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Secretion expression of SOD1 and its overlapping function with GSH in brewing yeast strain for better flavor and anti-aging ability

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Abstract Superoxide dismutase (SOD) is a significant antioxidant, but unlike glutathione (GSH), SOD cannot be secreted into beer by yeast cells during fermentation, this directly leads to the limited application of SOD in beer anti-aging. In this investigation, we constructed the SOD1 secretion cassette in which strong promoter *PGK1p* and the sequence of secreting signal factor from *Saccharomyces cerevisiae* were both harbored to the upstream of coding sequence of *SOD1* gene, as a result, the obtained strains carrying this cassette successfully realized the secretion of SOD1. In order to overcome the limitation of previous genetic modification on yeast strains, one new comprehensive strategy was adopted targeting the suitable homologous sites by gene deletion and *SOD1* + *GSH1* co-overexpression, and the new strain ST31 ($\Delta adh2::SOD1$ + $\Delta ilv2::GSH1$) was constructed. The results of the pilot-scale fermentation showed that the diacetyl content of ST31 was lower by 42 % than that of the host, and the acetaldehyde content decreased by 29 %, the GSH content in the fermenting liquor of ST31 increased by 29 % compared with the host. Both SOD activity test and the positive and negative staining assay after native PAGE indicated that the secreted active SOD in the fermenting liquor of ST31 was mainly a dimer with the size of 32,500 Da. The anti-aging indexes such as the thiobarbituric acid and

the resistance staling value further proved that the flavor stability of the beer brewed with strain ST31 was not only better than that of the original strain, but also better than that of the previous engineering strains. The multi-modification and comprehensive improvement of the beer yeast strain would greatly enhance beer quality than ever, and the self-cloning strain would be attractive to the public due to its bio-safety.

Keywords SOD1 · Secretion expression · GSH · Brewing yeast · Flavor · Anti-aging

Introduction

Flavor stability is an important index for alcoholic beverage such as wine and beer, and flavor components have a significant effect on their quality [33]. However, oxidation which can produce off-flavor components such as aldehyde often occurs during beer storage. As a product brewed with various ingredients, beer has different antioxidant components, for example: ferulic acid and polyphenol derived from malt and hops; SO_2 and glutathione (GSH), small molecules secreted by yeast, and so on. Based on yeast strain profile, several researches in relationship to beer flavor stability have been studied, and both SO_2 and GSH were reported to be important antioxidants responsible for the antioxidant activity of lager beer [41, 47]. SO_2 is produced by yeast during reductive sulfate assimilation [47]. Mutants which can produce higher levels of SO_2 have been obtained by over-expression of *SSU1* and/or *MET14* [5] or by disruption of *MET10* [14]. However, these yeasts were obtained from heterologous recombinant DNA such as antibiotics gene which cannot be applied commercially. In addition, although breeding

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of *Saccharomyces* strains which produce high levels of SO$_2$ is relative effective, it is accompanied by the undesirable H$_2$S produced as an intermediate in this pathway. Compared with SO$_2$, antioxidant GSH is more important to beer flavor. Since GSH also plays significant role in protein synthesis, DNA synthesis and amino acid transportation [6, 24], increase of GSH expression is helpful to improve the performance of both yeast cells and beer. GSH is synthesized by two sequential reactions in *Saccharomyces cerevisiae*, and *GSH1* gene encodes for γ-glutamylcysteine synthetase (EC 6.3.2.2) catalyzing the rate-limiting reaction during GSH synthesis [22]. Cloning of *GSH1* gene and its over-expression in *S. cerevisiae* have been reported by some researchers [8, 46], and the content of GSH in the beer fermented with these transformants increased, however, most of these transformants were hazardous for application in the beer industry because exogenous DNA such as bacteria gene has been carried into the host cells. In recent years, we have constructed several self-cloning strains with *GSH1* gene over-expression and some other genes' deletion [38–41] through self-cloning technique; as a result, the antioxidability of the beer brewed with these strains were improved. During the self-cloning genetic operation, all the knocked-in and knocked-out genes are from beer yeast strain, and the engineering strains harbored no heterologous DNA; therefore, the strains modified by self-cloning technique are considered to be secure and easily accepted by public [1]. In spite of the advantage of the self-cloning strains, in our previous studies, the increased content of GSH was not high enough, one reason might be that we only expressed one more copy of GSH in the transformants; hence, more investigations are necessary for improving yeast strains further. In order to increase the content of antioxidants, expression more copies of genes for GSH or other antioxidant such as SOD might be available.

Superoxide dismutase (SOD) is a kind of enzymatic antioxidant; it is significant just like nonenzymatic antioxidant glutathione GSH. SOD is regarded as a fundamental defense agent against reactive oxygen species [4, 16, 30, 31]. There are two forms of SOD identified in *S. cerevisiae*, one is copper-and-zinc-containing SOD (CuZnSOD or SOD1) and the other is manganese-containing SOD (MnSOD or SOD2) [23]. SOD1 locates mainly in the cytosol and is also found in the mitochondrial intermembrane space [32], accounting for 90–95 % of the total superoxide dismutase activity [42]. Both superoxide dismutase and glutathione are abundant and ubiquitous, and previous work also found that glutathione and SOD provided overlapping defensive function [11, 20]; however, SOD can hardly contribute antioxidability to beer because it localizes in yeast cells and cannot enter into beer spontaneously. We constructed the engineering yeast strain and made SOD

secrete into beer [21]; regretfully, the vitality of the strain was not stable, so a new strategy had to be considered.

In addition to the role of antioxidants, beer flavor stability also depends on some crucial flavor components such as diacetyl and acetaldehyde. Diacetyl is an intermediate compound during the biosynthesis of isoleucine–valine in yeast. Many methods have been adopted for decreasing the diacetyl content during the production of beer, including improvement of fermentation process and genetic modification of yeast strain. For example, expression of heterologous gene like α-acetolactate decarboxylase gene in yeast [2, 29, 44]; or over-expression or deletion of homologous genes just like *ILV2*, *ILV3* and *ILV5* could result in the decrease of diacetyl content in beer [19, 34, 35]. Acetaldehyde is an intermediate in ethanol biosynthetic pathway of yeast. As the acetaldehyde content in Chinese beer is usually higher (3–8 mg/L) than in overseas fine beer (<2 mg/L) [36], researchers have paid more attention on acetaldehyde metabolism of yeast in order to control the acetaldehyde content in beer. Several genes in *S. cerevisiae* are related to acetaldehyde synthesis, such as pyruvate decarboxylase gene, aldehyde dehydrogenase gene and alcohol dehydrogenase gene (*ADH*); of these, the *ADH*2 gene is considered as an important gene for regulating the acetaldehyde content in beer because alcohol dehydrogenase II (YADH-2) encoded by *ADH*2 catalyzes ethanol into acetaldehyde [25], which has less effect on the metabolic balance in yeast cells. Some genetic engineering yeast strains with low content of diacetyl or acetaldehyde have been constructed, but they are potentially dangerous for industrial application due to the existence of bacteria gene [13, 17, 36]; while other engineering strains can only be used in laboratory because of their auxotrophic or haploid characteristics ([28], *Saccharomyces* Genome Deletion Project web page). Several self-cloning strains with low content of diacetyl or acetaldehyde have ever been constructed in our laboratory, however, only one gene was deleted in the previous recombined strains [39–41], and the beer flavor was not improved comprehensively. Hence, further and extensive modification is necessary, for example, co-deletion of both *ILV2* and *ADH2* gene which are responsible for off-flavor components might be helpful to improve the strain greatly. In addition, *ILV6* deletion has been reported to reduce diacetyl in some lager beer yeast [7]; this extends the field for diacetyl regulation and may be suitable for other yeast strains' modification.

In order to make full use of the better effect of oxidation resistance offered by overlapping function of the antioxidants, new and comprehensive strategy was designed in this study. We adopted the strategy of multi-gene regulation in one industrial yeast strain, for example, SOD1 with secretion ability and GSH were both over-expressed, at the same time both *ILV2* and *ADH2* gene were deleted in the same yeast strain. In order to avoid the shortcoming

Table 1 Strains and plasmids used in this study

Strains or plasmids	Relevant genotype	Reference or source
Strains		
E.coli. DH5α	*sup*E44 Δ*lac*U169(φ80*lac*ZΔM15) *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1	Stratagene
QY31	Wild-type yeast strain	Tsingtao Beer Company (Qingdao, China)
T31-2	Recombined strain (Δ*ilv2: :GSH1*)	[40]
S31	Recombined strain (Δ*adh2: : SOD1*)	This work
ST31	Recombined strain (Δ*adh2::SOD1* + Δ*ilv2: :GSH1*)	This work
Plasmids		
pPIC9	Cloning vector, *amp*	Invitrogen
pMP1	Cloning vector, *amp*	[49]
pYCUP	Recombined plasmid, *URA3 amp*	[48]
pADH	Recombined plasmid, *URA3 amp*	[41]
pMPS1	Recombined plasmid, *amp*	This work
pAS1C	Recombined plasmid, *URA3 amp*	This work

Table 2 Oligonucleotide primers used in PCR amplification

Primers	Sequence 5′ → 3′
α-L	CCA<u>GAATTC</u>CCAAACGATGAGATTTCCTTC (EcoRI)
α-R	CGA<u>TCTAGA</u>TACGTAAGCTTCAGCCTC (XbaI)
SOD1-L	CAC<u>TCTAGA</u>ATGGTTCAAGCAGTCGCAG (XbaI)
SOD1-R	CTA<u>GAGCTC</u>GGACCCCTCAAGACCCCTC (SacI)
CUP1-L	CGCTATACGTGCATATGTTC
CUP1-R	ATCTGTTGTACTATCCGCTT
ADH2-L	GCTGTTATGTTCAAGGTC
ADH2-R	TTCAGAGGAGCAGGACAA

All the primers are designed according to the sequence of model strain *Saccharomyces cerevisiae*. The sequences of restriction enzyme's site (underlined) and the protective bases are added to the upstream of those primers which are used in plasmid construction

that emerged in our previous strains such as the unstable vitality, the suitability between modified genes was considered and a different locus for *SOD1* insertion was adopted in the new design. Consequently, the performance of the new constructed strain improved, and the flavor and anti-aging property of the beer brewed with the modified strain were enforced greatly; this kind of beer yeast strain was not reported by other researchers by now.

Materials and methods

Strains, plasmids and cultivation conditions

The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α was used as the host for plasmid construction. Industrial brewer's yeast strain QY31 was provided by Tsingtao Beer Company (Qingdao, China).

E. coli strain was grown at 37 °C in Luria–Bertani medium [26] supplemented with ampicillin (100 mg/L) when necessary. The yeast strain for transformation was grown at 28 °C in YEPD medium (1 % yeast extract, 2 % peptone and 2 % glucose). YEPD or 12°P wort was used as the medium for culture or fermentation of the yeast strains.

DNA manipulation, analysis and construction of plasmids

The primers used in this study are listed in Table 2. Genomic DNA of yeast strains was prepared as described by Burke et al. [3]. Plasmid DNA was prepared from *E. coli* as described by Sambrook and Russell [26].

Copper-and-zinc-containing superoxide dismutase gene, *SOD1*, was amplified from the genomic DNA of QY31 with primers SOD1-L/SOD1-R using PCR. Alpha factor leader sequence was amplified from cloning vector pPIC9 (Invitrogen) with primers α-L/α-R using PCR. PCR for amplification of these DNA fragments was performed in 50 µL volume with 25 µL 2× Pfu PCR MasterMix, 400 ng of template DNA and 0.4 µM primers. Cycle conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 55 °C for 1 min, 72 °C for 2 min and finally 72 °C for 15 min.

The purified PCR product α-factor and *SOD1* were digested, respectively, with restriction enzymes *Eco*RI-*Xba*I and *Xba*I-*Sac*I, and then cloned into EcoRI-SacI digested pMP1 [49] to construct plasmid pMPS1 (Fig. 1). The purified *CUP1* fragment which was digested from pYCUP [48] with KpnI-HindIII and fragment *PGK1p+α-factor+SOD1* obtained by digesting plasmid pMPS1 with SacI-KpnI were ligated into the SacI-HindIII sites of pADH [41] by T4 DNA ligase to construct plasmid pAS1C (Fig. 2). The constructed plasmids were analyzed using different restriction enzymes and sequence analysis.
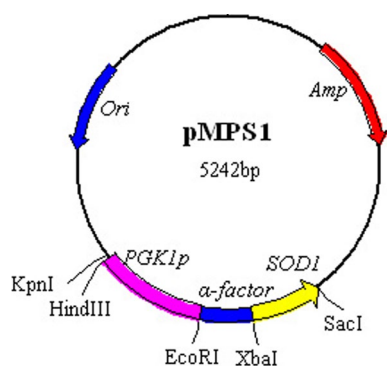
Fig. 1 Map of plasmid pMPS1. Main elements of pMPS1: *PGK1p* promoter, α-factor, *SOD1*
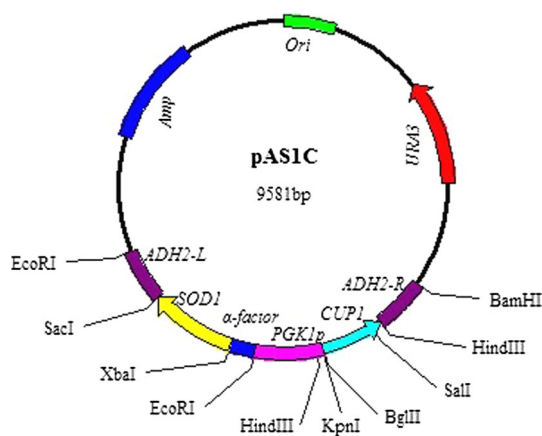

Fig. 2 Map of plasmid pAS1C. Main elements of pAS1C: part of *ADH2* sequence as the left and right arms for homologous recombination (*ADH2-L, ADH2-R*), *PGK1p* promoter + α-factor + *SOD1*, *CUP1* gene as marker for screening

DNA fragments for recombination was amplified from plasmid pAS1C with primers ADH2-L/ADH2-R using PCR, which was carried out in 100 μL volume with 50 μL 2× Long Taq PCR MasterMix, 800 ng of template DNA and 0.4 μM primers. PCR analysis of yeast recombinants was carried out in 10 μL volume with 5 μL 2× Long Taq PCR MasterMix, 160 ng of template DNA and 0.4 μM primers. Cycle conditions were 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 55 °C for 1 min, 72 °C for 4 min and finally 72 °C for 15 min.

The sequence of the constructed plasmids and PCR fragments of recombinants genomic DNA were analyzed using automated DNA sequencer.

Transformation and screening of yeast strains

The DNA fragments prepared by PCR for yeast transformation were purified according to the DNA Gel Extraction Kit, and used for yeast recombination using the lithium acetate method as described [15, 27]. The recombined strains were selected on YEPD plate with copper sulfate (CuSO$_4$).

Analysis of enzyme activity and genetic stability

The recombined strains and their host were cultivated in 50 mL YEPD at 28 °C for 2 days, and the yeast cells and fermenting liquor were then used for preliminary assay including enzyme activity of alcohol dehydrogenase II (YADH-2) and superoxide dismutase (SOD). SOD activity was assayed as described by Xie et al. [43]; YADH-2 activity was measured as described by Wang et al. [41].

Yeast recombinants for stability analysis were successively transferred into YEPD medium for 50 generations, and the 50th generation strains were used for copper-resistance stability by plate streaking and also for identification of genome DNA by PCR as described by Wang et al. [41]. YADH-2 and SOD enzyme activity of the 50th generation strains were also detected.

Fermentation and analysis of metabolites

The host and recombinants were first grown in 5 mL of 12° P wort at 25 °C for 30 h, then they were, respectively, inoculated into 60 mL of wort (12°P) at a ratio of 1:50. After cultivation at 25 °C for 60 h, these strains were inoculated into 480 mL of 12° P wort in 1-L triangle bottles and fermented at 10 °C for 16 days. Samples were assayed every day.

The initial activation and propagation for the pilot-scale brewing was performed similarly to the above and the conical flasks with fermentation bungs was firstly used for fermentation at 10 °C for 10 days. During the fermentation, the flasks were weighed every day, and the difference of the flasks in weight on two adjacent days during fermentation was the CO$_2$ reduction. The yeast pellets centrifuged from the conical flasks after fermentation were inoculated into a 6-L European Brewery Convention (EBC) tube with 5 L 12° P wort and fermented at 10 °C for 12 days.

SOD activity in the fermented liquor was detected as described by Xie et al. [43] and was also assayed using positive and negative staining methods after polyacrylamide gel electrophoresis (PAGE) [45]. GSH content was measured as described by Wang et al. [41].

Attenuation degree and real extract concentration in the filtrate of the fermenting liquor were measured using Beer Analyzer. Acetaldehyde and diacetyl content were measured using gas chromatography. GSH content was determined using 5, 5′-dithiobis (2-nitrobenzoic acid) (DTNB) method [8]. The thiobarbituric acid (TBA) value was measured as described by Grigsby and Palamand [12]. The flavor freshness period was assessed using the resistance

staling value (RSV): RSV = $1/4$ $(12/\Delta TBA_{12} + 24/\Delta TBA_{24} + 36/\Delta TBA_{36} + 48/\Delta TBA_{48})$. ΔTBA_{12-48} is the difference in TBA between the control that is kept at 0 °C and the samples that were kept at 60 °C for 12, 24, 36 and 48 h, respectively, before TBA measurement.

Results

Construction of plasmids, selection of transformants and enzyme activity assay

The plasmids pMPS1 and pAS1C (Figs. 1, 2) were constructed as described in the above methods. After PCR amplification when using pAS1C as template and ADH2-L/ADH2-R as primers, we got the knock-in/knock-out cassette ASC, a 4426-bp of DNA fragment with *ADH2-R + CUP1 + PGK1p + α-factor + SOD1 + ADH2-L* (Fig. 3). This cassette was then used for recombination in two host strains, respectively. When industrial brewing yeast QY31 was used as the host, the recombinant was selected on the YEPD plate with 7.5 mM $CuSO_4$ and self-cloning strain S31 was obtained. When our previous recombinant strain T31-2 [40] was used as the host, we got self-cloning strain ST31 on the YEPD plate with 9.5 mM

Fig. 3 DNA cassettes in self-cloning strains. Strain S31: *Δadh2::SOD1+CUP1*. Strain T31-2: *Δilv2::GSH1+CUP1*. Strain ST31: *Δadh2::SOD1+CUP1*, *Δilv2::GSH1*

$CuSO_4$, in which *ILV2* was interrupted by *GSH1* integration and *ADH2* was interrupted by *SOD1* integration (Fig. 3). Superoxide dismutase (SOD) activity was tested out in fermenting liquor of strains S31 and ST31, while alcohol dehydrogenase II (YADH-2) activity in S31 and ST31 cells decreased (data not shown). These results indicated that the recombination might take place at the correct locus.

PCR verification, sequence analysis and genetic stability

The different primers' combination for different DNA fragments with various sizes was used in PCR amplification to verify gene deletion and insertion in the transformants. When using SOD1-L/ADH2-L, α-R/CUP1-R and CUP1-L/ADH2-R as primer pairs to verify fragment ASC in the genome DNA of recombined strain S31 and ST31, there were different PCR products which were 1,155, 2,708 and 1,609-bp in size, respectively. The above results were in accordance with theoretical values, and indicated that *ADH2* gene might be disrupted by *PGK1p + α-factor + SOD1 + CUP1* integration. By contrast, there were no PCR products for these three primer pairs using the host genome as template. When primer pair ADH2-L/ADH2-R and DNA template of recombined strain S31 and ST31 genome were used for PCR assay, the size of PCR products was 4,426 bp, which was also in accordance with theoretical value as the above, while the PCR products for the host with primers ADH2-L/ADH2-R was 2,501 bp DNA in length (Fig. 4).

The PCR products using ADH2-L/ADH2-R as primer pair and transformants genome as template were also tested by sequence analysis, the results displayed identity to reported sequences as theoretical anticipation, and further proved that *ADH2* gene was successfully knocked out by *PGK1p + α-factor + SOD1 + CUP1* knock-in in strain S31 and ST31.

Both S31 and ST31 strains were analyzed for their stability. Copper-resistance stability was detected by observing the growth of randomly selected 50 single-grown
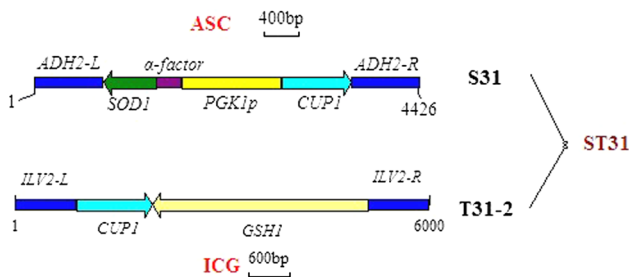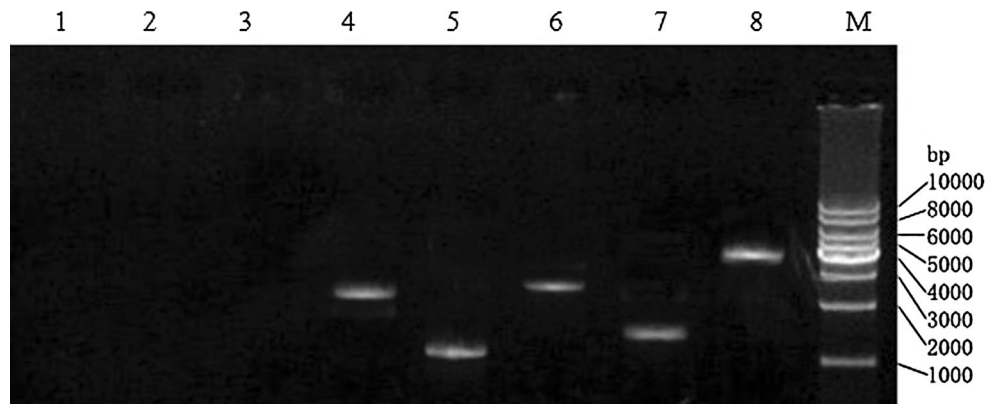

Fig. 4 PCR analysis of transformant and host genome. Genome DNA templates: *1–4* host QY31; *5–8* ST31. Primers: *1, 5* SOD1-L/ADH2-L; *2, 6* α-R/CUP1-R; *3, 7* CUP1-L/ADH2-R; *4, 8* ADH2-L/ADH2-R. *M* marker. The results of strain S31 is the same as that of ST31 (not shown)
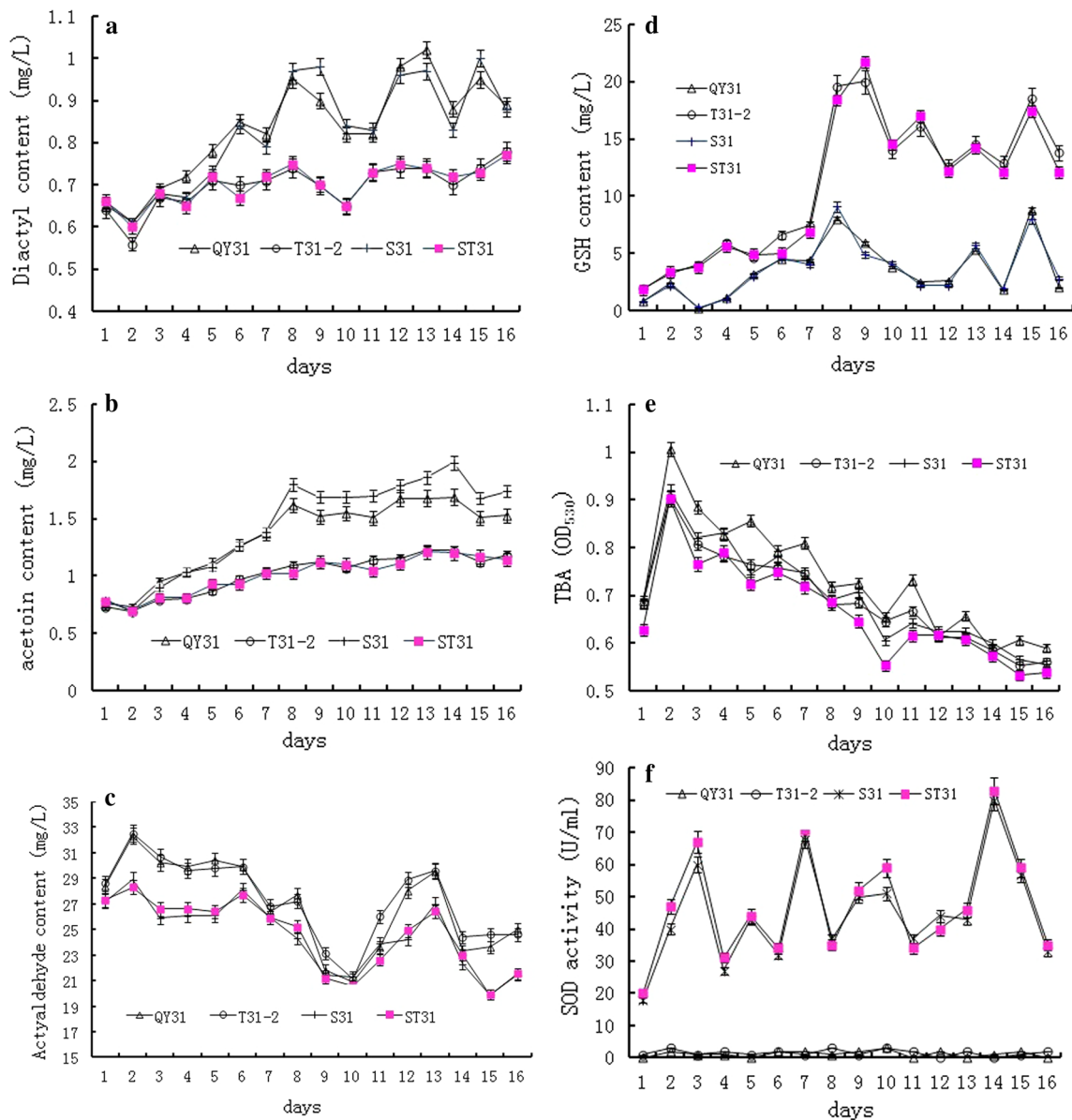
Fig. 5 Parameters' comparison during triangle bottle fermentation of transformants and the host. **a–f** are, respectively, the results of the content of diacetyl, acetoin, acetaldehyde, GSH, the thiobarbituric acid (TBA) and SOD activity of transformants and the host

colonies of the 50th generation recombinants on YEPD with 7.5 and 9.5 mM CuSO4, respectively, and the results of increased resistance suggested that the over-expression of the inserted *CUP1* gene was genetically stable. The results of PCR verification for genome DNA of the 50th generation of the recombinants S31 and ST31 were consistent with that of the first generation, which further indicated the genetic stability of *ADH2* deletion and integration of *SOD1* and *CUP1* in these recombined strains. The results of enzyme activity of SOD and YADH-2 of the 50th generation recombinants were consistent with that of the first generation, this also illustrated that these constructed strains were genetically stable.

Fermentation test in triangle bottles

The different comparisons on physical–chemical indexes among self-cloning strains S31 and ST31 constructed in this study, previously constructed T31-2 [40] and the original host QY31 were carried out during 16 days preliminary fermentation in triangle bottles. The results indicated that S31 gave better indexes including acetaldehyde content and SOD activity as anticipated (Fig. 5c, f), and its antioxidant index such as the thiobarbituric acid (TBA) was also better than that of the host (Fig. 5e), but the off-flavor component acetoin from S31 was higher than that of the host (Fig. 5b). T31-2 presented advantage in diacetyl, acetoin and GSH

Table 3 Performance parameters in pilot-scale brewing

Parameters	In conical flasks		In EBC tube	
	QY31	ST31	QY31	ST31
Real attenuation (%)	66.95 ± 0.05	67.23 ± 0.04	67.05 ± 0.04	67.23 ± 0.05
Real extract (%)	4.44 ± 0.02	4.39 ± 0.02	4.26 ± 0.03	4.39 ± 0.03
Diacetyl (μg/L)	222 ± 5	122 ± 4	191 ± 3	111 ± 3
Acetaldehyde (mg/L)	10.75 ± 0.5	7.53 ± 0.4	9.95 ± 0.5	7.02 ± 0.3
GSH (mg/L)	7.41 ± 0.8	9.55 ± 0.6	7.55 ± 0.5	9.72 ± 0.8
SOD (U/ml)	–	66 ± 3	–	60 ± 4
TBA (OD$_{530}$)	–	–	0.63 ± 0.04	0.51 ± 0.03
RSV	–	–	415 ± 6	522 ± 5

TBA the thiobarbituric acid, *RSV* the resistance staling

(Fig. 5a, b, d), and its TBA value was a little better than the host (Fig. 5e); however, its acetaldehyde content was still in a higher level (Fig. 5c). Among these transformants, ST31 was not only superior to the original host, but also better than the other two engineering strains both in decreasing the concentration of diacetyl, acetoin and acetaldehyde and in increasing the anti-aging capability which were reflected by GSH content, SOD activity and TBA value (Fig. 5a–f). Besides the contribution of lower content of diacetyl, acetoin and acetaldehyde, the increased content of antioxidants GSH and SOD1 might be important factors offering the change of TBA value for ST31.

Compared with the wild-type host strain QY31 and the engineering strain S31 and T31-2 with only one gene deletion and one gene over-expression, the multi genes modified strain ST31 presented advantageous performance comprehensively during the fermentation in triangle bottles; hence, ST31 was chosen for further fermentation test.

Pilot-scale brewing

The fermentation ability of the newly constructed strain ST31 was not affected by the genetic modification according to the results as follow. Firstly, CO_2 reduction of recombinant ST31 during conical flask fermentation for the pilot-scale brewing was analogous to that of the host; secondly, the assay of attenuation further confirmed that ST31 presented a similar degree of fermentation compared with the host (Table 3). Both of the above results indicated that integration of *SOD1* and *GSH1* into the internal of *ADH2* and *ILV2* in our yeast strains did not change their fermentation performance.

After conical flask fermentation, the diacetyl content in the fermenting liquor of ST31 decreased by 45 % compared with that of the host. The acetaldehyde content of ST31 was 30 % lower than that of the host. In comparison with the host, the GSH content in the fermenting liquor of ST31 increased by 29 %, and the SOD activity also presented in the fermenting liquor of ST31 (Table 3).
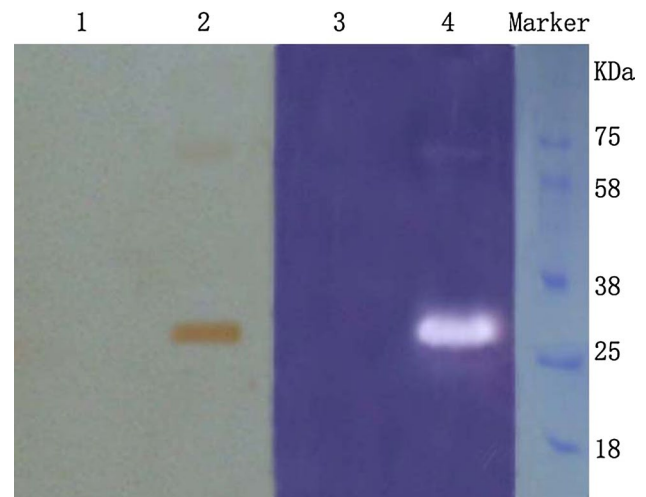

Fig. 6 SOD1 activity of ST31 assayed by positive and negative staining methods. Positive staining: *1, 2*; negative staining: *3, 4*. Strains: *1, 3* host; *2, 4* ST31

After EBC tube fermentation, real attenuation and real extract of the recombinants were similar to that of the host, the diacetyl content of ST31 decreased by 42 %, and the acetaldehyde content was lower by 29 % compared with that of the host (Table 3). The difference of GSH content in fermenting liquor from the EBC tube between the host and ST31 is similar to that of previous conical flasks process, and so did SOD activity.

SOD activity in the fermented liquor of ST31 was also tested by positive and negative staining methods. Both staining results after native PAGE indicated that there was active secreted SOD in the fermented liquor of ST31, which was different from that of the host. The secreted SOD was mainly dimer with about 32,500 Da of molecular weight, and there was also a small quantity of tetramer (Fig. 6).

In terms of the results shown in Table 3, the decreased TBA value of the ST31 reflected that the antioxidant capacity of fermenting liquor from ST31 was increased. The increased RSV value of ST31 (Table 3) provided further

evidence that the flavor freshness performance of ST31 was better than that of the host.

Discussion

Beer that is particularly high in antioxidants may have more stable sensory characteristics, such as better flavor and aroma, which should result in higher quality and longer shelf life. There are several different compounds in beer contributing to anti-aging effect. Besides that some components from malt and hops may partly play the role in promoting the antioxidant activity in beer, GSH from yeast also can offer antioxidant capacity for beer.

GSH is not only a significant antioxidant against the toxic effects of O_2 and other oxidative compounds, it also plays an important role in DNA synthesis, protein synthesis and post-translational modification [10, 24]. As a smaller molecular, GSH in yeast cells can release into the fermenting liquor automatically. Both the multi-functions of GSH and its easy accessibility to the beer make it more important in improving beer anti-aging ability; this has been proved by our previously work. However, this improvement is relatively limited. One reason might be that the added copy of *GSH1* gene was less; another reason might be that much of the over-expressed GSH might stay in the yeast cells, and only a small quantity of GSH is secreted into the fermenting liquor, which were not effective enough for better anti-aging ability. Hence, further extensive strategies or other antioxidant modifications are necessary.

Similar to GSH, SOD is not only a fundamental antioxidant, it is also an important element of a protein network that ensures proper functions of mitochondria [18]; but unlike GSH, SOD cannot secrete from yeast cells into the beer freely, therefore secretion strategy should be considered if we want to increase the antioxidability of beer by over-expression SOD.

In order to make SOD play its antioxidant role in beer, we constructed the engineering yeast strain which realized SOD secretion into beer by interrupting *ILV2* at the same time. Unfortunately, the recombined strain only gave better anti-aging ability during the earlier experimental period, and we found that the strain had no vitality stability later. The reason for this phenomenon was still unclear; one surmise may be the unsuitability between *SOD1* and the interrupted *ILV2*. Considering the complexity of interaction of different genes or DNA fragment, new comprehensive strategy was adopted in this investigation. In order to avoid the negative role of *SOD1* expression with *ILV2* deletion on yeast cells growth, we inserted *SOD1* to the locus of internal of *ADH2* gene in this study. Since Swiegers et al. [33] has reported that when the strong promoter such as promoter of 3-phosphoglycerate kinase of *S. cerevisiae*

(*PGK1p*) was used to construct engineering wine yeast strain, wine flavor could be improved, we also adopted *PGK1p* to enhance SOD1 expression in this study. In the meantime, we inserted signal DNA element of *S. cerevisiae* to the upstream of coding sequence *SOD1* gene for SOD1 secretion. The new strategy was successful, and we surmised that the locus of *ADH2* gene is suitable for *SOD1* insertion.

Besides a little of improvement on anti-aging ability in our previous engineering strains, another shortcoming is that the main flavor indexes such as diacetyl and acetaldehyde were not improved at the same strain. The strategy of double genes' deletion and over-expression should probably make the flavor and the antioxidability of the beer better because the content of crucial off-flavor components would decrease and the content of two kinds of antioxidants increased. In addition, the overlapping function of SOD1 and GSH would offer the beer more antioxidant ability. As we anticipated, the constructed strains with both *ADH2* and *ILV2* disruption and over-expression of *GSH1* and *SOD1* had good performance, which is reflected in Fig. 5a–f and Table 3. No matter whether the fermentation is in triangle bottles or in pilot scale, SOD1+GSH co-overexpression resulted in the better effect of oxidation resistance than respective function of SOD1 and GSH.

In summary, the performance of the beer yeast strain was greatly improved by over-expression of SOD1 + GSH and by decrease of off-flavor components such as diacetyl and acetaldehyde simultaneously. The comprehensive modification in strain ST31(Δ*adh2::SOD1* + Δ*ilv2::GSH1*) not only provided a better flavor for the beer, but also made beer flavor more stable due to the co-effect of SOD1 and GSH. All the improved performance will be helpful to enhance beer quality greatly and be attractive to consumer, which will probably bring more profits for the beer industry. On the other hand, no heterologous DNA was brought into the brewing yeast strain during all the genetic modification; hence, the bio-safety of the engineering strain is fine.

Both flavor and antioxidant capacity are important to alcoholic beverage, and the development of biotechnology and exploitation of yeast metabolic mechanism have extended regulation of industrial strains in many ways. Besides the modification of *ILV2, ADH2, GSH1* and *SOD1* manipulated in our study, some other genes and global factors affecting beer or wine yeast strain have been reported such as *ILV6, SOD2, HSP12*, etc. [7, 9]; inhibitor of alcohol dehydrogenases (ADH) such as 4-methylpyrazole was also reported to be used in beer flavor regulation [37]. All these investigations will be very helpful to more full modification of yeast strain in later research; as a result, a diversity of beer or wine with better quality will be produced in the future.

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