 50-μl volume with 25 μl of Pfu or *Taq* mastermix (Tiangen Biotech (Beijing) Co., Ltd, Beijing, China), 120 ng of template DNA, and 0.2 μM primers. Cycle conditions were 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 2 min 30 s, and finally 72°C for 15 min.

**Determination of the Number of Calories**

The number of calories in beer is due to the presence of alcohol and carbohydrates. The ASBC (American Society of Brewing Chemists) provides a formula for calculating the number of calories in beer [1]:

$$\text{Cal. per 12 oz beer}=[(6.9\times ABW)+4.0\times(RE-0.1)]\times FG\times 3.55. \quad (1)$$

The caloric contribution of ethanol is determined from the alcohol-by-weight (ABW) and the known value of 6.9 cal/g of ethanol. The caloric contribution from the carbohydrates present is determined from the real extract (RE) and the known value of 4.0 cal/g for carbohydrates. An empirically derived constant (0.1) accounts for the ash portion of the extract. These terms provide the calories per 100 g of beer. This is easily converted to calories per 100 ml of beer by accounting for the final gravity [FG, in (g/ml)]. In turn, 100 ml is converted to 12 oz by a scalar value [3.55, in (ml/oz)].

**Preservative Qualities Assay**

The preservative qualities of the fermentation broths, and the corresponding antiaging characteristics of the various strains, were determined by measuring both the thiobarbituric acid (TBA) value and resistant staling value (RSV) using a spectrophotometric method [10]. These two values are two significant indexes reflecting beer freshness.

**Fermentation Test and Pilot-Scale Brewing**

The fermentation test was performed as described by Wang *et al*. [32]. The fermentation was carried out at 12°C for 10 days first in

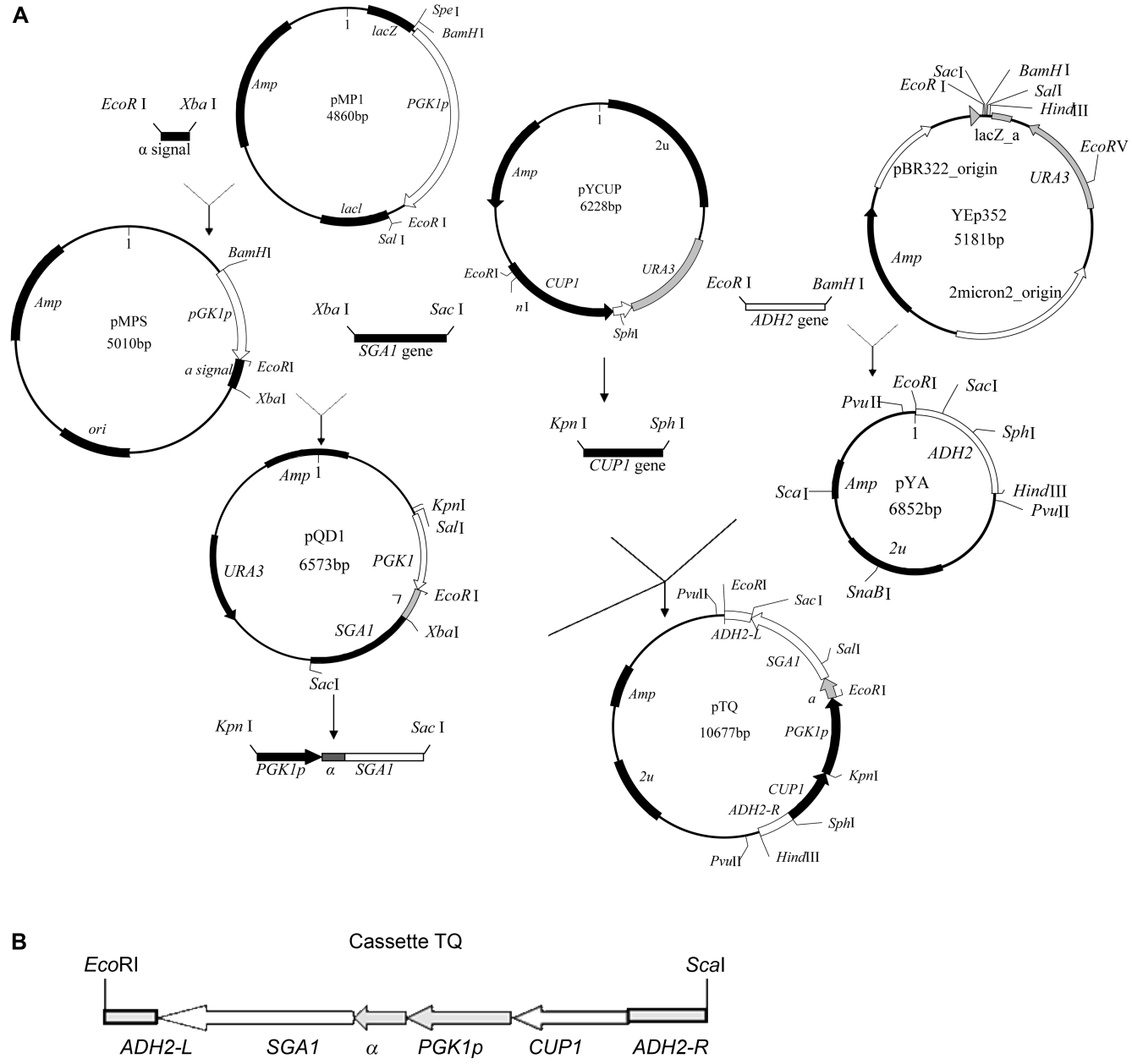


**Fig. 1.** The construction of related plasmids and recombinant strains.

conical flasks. The yeast pellets from conical flasks were harvested for the pilot-scale brewing, which was carried out in a 6-l European Brewery Convention (EBC) tube with 5-l 10 °P wort at 10°C for 15 days. Real extract, attenuation degree, and alcohol content in the fermenting filtrate were measured using an Alcolyzer Plus Beer machine WBA-505B (Kyoto Electronics Manufacturing Co., Ltd, Tokyo, Japan). Acetaldehydes, diacetyl, and main residual sugars content were measured by GC/MS as described by Landaud *et al.* [11]. The fermentation broths were refrigerated at 4°C for 3 days. A comparative and qualitative test to evaluate the sensorial characteristics of the broths was then conducted on-site by six tasting experts, sent in from the division of the Tsingtao Brewery Co., per company quality control procedures.

## Results and Discussion

### Construction and Selection of Recombinant Strains

Since the higher caloric content in beer is mainly caused by the absence of amylolytic activity in brewing yeast, modification of the yeast strain by offering amylolytic properties is a better way to solve this problem. In our previous study, the *LSD1* gene was introduced into the industrial brewing yeast YSF5 and the engineering strain T1 was constructed. The properties of T1 have been improved; however, only *LSD1* expression could not decrease the caloric content efficiently, and therefore, another amylolytic

gene, *SGA1*, was modified in this study by enhancing its expression with a strong promoter. In the meantime, the alcohol dehydrogenase gene (*ADH2*) was disrupted in order to decrease the acetaldehyde content in beer.

Recombinant strains were generated from the transformation of T1 with the 5.0-kb expression cassette TQ (*adh2*Δ::*SGA1*) by homologous recombination, and 28 recombinant strains were selected for resistance to higher copper concentrations (9 mM $CuSO_4$) and sequentially named using the nomenclatures TQ1, TQ2, up to TQ28.

The different enzyme activities of these 28 recombinant strains and T1 cultivated in YPD medium were measured. The alcohol dehydrogenase activities of the recombinant strains were 47.01–55.78% (8.10–9.61 U/mg) lower than that of the host strain T1 (17.23 U/mg). All 28 recombinant strains showed high glucoamylase activity, whereas insignificant glucoamylase activity was detected in the host strain T1 (data not shown). TQ1 was chosen for further research because of its high glucoamylase activity and low alcohol dehydrogenase activity.

**Genetic Stability Analysis**

All 100 single colonies of the 1st, 4th, 8th, 12th, and 15th generations of the recombinant strain TQ1 grew on the YPD plate in the presence of the selection marker, 9 mM $CuSO_4$, whereas the host strains T1 and YSF5 did not. Such long-term stable growth in copper indicated that the *CUP1* gene stably integrated into the *ADH2* locus, enabling the TQ1 recombinant strain to exhibit copper resistance. Likewise, evidence of long-term stability in enzymatic expression, *via* measured alcohol dehydrogenase, glucoamylase, and dextranase activities of each generation (1st, 4th, 8th, 12th, and 15th) also indicated stable integration (data not shown).

**PCR Verification and Enzymes Analysis of the Recombinant Strain**

The flanking *ADH2* sequences were designed for homologous recombination targeting the *ADH2* allele of the T1 chromosome. The *SGA1* and *CUP1* genes were integrated into the *ADH2* locus of the yeast genomic DNA, which was confirmed by PCR (Fig. 2) using different primer pairs (Table 2) on the TQ1 genomic DNA. The sizes of the fragments were in agreement with the predicted PCR fragment sizes, with the exception of the PCR product using the primer pair ADH2-L/ADH2-R. This exceptional PCR product was 5.00 and 1.70 kb in size, indicating that only one copy of *ADH2* was deleted and that an additional copy of the wild-type *ADH2* was still present. Although it would have been ideal to disrupt both copies of the wild-type *ADH2* gene, previous studies [30, 32] showed that one copy of the pair of *ADH2* gene was more problematic to disrupt than the other. According to our previous studies on this gene (unpublished), totally knocking out the *ADH2* gene would affect the biomass of the yeast. Thus, the *ADH2* gene might be essential for yeast to grow. In this work, because only one of the two copies of the *ADH2* gene was disrupted, alcohol dehydrogenase activity in the recombinant strain TQ1 was still detected. However, this activity was reduced to approximately half of that observed in the T1 and the parental YSF5 strains, which both had two intact copies of the *ADH2* gene.

Moreover, the sizes of the amplified PCR fragments using primers LSD1-L/LSD1-R and ILV2-L/ILV2-R on the TQ1 template were as anticipated. The dextranase and α-acetolactate synthase activities of strain TQ1 were similar to those of the T1 (data not shown), indicating that the disruption of *ADH*2 gene by *SGA1* in the T1 strain did not affect the expression of dextranase and α-acetolactate

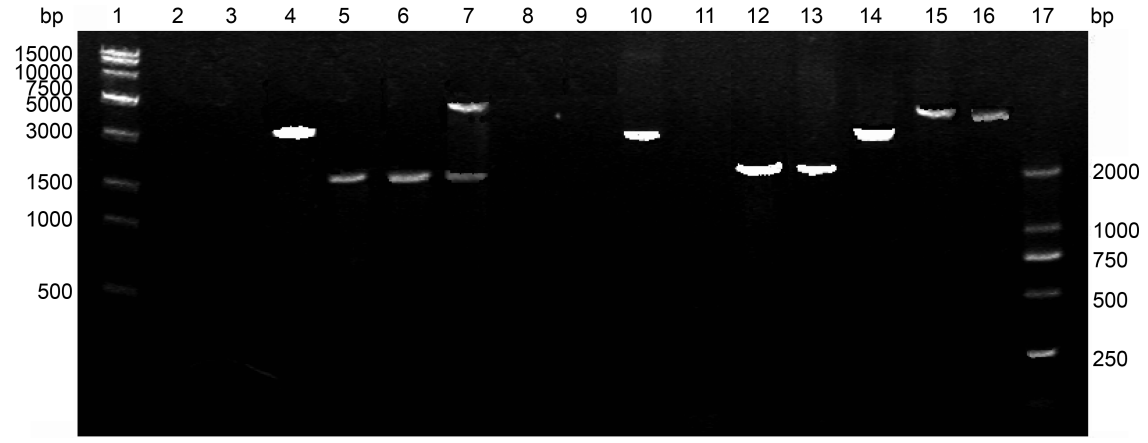


**Fig. 2.** PCR verification of the recombinant strains.

Lane 1: Marker D15000; Lane 2: YSF5 (PGK1-L/SGA1-R); Lane 3: T1 (PGK1-L/SGA1-R); Lane 4: TQ1 (PGK1-L/SGA1-R); Lane 5: YSF5 (ADH2-L/ADH2-R); Lane 6: T1 (ADH2-L/ADH2-R); Lane 7: TQ1 (ADH2-L/ADH2-R); Lane 8: YSF5 (CUP1-L/PGK1-R); Lane 9: T1 (CUP1-L/PGK1-R); Lane 10: TQ1 (CUP1-L/PGK1-R); Lane 11: YSF5 (LSD1-L/LSD1-R); Lane 12: T1 (LSD1-L/LSD1-R); Lane 13: TQ1 (LSD1-L/LSD1-R); Lane 14: YSF5 (ILV2-L/ILV2-R); Lane 15: T1 (ILV2-L/ILV2-R); Lane 16: TQ1 (ILV2-L/ILV2-R); Lane 17: marker D2000.

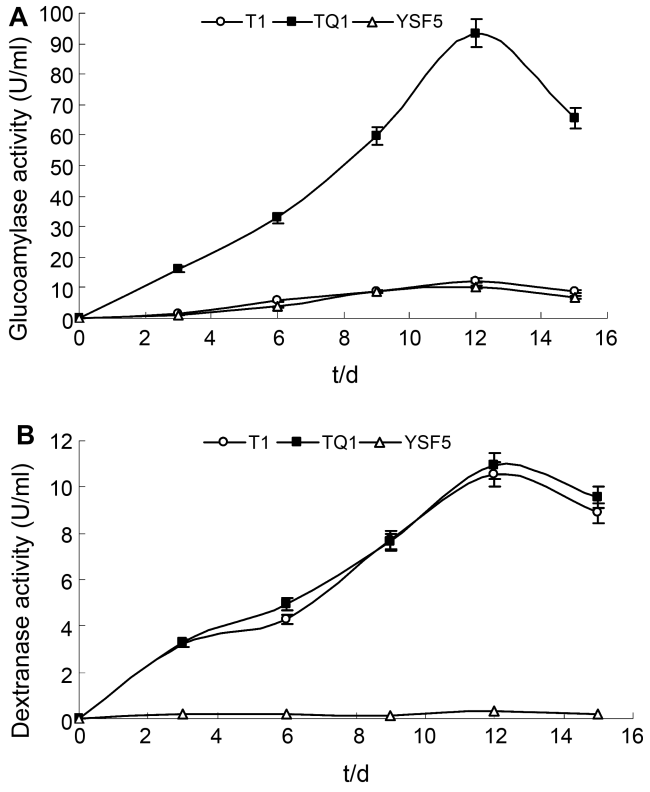


**Fig. 3.** Assay of glucoamylase (**A**) and dextranase (**B**) activities present in the TQ1 (■), T1 (○), and YSF5 (△) yeast strains during EBC tube fermentation.
Values represent the means of three replications.

synthase. The results of the PCR reactions using the genomic DNA of YSF5 and T1 as templates are also shown in Fig. 2, and their amplified fragment sizes were as anticipated. This verified that the DNA fragments were inserted into the host strain T1's genome.

**Fermentation Test and Pilot-Scale Brewing**

Recombinant strain TQ1, its host strain T1, and the parental industrial brewing yeast strain YSF5 were tested in comparative pilot-scale fermentations. The data obtained were analyzed by a one-way analysis of variance (ANOVA).

During EBC tube fermentation, different enzymes activities were detected (Fig. 3). Firstly, significantly higher levels of glucoamylase activity were detected in the TQ1 strain, compared with T1 and YSF5, with the highest 93.26 U/ml being observed in TQ1 fermentation broth on the 12th day of fermentation. In contrast, insignificant glucoamylase activity was detected in both T1 and YSF5 fermentation broths through the low-level expression of glucoamylase. However, under the control of the strong *PGK1* promoter, the *SGA1* gene was highly expressed in the recombinant strain TQ1, and *via* α signal sequence, its glucoamylase could easily be secreted into the fermentation broth. Hence, the glucoamylase activity detected in the

**Table 3.** Glucoamylase activities of TQ1, T1, and YSF5 in fermentation tests from conical flasks (mean±SD, $n$=3).

| Strain | Glucoamylase activity | |
|---|---|---|
| | Intracellular (U/mg) | Fermentation broth (U/ml) |
| YSF5 | 2.07±0.02 | 1.02±0.01 |
| T1 | 2.36±0.09 | 1.49±0.03 |
| TQ1 | 9.59±0.12 | 36.47±0.23 |

TQ1 fermentation broth from conical flasks exceeded that measured intracellularly by nearly 4-fold (Table 3). This value was higher than the heterologous glucoamylase gene expression levels reported by Liu *et al.* [12]. Besidesthis, high levels of dextranase acitivity were detected in both TQ1 and T1 fermentations, unlike in YSF5 where none was detected. This also indicated that the dextranase gene was steadily expressed in strain TQ1 in despite of *SGA1* expression and *ADH2* disruption.

At the end of the EBC tube fermentation, the concentrations of certain key residual saccharides were measured. Saccharides such as glucose, sucrose, maltose, and fructose were exhausted and not detected. The main reduction detected was the concentration of residual maltotriose using the GC/MS test (Table 4). The residual maltotriose concentration measured in the TQ1 fermentation broth (0.69 mg/l) was 18.82% and 13.75% ($F$=20.04, $P$<0.01) lower than that observed for YSF5 (0.85 mg/l) and T1 (0.80 mg/l). Liu *et al.* [14] reported that a mutant brewing yeast strain expressing the glucoamylase (*GLA*) gene was able to ferment maltotriose. In this study, the host strain T1 expressing the *LSD1* gene fermented maltotriose. In contrast, the recombinant strain TQ1 coexpressing both the *LSD1* and *SGA1* genes showed a significantly greater capacity to metabolize maltotriose in the fermentation broth. This resulted in a greater reduction of carbohydrate and caloric contents. The fermentation degree was therefore increased to 74.48% for TQ1 compared with that for YSF5 (67.47%) and T1 (70.16%). Correspondingly, the real extract concentration in the fermentation broth of TQ1 was reduced to 3.52% compared with that of YSF5 (4.54%) and T1 (4.18%). Whereas single expression of the *LSD1* gene improved the fermentation degree and reduced the residual saccharide concentrations, the coexpression of the *SGA1* and *LSD1* gene ensured further degradation of the residual saccharides and increased the fermentation degree.

Decreasing the real extract concentration resulted in a lower calorie level [see Eq.(1)]. The ethanol concentration present in the TQ1 fermentation broth was similar to that of T1 and YSF5. Using Eq. (1), TQ1 produced the least amount of calories per 12 oz fermentation broth (259.47 cal.), which was 12.00% and 9.48% ($F$=29.31, $P$<0.01) lower than that of YSF5 (294.86 cal.) and T1 (286.65 cal.). Consequently, beer fermentation using the recombinant

**Table 4.** Parameters of the fermenting liquor from EBC fermentation tube (mean±SD, $n$=3).

| Parameters | YSF5 | T1 | TQ1 | F | $P$-value |
|---|---|---|---|---|---|
| %Real extract | 4.54±0.02 | 4.18±0.03 | 3.52±0.06 | 36.55 | $4.36e^{-4}$ ,S |
| %Real attenuation | 67.47±0.02 | 70.16±0.03 | 74.48±0.31 | 333.18 | $7.11e^{-7}$ ,S |
| Fructose (g/l) | - | - | - | - | - |
| Glucose (g/l) | - | - | - | - | - |
| Sucrose (g/l) | - | - | - | - | - |
| Maltose (g/l) | - | - | - | - | - |
| Maltotriose (g/l) | 0.85±0.001 | 0.80±0.001 | 0.69±0.001 | 20.04 | $1.72e^{-3}$ ,S |
| Acetaldehyde (ppm) | 9.19±0.01 | 8.80±0.01 | 7.97±0.01 | 261.64 | $1.46e^{-6}$ ,S |
| Diacetyl (ppb) | 147.00±4.00 | 134.00±1.00 | 130.33±10.33 | 45.02 | $2.44e^{-4}$ ,S |
| Pentanedione (ppb) | 130.00±37.00 | 80.33±9.33 | 62.67±25.33 | 153.06 | $7.1e^{-6}$, S |
| Calorie per 12 oz fermentation broth | 294.86±48.53 | 286.65±33.44 | 259.47±23.39 | 29.31 | $8.01e^{-4}$ ,S |
| TBA value ($OD_{530}$) | 0.56±0.001 | 0.51±0.001 | 0.47±0.001 | 41.57 | $6.50e^{-3}$ ,S |
| RSV | 436.58±51.70 | 497.39±11.98 | 479.54±29.99 | 62.58 | $3.58e^{-3}$ ,S |

S, significant ($P$<0.01).

strain TQ1 represents an improved approach to reduce beer caloric content with consumer health in mind.

Since both the *ILV2* and *ADH2* genes were disabled in the TQ1 recombinant strain, primary off-flavor compound concentrations were also reduced in its final fermentation broth. The reduced concentration of pentanedione from TQ1 (62.67 ppb) was 51.79% and 21.98% (F=153.06, $P$<0.01) lower compared with that from YSF5 (130.00 ppb) and T1 (80.33 ppb). The reduced concentration of diacetyl from TQ1 (130.33 ppb) was 11.34% (F=45.02, $P$<0.01) lower compared with that from YSF5 (147.00 ppb) and similar to that from T1 (134.00 ppb). The similarity between the diacetyl concentration from TQ1 and that from T1 was expected because the *ILV2* gene was disrupted and inactivated in both strains.

In addition, the reduced acetaldehyde concentration from TQ1 (7.97 ppm) was 15.23% and 9.43% (F=261.64, $P$<0.01) lower compared with that from YSF5 (9.19 ppm) and T1 (8.80 ppm), respectively (Table 4). Surprisingly, a small decrease in the concentration of acetaldehyde was observed from T1 compared with YSF5, even though its *ADH2* gene was still functional. This observation is consistent with our previous study on gene modification (unpublished) and may result from the effects that other gene disruptions have on both the expression of *ADH2* and/or its associated metabolic pathway. Since the *ADH2* gene in the TQ1 recombinant strain was only partially disrupted, the decrease in acetaldehyde concentration in its fermentation broth was only 15.23%. To our knowledge, no other publications exist that show a reduction when both *ADH2* allele copies of industrial brewing *S. cerevisae* yeast strain are otherwise disrupted. Moreover, TQ1 most likely had to adjust its gene regulation to survive since a number of manipulations were done to its genomic DNA. Such an adjustment may further explain the modest observed reduction in acetaldehyde concentration. Nevertheless, the TQ1 fermentation broth still had an improved flavor when compared with the products derived from its host strain T1 and parental industrial brewing yeast strain YSF5. In the evaluation of the sensorial characteristics of the refrigerated fermentation broths, all six experts from Tsingtao Brewery Co., Ltd considered the beer brewed from the TQ1 strain better tasting than those from T1 and YSF5. This improvement most likely resulted from the lower acetaldehyde, diacetyl, and pentanedione concentrations in the TQ1 broth. Although not as high as previous reports [30, 33], the reduction in acetaldehyde concentrations in the TQ1 and T1 fermentation broths still resulted in an enhanced beer freshness, as evidenced by TBA value and RSV (Table 4). Based on these two significant indexes reflecting beer staling and freshness, both the TQ1 and T1 strains generated beer with increased antioxidizability and flavor freshness period compared with beer brewed using the YSF5 strain.

In conclusion, the reduction of off-flavor compounds and residual maltotriose concentrations in the fermentation broth of TQ1 indicated that a recombinant strain with *SGA1*/*LSD1* coexpression and *ILV2*/*ADH2* deletion generates beer with more desirable characteristics compared with both the parental strain YSF5 and the host strain T1. Therefore, TQ1 appears more suitable for commercial use in the brewing industry based on its product's low caloric content and improved flavor. In addition, the construction of this recombinant strain using the yeast-derived TQ expression cassette and excluding non-heterogeneous DNA, bacterial plasmid sequences, and drug-resistant markers, ensures that it is safe for large-scale beer production.

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