

Construction of recombinant industrial brewer's yeast with lower diacetyl production and proteinase A activity

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Abstract The characteristic buttery taste of diacetyl has long been a major problem in the brewing industry, and the foam stability of unpasteurized beer is often influenced by proteinase A (PrA), which is encoded by *PEP4* and released from yeast cells into beer during brewing. A recombinant industrial brewer's yeast strain that reduces the diacetyl content of beer and improves foam stability was constructed. We constructed a *PGK1p-ILV5-PGK1t* expression cassette, which was introduced into one of the *PEP4* alleles via PCR-mediated homologous recombination. Then, the second *PEP4* allele was disrupted using the *Cre-loxP* recombination system, and the recombinant strain was designated as S-CSIK12. The results show that the diacetyl production of S-CSIK12 is always lower than that of the host strain at all stages of beer fermentation. In addition, brewing with S-CSIK12 reduced the PrA activity of the final beer by 44 % compared with that using the wild-type strain. The head retention of the beer brewed with S-CSIK12 (260 ± 2 s) was better than that of the host strain S-6 (212 ± 3 s). Considering that more PrA is released from yeast cells during the final stage of main fermentation and that the timing of yeast cropping is determined by diacetyl reduction, brewing with strains that have low diacetyl production also reduced the PrA activity of the beer and improved its head retention. The present study provides reference for the brewing industry as well as research on the diacetyl reduction and foam stability of beer.

Keywords Proteinase A (PrA) · Diacetyl · Head retention · Industrial brewer's yeast

Abbreviations

PrA	Proteinase A
<i>PEP4</i>	A kind of gene that encodes proteinase A
<i>ILV5</i>	A kind of gene that encodes reductoisomerase
RI	Acetohydroxyacid reductoisomerase
ILV pathway	Isoleucine–valine pathway
ALDC	α -Acetolactate decarboxylase
<i>ILV2</i>	A kind of gene that encodes acetolactate synthase I/III large subunit
<i>ILV3</i>	A kind of gene that encodes dihydroxyacid dehydratase
<i>ILV6</i>	A kind of gene that encodes acetolactate synthase I/III small subunit
LTP1	Lipid transfer protein 1
<i>GPD1</i>	A kind of gene that encodes glycerol-3-phosphate dehydrogenase
ROX	Reference dye for real-time PCR
HK	Hexokinase
Pfk	Phosphofructokinase
PYKi	Pyruvate kinase
S-6	Wild-type industrial brewer's yeast
S-CSK10	Two <i>PEP4</i> allele disruption strain
S-CSIK12	Two <i>PEP4</i> allele disruption and <i>ILV5</i> overexpression strain

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Introduction

Diacetyl (2,3-butanedione) has an unpleasant butter-like flavor, and its content is crucial to beer maturation. This compound is particularly undesirable in lagers, and the

threshold for diacetyl is 0.15 ppm or even lower [1, 2]. Diacetyl is a vicinal diketone formed by the non-enzymatic oxidative decarboxylation of α -acetolactate that diffuses from the isoleucine–valine (ILV) biosynthetic pathway in yeast during the main fermentation [3]. Diacetyl is reabsorbed into yeast cells and reduced to acceptable levels during maturation. Diacetyl removal is the major reason for the 1–3-week maturation period of beer. Therefore, preventing diacetyl formation would help shorten the maturation process. Numerous strategies have been proposed to eliminate this costly process, including improvement of the fermentation craft, genetic approaches to decrease the diacetyl content in yeast during beer production [4], and the elimination of the diacetyl production from its precursor α -acetolactate. Two approaches have been applied. First, α -acetolactate decarboxylase (ALDC) can be added to green beer, which will convert α -acetolactate into acetoin. Second, the genes that encode ALDC from different bacterial species can be expressed in brewer's yeast using genetic methods. The genetically modified brewer's yeast would then decrease the diacetyl content during beer production [5–8].

Diacetyl is a side product of the ILV pathway. Blocking the formation of α -acetolactate and increasing the flux toward valine formation would reduce the diacetyl concentration in green beer [9]. Several genes are closely related to diacetyl content in the ILV biosynthetic pathway. Previous studies on brewers' yeast strains with *ILV2* and *ILV6* gene deletions, as well as overexpression of the *ILV5* gene or both of *ILV3* and *ILV5* result in decreased diacetyl concentrations (40–70 %) during beer production [9–11].

Foam stability is a critical characteristic of beer quality, and a solid head of foam is essential to the presentation of a well-crafted beer [12, 13]. During beer production, raw materials and the brewing process play major roles in the development and maintenance of foam. The most important compounds involved in beer foam formation are proteins and polypeptides. In terms of beer foam stability, several beer proteins have been identified as foam positive, such as protein Z, lipid transfer protein 1 (LTP1), and various hordein-derived polypeptides [14–16]. Researchers have also shown that beers with different heads of foam influence the taste in sensory evaluation tests [17].

Yeast proteinase A (PrA, EC3.4.23.25) is detrimental to beer foam, especially in unpasteurized beer [18–21]. PrA is a vacuolar aspartic proteinase encoded by the *PEP4* gene [22, 23] that can be excreted into beer under stress conditions or released through yeast autolysis or mechanical damage [19, 24, 25]. During beer production, yeast cells release PrA that actively degrades LTP1, which decreases beer head retention [26]. Previous studies have shown that deleting the *PEP4* gene in industrial brewer's yeast helps maintain beer foam stability [20, 21, 26, 27].

ILV5 gene overexpression decreases diacetyl production by 50–60 % compared with the control strain and significantly shortens the maturation period of beer production [28, 29]. In addition, PrA is primarily released from yeast cells during the final stage of fermentation and maturation because of nutrition-deficient conditions, especially under nitrogen starvation; pressure and temperature are also thought to affect yeast cell vitality and subsequent PrA excretion [30]. Yeast strains should be removed once the diacetyl concentration in the worts decreases below the taste threshold during beer fermentation. Otherwise, the low-vitality yeast and enzymes released will adversely affect beer flavor and foam stability. Hence, shortening the maturation time would decrease the PrA activity of beer. In the current study, we constructed a recombinant industrial brewer's yeast strain with partial *PEP4* allelic genes replaced with a constructed *ILV5* expression cassette to develop an improved strain with lower diacetyl production and PrA activity. The diacetyl content, PrA activity, and fermentation performance of the recombinant industrial brewer's yeast strains were examined.

Materials and methods

Strains, vectors, and cultivation conditions

The genetic properties of all strains and plasmids used in the present study are summarized in Table 1. The industrial brewer's yeast S-6 was obtained from the Yeast Collection Center of the Tianjin Key Laboratory of Industrial Microbiology, Tianjin University of Science and Technology, China.

Escherichia coli DH5 α strain was grown at 37 °C in Luria–Bertani broth (1 % NaCl, 1 % tryptone, and 0.5 % yeast extract) supplemented with ampicillin (100 mg l⁻¹). The yeast strain was grown at 28 °C in YEPD medium (1 % yeast extract, 2 % peptone, and 2 % glucose). To select the Geneticin (G418)-resistant yeast strains after transformation, the YEPD plate was supplemented with 800 mg l⁻¹ G418. To select Zeocin-resistant yeast strains, 500 mg l⁻¹ Zeocin (Promega, Madison, United States) was added to the YEPD plates for yeast culture. Then, the YEPG medium (1 % yeast extract, 2 % peptone, and 2 % galactose) was used for *Cre* expression in the yeast transformants.

Plasmid construction

Plasmid DNA was prepared from *E. coli* DH5 α as described by Sambrook and Russell [31]. Genomic yeast DNA was prepared from industrial brewer's yeast S-6 as

Table 1 Strains and plasmids used in the current study

Strains or plasmids	Relevant characteristic	Reference or source
Strains		
<i>E. coli</i> . DH5 α	supE44 Δ lacU169(ϕ 80lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA	Stratagene
S-6	Wild-type industrial brewer's yeast	Hao et al. [45]
S-K10	<i>PEP4</i> (n-1)/ <i>pep4</i> (73, 750):: <i>loxP</i> - <i>KanMX</i> - <i>loxP</i>	This work
S-CK10	<i>PEP4</i> (n-1)/ <i>pep4</i> (73, 750):: <i>loxP</i>	This work
S-SK10	<i>PEP4</i> (n-2)/ <i>pep4</i> (73, 750):: <i>loxP</i> / <i>pep4</i> (369, 412):: <i>loxP</i> - <i>KanMX</i> - <i>loxP</i>	This work
S-CSK10	<i>PEP4</i> (n-2)/ <i>pep4</i> (73, 750):: <i>loxP</i> / <i>pep4</i> (369, 412):: <i>loxP</i>	This work
S-IK12	<i>PEP4</i> (n-1)/ <i>pep4</i> (73, 750):: <i>PGK1p</i> - <i>ILV5</i> - <i>PGK1t</i> - <i>loxP</i> - <i>KanMX</i> - <i>loxP</i>	This work
S-CIK12	<i>PEP4</i> (n-1)/ <i>pep4</i> (73, 750):: <i>PGK1p</i> - <i>ILV5</i> - <i>PGK1t</i> - <i>loxP</i>	This work
S-SIK12	<i>PEP4</i> (n-2)/ <i>pep4</i> (73, 750):: <i>PGK1p</i> - <i>ILV5</i> - <i>PGK1t</i> - <i>loxP</i> / <i>pep4</i> (369, 412):: <i>loxP</i> - <i>KanMX</i> - <i>loxP</i>	This work
S-CSIK12	<i>PEP4</i> (n-2)/ <i>pep4</i> (73, 750):: <i>PGK1p</i> - <i>ILV5</i> - <i>PGK1t</i> - <i>loxP</i> / <i>pep4</i> (369, 412):: <i>loxP</i>	This work
Plasmids		
pPGK1	Ap ^r , containing the <i>PGK1_p</i> - <i>PGK1_t</i> expression cassette	Lilly et al. [32]
pUC19	Ap ^r , cloning vector	Invitrogen
pUG6	<i>E. coli</i> / <i>S. cerevisiae</i> shuttle vector, containing <i>Amp</i> ⁺ , <i>loxP</i> - <i>kanMX</i> - <i>loxP</i> disruption cassette	Gueldener et al. [33]
pSH-Zeocin	Zeo ^r , Cre expression vector	This work
pUC-KAR	Ap ^r , Kan ^r containing <i>PEP4</i> A- <i>loxP</i> - <i>KanMX</i> - <i>loxP</i> - <i>PEP4</i> R	This work
pUC-SKAR	Ap ^r , Kan ^r containing <i>PEP4</i> SA- <i>loxP</i> - <i>KanMX</i> - <i>loxP</i> - <i>PEP4</i> R	This work
pUC-IKAR	Ap ^r , Kan ^r containing <i>PEP4</i> A- <i>PGK1p</i> - <i>ILV5</i> - <i>PGK1t</i> - <i>loxP</i> - <i>KanMX</i> - <i>loxP</i> - <i>PEP4</i> R	This work

pep4 (73, 750) indicated the target site of the first *PEP4* allele disruption; *pep4* (369, 412) indicated the target site of the second *PEP4* allele disruption

described by Sambrook and Russell [31]. The primers used in this study are listed in Table 2.

An upstream homologous fragment of the *PEP4* gene was amplified by PCR using S-6 genomic DNA as templates with the A-UP and A-DOWN primers. A downstream homologous fragment was similarly amplified using the PR-U and PR-D primers. Then, the PCR products were digested with the appropriate endonucleases and cloned into the pUC19 cloning vector (Invitrogen, China) at *Hind* III and *Kpn* I sites to construct plasmid pUCAR. An *Xho*I fragment of the *ILV5* gene was amplified from S-6 genomic DNA by PCR using the primers ILV-U and ILV-D to construct the recombinant plasmid. The *ILV5* fragment was cloned into vector pPGK1 [32] after both were digested with *Xho*I, resulting in pPILV. The fragment containing the *PGK1* promoter, the *ILV5* gene, and the *PGK1* terminator was amplified from pPILV using the primers PGK1p and PGK1t. The PCR product was digested with *Pst*I and inserted into pUCAR to construct pIAR, which contains the *ILV5* expression cassette. Finally, the *KanMX* cassette amplified by PCR using pUG6 [33] as the template with the primers K-U and K-D was cloned into pUCAR and pPILV after digestion with the appropriate endonucleases to produce the final plasmids, which were designated as pKAR and pIKAR (Table 1). Based on the aforementioned strategy, the pKSAR plasmid (Table 1) was constructed by inserting the upstream homologous fragment *PEP4* SA, the

KanMX cassette, and the downstream homologous fragment *PEP4* SR into the pUC19 cloning vector. The homologous fragments of this plasmid were amplified using the designed retractive primers, as previously described [34].

Disruption and transformation strategy

Industrial brewer's yeast strains are diploid or polyploid. Thus, a retractive primer disruption strategy was used to repeat the deletion of the *PEP4* gene efficiently as previously described [34]. The *PEP4* A-*loxP*-*KanMX*-*loxP*-*PEP4* R and *PEP4* A-*PGK1p*-*ILV5*-*PGK1t*-*loxP*-*KanMX*-*loxP*-*PEP4* R disruption cassettes were amplified from the plasmids pKAR and pIKAR, respectively. The disruption strategy is shown in Figs. 1a, b. A *Cre*-*loxP* recombination system with gene disruption strategy was used to rescue the *KanMX* cassette. Then, *PEP4* SA-*loxP*-*KanMX*-*loxP*-*PEP4* SR disruption cassette was used to disrupt the second copy of the *PEP4* gene. The retractive primer disruption strategy is shown in Fig. 1c.

Brewer's yeast transformation was performed using the lithium acetate/PEG method [35]. Transformants were screened on YEPD plate containing 800 mg l⁻¹ G418. PCR was applied to verify the recombinant strains with accurate site integration. Several different pairs of primers were designed, and the primer binding regions are shown in

Table 2 Primers used in the present study

Primer	Sequence (5' → 3')	Restriction site
For plasmid construction		
A-UP	CCCAAGCTTCGCTGCTATTTATTCATTCCACC	<i>HindIII</i>
A-DOWN	CCCAAGCTTGACTTTTGCAGCAACTTGGT	<i>HindIII</i>
PR-U	GGGGTACCGACGAGTCTAAGTTCAAGGGCGATAT	<i>KpnI</i>
PR-D	GGGGTACCATTTACGCAAGAAGGCATCACC	<i>KpnI</i>
ILV-U	CTGCTCGAGATGTTGAGAACTCAAGCCG	<i>XhoI</i>
ILV-D	CCGCTCGAGTTATTGGTTTTCTGGTCTC	<i>XhoI</i>
PGK1p	AACTGCAGTCTAACTGATCTATCCAAAAC	<i>PstI</i>
PGK1t	AACTGCAGTAACGAACGCAGAATTTTC	<i>PstI</i>
K-U	CGGGATCCAGCTGAAGCTTCGTACGC	<i>BamHI</i>
K-D	CGGGATCCGCATAGGCCACTAGTGGATCTG	<i>BamHI</i>
SA-UP	CCCAAGCTTACACGAGTTGTCCGATGAGATG	<i>HindIII</i>
SA-DOWN	AACTGCAGCCACATTCGTTACTTGGAAC	<i>PstI</i>
SPR-U	GGGGTACCCATCAAGCTACAAAGCTAATGG	<i>KpnI</i>
SPR-D	CGGAATTCCTCACCGCCATTTTCAGTATC	<i>EcoRI</i>
For PCR verification		
YZ-SUP	GGCTGTAACCCGTCTTATGCCTTCC	–
YZ-SDOWN	CTAAACCCTTTCCCGCCATTC	–
NYZ-AUP	CTGTCAAGGAGGGTATTCTGG	–
NYZ-ADOWN	GCTGATACGGATCTTAGTGGG	–
YZ-2A	ACAACCTATTAATTTCCCCTCG	–
YZ-AUP	TTTTGATGACGAGCGTAATGGC	–
YZ-ADOWN	GGTGAAGGAAGGGATCGGATTT	–
For real-time PCR		
ILV5-F	TTTGTACTTCTCCCACG	–
ILV5-R	GAGTTAATACCACGACC	–
G1-F	TGCCCCGTATCTGTAGC	–
G1-R	AGCACCAACTTCAAAACCC	–

Fig. 1. The genetic stability of the yeast strain was determined using the method of Cha et al. [36].

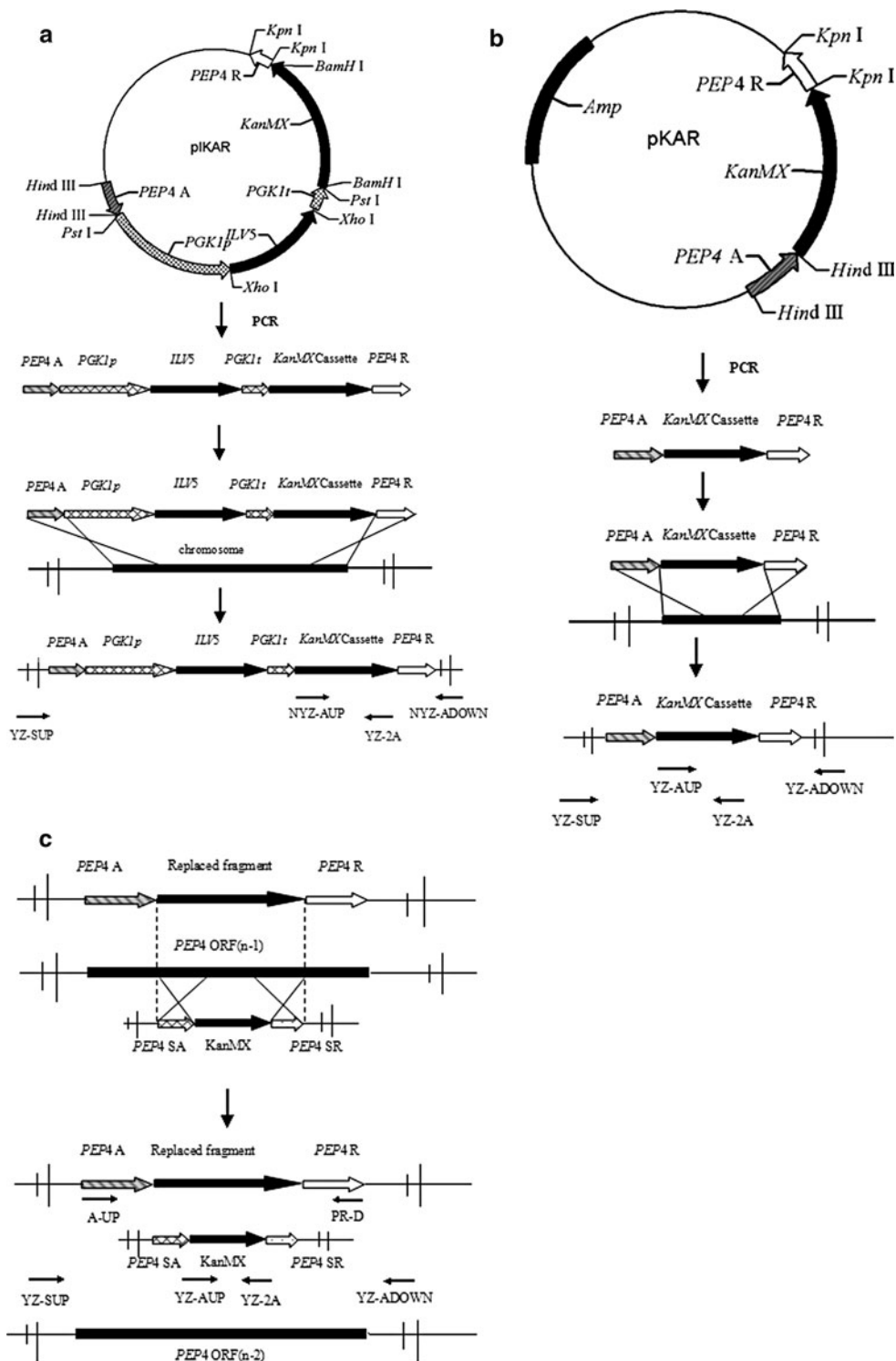
Construction of recombinant industrial brewer's yeast

The DNA fragment of *PEP4 A-PGK1p-ILV5-PGK1t-loxP-KanMX-loxP-PEP4 R* was amplified and transformed into the industrial brewer's yeast S-6. The fragment was integrated into the chromosome at the *PEP4* locus of S-6 by homologous recombination to construct single *PEP4* allele disruption and *ILV5* overexpression strains (Fig. 1a). The transformants were screened on G418 selective plates after transformation and verified by PCR using primer pairs YZ-SUP/YZ-SDOWN and NYZ-AUP/NYZ-ADOWN. Amplified 1,625- and 1,235-bp DNA fragments were obtained, respectively (Fig. 2). These results proved that the constructed DNA was inserted into the *PEP4* locus correctly. After the PCR verification, 10 verified transformants were selected randomly for initial fermentation tests. One transformant with the lowest PrA activity and diacetyl content was selected and was designated as S-1K12. We

also constructed single *PEP4* allele disruption strains, which resulted from transforming *PEP4 A-loxP-KanMX-loxP-PEP4 R* disruption cassette into S-6 using the same method described above (Fig. 1b). The strains were verified using the primers YZ-SUP/YZ-2A and YZ-AUP/YZ-ADOWN obtained from the amplification of the 1,626- and 1,751-bp DNA fragments (Fig. 2). A recombinant strain with the lowest PrA activity and diacetyl content was selected and designated as S-K10.

Cre recombinase was expressed and *KanMX* was excised after introducing the plasmid pSH-Zeocin into S-1K12 and S-K10. Loss of pSH-Zeocin changed the genotype of the mutants into *PEP4* (n-1)/*pep4* (73, 750)::*PGK1p-ILV5-PGK1t-loxP* and *PEP4* (n-1)/*pep4* (73, 750)::*loxP*, which were designated as S-C1K12 and S-CK10, respectively. The DNA fragment of *PEP4 SA-loxP-KanMX-loxP-PEP4 SR* disruption cassette was used to transform S-C1K12 and S-CK10 to delete the second *PEP4* allele. To verify whether *KanMX* was correctly integrated into the second *PEP4*, the primer pairs YZ-SUP/YZ-2A and YZ-AUP/YZ-ADOWN were used, and the

Fig. 1 Strategy for homologous recombination in a brewers' yeast strain and the primer binding region for PCR confirmation of successful homologous recombination. The *horizontal arrows* indicate the primer sites. **a** The homologous recombination of *PEP4 A-PGK1p-ILV5-PGK1t-KanMX-PEP4 R* with the brewers' yeast S-6. **b** The homologous recombination of *PEP4 A-KanMX-PEP4 R* with the brewers' yeast S-6. **c** Retractive primer design strategy for deleting the second copies of the *PEP4* gene in the brewers' yeast. Homologous sequences located on the outboard side of the *dotted line* were used for deleting the first allele, whereas those located on the inboard side of the *dotted line* were used for deleting the second allele. Homologous regions were segregated by the *dotted line*



1,932- and 2,092-bp amplification fragments indicated correct recombination (Fig. 2). To verify further the disruption of the second *PEP4* allele, A-UP/PR-DOWN was used as primers with the genome of two *PEP4* allele disruption transformants and those of two *PEP4* allele disruption and *ILV5* overexpression transformants as templates. The PCR products were 3,042 + 887 + 1,407 bp and 3,042 + 3,851 + 1,407 bp,

respectively (Fig. 2). A 3,042-bp fragment indicated amplification of the sequence that includes *PEP4 SA-loxP-KanMX-loxP-PEP4 SR* for both yeasts. By contrast, a 3,851-bp fragment indicated *PEP4 A-PGK1p-ILV5-PGK1t-loxP-PEP4 R* for S-SIK12 and 887-bp fragment indicated *PEP4 A-loxP-PEP4 R* for S-SK10. A 1,407-bp fragment indicated the presence of intact *PEP4* because the same fragment was

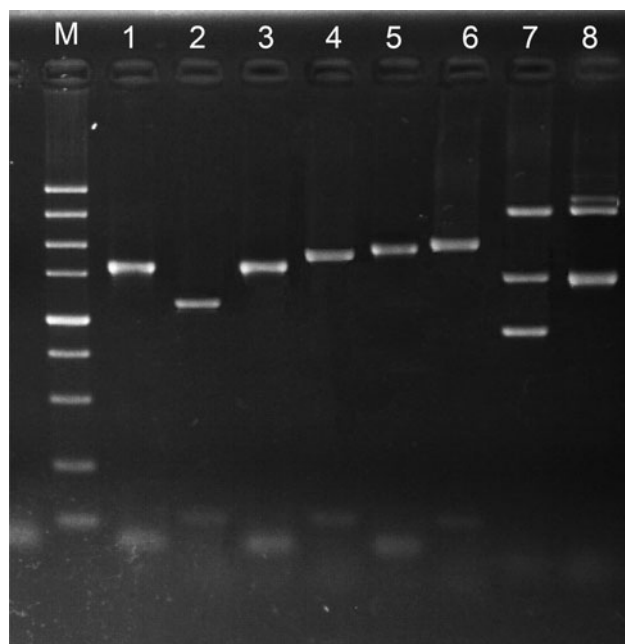


Fig. 2 PCR analysis of the yeast recombinants. DNA templates: genome of the single *PEP4* allele disruption and *ILV5* overexpression transformants (lanes 1 and 2), genome of the single *PEP4* allele disruption transformants (lanes 3 and 4), genome of the two *PEP4* allele disruption and *ILV5* overexpression transformants (lanes 5, 6, and 8), genome of the two *PEP4* allele disruption transformants (lane 7). Primers: YZ-SUP/YZ-SDOWN (lane 1), NYZ-AUP/NYZ-ADOWN (lane 2), YZ-SUP/YZ-2A (lanes 3 and 5), YZ-AUP/YZ-ADOWN (lanes 4 and 6), and A-UP/PR-D (lanes 7 and 8)

obtained when the primer pair A-UP/PR-DOWN was used to amplify the S-6 DNA template. S-SIK12 and S-SK10 were selected from 20 verified transformants through an initial fermentation test.

All of the 50 single-grown colonies of the 10th generation verified that the recombinant strain could grow on YEPD with 800 mg l^{-1} G418. These results indicate that the *KanMX* gene might have been integrated into the *PEP4* locus, which increased the kanamycin resistance of the recombinant strains, with their genetic stability reaching 100 %.

Finally, the *KanMX* gene was excised using the *Cre-loxP* recombination system to obtain the mutants S-CSIK12 and S-CSK10.

Analytical methods

Headspace gas chromatography coupled with electron capture detection was used to measure diacetyl content [37]. Before analysis, the samples were heated to $60 \text{ }^\circ\text{C}$ for 1 h to convert completely α -acetolactate into diacetyl. Assay for the *ILV5* gene product acetohydroxyacid reductoisomerase (RI) activity has been described previously [28]. PrA activity was assayed using a fluorescent method [30]. Head retention was determined with a NIBEM-T

foam stability tester (Haffmans, VENLO Holland) according to the manufacturer's instructions. The esters and higher alcohols were measured using headspace gas chromatography coupled with flame ionization detection [37]. Headspace instrument conditions were as follows: Samples were heated for 20 min at $60 \text{ }^\circ\text{C}$ in the headspace autosampler before injection; sample temperature, $60 \text{ }^\circ\text{C}$; needle temperature, $95 \text{ }^\circ\text{C}$; transfer line temperature, $105 \text{ }^\circ\text{C}$; GC-cycle time, 26 min; equilibration time, 20 min; pressurize time, 1 min; injection time, 1 min; withdrawal time, 0.5 min; GC conditions were as follows: The oven temperature was held at $35 \text{ }^\circ\text{C}$ (3 min), followed by an increase to $80 \text{ }^\circ\text{C}$ (0.5 min) at a rate of $10 \text{ }^\circ\text{C}$ per min, then increasing to $220 \text{ }^\circ\text{C}$ (0.5 min) at a rate of $10 \text{ }^\circ\text{C}$ per min. The FID temperatures were kept constant at $240 \text{ }^\circ\text{C}$. The analyses were performed in triplicate. Statistical analysis was done by using the Student's *t* test.

Real-time PCR

Yeast strains were cultured in a YEPD medium for 18 h and collected for RNA extraction. The mRNA was extracted using a yeast RNA kit (Omega, Madison, United States), and the changes in *ILV5* gene expression were assessed via real-time quantitative PCR (qRT-PCR) using an Ultra SYBR Two-Step qRT-PCR kit with ROX (reference dye for real-time PCR) (CW BIO, China). The primers used to amplify the small parts of *ILV5* and the reference gene *GAPDH* (*GPD1*) are listed in Table 1. The $\Delta\Delta\text{C}$ (t) method was applied for the relative quantification of transcription of *ILV5* in the overexpressing strains compared with the reference strain.

Fermentation test

The yeast strains were first grown in 5 ml of 11°P wort at $28 \text{ }^\circ\text{C}$ for 24 h, and the suspension was inoculated into 50 ml of wort (11°P). Then, the preculture was incubated without shaking at $16 \text{ }^\circ\text{C}$ for 48 h to adapt the yeast to a lower temperature. After this cultivation process, it was inoculated into 500 ml of 11°P wort in conical flask with fermentation bung. The density of the inoculum was approximately 2×10^7 cells ml^{-1} . Fermentation was conducted at $10 \text{ }^\circ\text{C}$ for approximately 15 days. After the diacetyl level in the wort decreased below the taste threshold, the yeast was cropped immediately, and an additional 10 days of cold conditioning process was started. Apparent wort extracts were recorded daily. The diacetyl concentration and the PrA activity of the fermented worts during brewing were assayed at different stages of fermentation such as the vigorous stage of main fermentation (day 4), after yeast cropping (days 13–15), after cold conditioning (days 23–25), and other stages. The esters and

higher alcohols were assayed after cold conditioning. Microscale fermentation (5 l) and pilot scale brewing (100 l) trials were conducted to evaluate the flavor and taste of the finished beer products brewed with the host strain and the recombinant strains [46]. The finished beer samples were evaluated by an expert panel consisting of six tasting experts from the Tianjin University of Science and Technology Beer Fermentation Pilot Base.

Results and discussion

Diacetyl content assay during beer fermentation

The diacetyl production of the host strain (S-6), the two *PEP4* allele disruption and *ILV5* overexpression strain (S-CSIK12), and the two *PEP4* allele disruption strain (S-CSK10) were measured at different stages of fermentation. Samples were taken for analysis on days 2, 4, 6, 8, 10, 11, 12, 13, 14, and 15. The fermentation was performed in triplicate for each strain. The results show that the peak diacetyl production of each strain was observed on day 6 of fermentation (Fig. 3). The peak diacetyl content of the beer brewed with S-6, S-CSIK12, and S-CSK10 was 0.855, 0.581, and 0.946 mg l⁻¹, respectively. Then, the diacetyl concentration decreased moderately and dropped to the 0.1–0.15 mg l⁻¹ threshold on days 13 (brewed with S-CSIK12) and 15 (brewed with S-6 or S-CSK10). As shown in Fig. 3, the diacetyl production of S-CSIK12 was always lower than that of S-6 and S-CSK10 at all sampling stages. This finding indicates that *ILV5* gene overexpression effectively decreases the diacetyl production in beer. The results support the conclusion by Villanueva et al. [10], who stated that the presence of extra *ILV5* copies in yeast strains decreases diacetyl production during wort fermentation. In this study, an extra copy of the *ILV5* gene was integrated into the brewer's yeast chromosome to produce a stable, decreased diacetyl production strain. The two copies of the *PEP4* disruption strain S-CSK10 produced slightly more diacetyl than the S-6 host strain during fermentation, as shown in Fig. 3. However, the explanation for this phenomenon is beyond the scope of the current knowledge. We speculate that altering a single gene could have some unexpected effects on a cell. Although the effect is not evident, the disruption of two *PEP4* alleles may have influence on diacetyl production by the yeast cells. Further studies are needed to elucidate this condition.

RI activity and the *ILV5* expression levels

The host strain (S-6), the two *PEP4* allele disruption, and *ILV5* overexpression strain (S-CSIK12) were grown in wort medium for 18 h. The RI activities of S-6 and

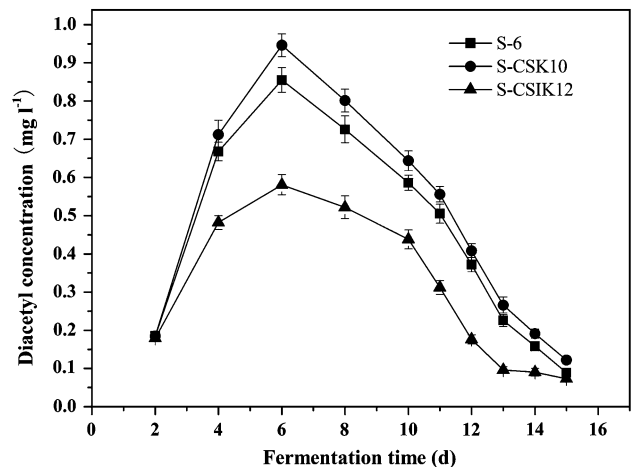


Fig. 3 Diacetyl content of the recombinant and host strains during fermentation. The values are the means of triplicate experiments with standard deviations lower than 5 %

S-CSIK12 were 0.38 and 0.62 μM NADPH oxidized per hour per milligram of protein. These results are the means of three independent experiments. Furthermore, the qRT-PCR results show that the cDNA of the recombinant *ILV5* overexpression strain (S-CSIK12) increased to 1.88-fold that of the host strain (S-6), as shown in Fig. 4. The real-time PCR results demonstrate higher *ILV5* expression levels and larger transcript sizes in S-CSIK12 than those of the control. The RI activity assay results indicate that *ILV5* gene overexpression decreased diacetyl production.

PrA activity and beer head retention

The wild-type brewer's yeast S-6 and the two recombinant strains S-CSIK12 and S-CSK10 were inoculated for investigation. The PrA activity of the worts was assayed on days 4 (vigorous stage of main fermentation), 13 (S-CSIK12 was cropped), and 15 (S-CSK10 and S-6 were cropped), and then after cold conditioning. The PrA activity gradually increased from the vigorous stage to the final stage of main fermentation (Fig. 5). However, the PrA activity slightly decreased after cold conditioning. The PrA activities of the three yeast strains were also compared during the different stages of brewing (Fig. 5). The PrA activities of the recombinant strains S-CSIK12 and S-CSK10 were always lower than those of the wild-type strain at all sampling stages. The PrA activities of the recombinant strains S-CSIK12 and S-CSK10 after cold conditioning were only 56 and 65 % compared to the wild-type strain.

The construction of a *PEP4* disruption strain was a logical approach to improving foam stability [26, 34]. First, a single *PEP4* allele disruption strain was constructed. Then, the PrA activity was determined by disrupting one copy of the *PEP4* gene of S-6; however, no significant

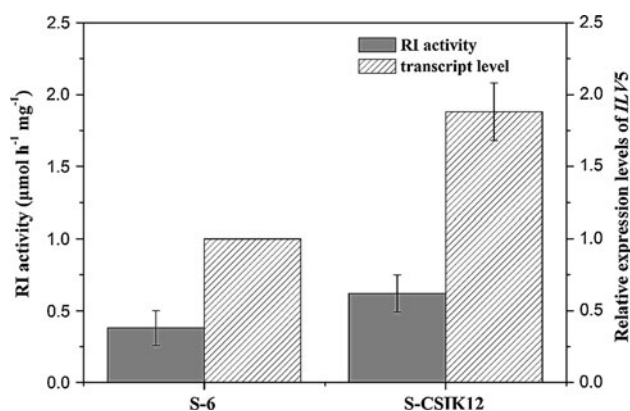


Fig. 4 Determination of *ILV5* gene expression levels in the recombinant strain S-CSIK12 using real-time PCR and the acetohydroxyacid reductoisomerase activity of the recombinant strain S-CSIK12 and the host strain S-6. The *GAPDH* (*GPD1*) gene was used as the internal control. RT-qPCRs and measurement of acetohydroxyacid reductoisomerase activity were performed with three biological replicates and technical duplicates of each sample. Error bars represent the standard deviation of the mean

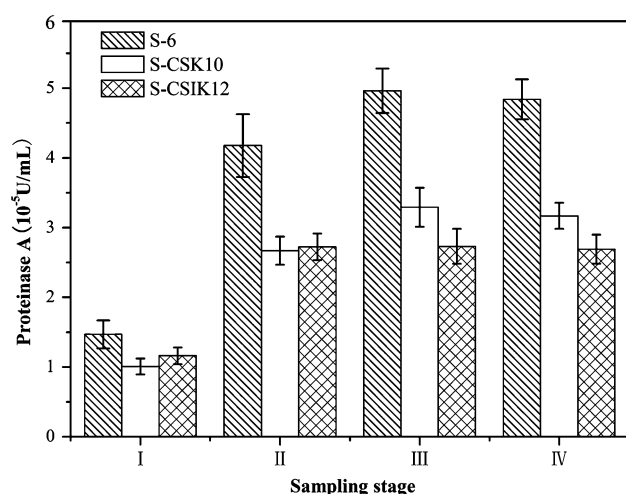


Fig. 5 Proteinase A activity of the recombinant strains and their host during fermentation. Sampling stage I: vigorous stage of main fermentation (day 4 of brewing); sampling stage II: after S-CSIK12 was cropped (day 13 of brewing); sampling stage III: after S-CSK10 and S-6 were cropped (day 15 of brewing); sampling stage IV: after cold conditioning (10 days after yeast cropping). The values are the means of triplicate experiments with standard deviations lower than 5 %

difference was observed between transformants and the host. All transformants exhibited at least 90 % PrA activity compared with the host (data not shown). Two *PEP4* allele disruption strains were then constructed by applying the *Cre-loxP* recombination system, and their PrA activities were significantly lower than that of the host strain. This decrease may have been caused by the tetraploidy of S-6. Three *PEP4* allele disruption strains and four *PEP4* allele disruption strains were also constructed, and their PrA

activities were even lower than that of the two *PEP4* allele disruption strains; however, a long growth lag phase and significantly reduced wort sugar consumption were observed when the third and fourth allelic *PEP4* genes were disrupted (data not shown). Thus, the three *PEP4* allele disruption strain and four *PEP4* allele disruption strains were not adopted because of their negative effects on beer fermentation. The qPCR results indicated that S-6 contained four copies of the *PEP4* gene (data not shown). The PrA activity test showed that a lower copy number of the *PEP4* gene in the mutants led to lower PrA activity production. In addition, we could not detect PrA activity when all copies of the *PEP4* gene were disrupted. Thus, we conclude that the PrA activity is related to *PEP4* gene copy number and ploidy of a yeast strain. Some authors have reported that the disruption of single *PEP4* alleles in brewer's yeast significantly decreases PrA activity [38, 39]. This discrepancy with the present results may have been caused by the different *PEP4* copy numbers and the evolutionary origins of the host strains. S-6 contained four copies of the *PEP4* gene, which might be more than its host strains. Previous studies on brewer's yeast have shown that their genomes are hybrid and polyploid, such that the copy numbers of certain genes in various brewer's yeast strains may range from haploid to tetraploid [40]. Brewer's yeast strains with different *PEP4* gene copy numbers might produce different amounts of PrA.

The stage following the end of diacetyl removal is an important stage of brewing. The worts during this stage of beer fermentation present a hostile environment for yeast cells because of nitrogen starvation, high ethanol concentrations, high osmotic pressure, and high carbon dioxide concentrations [19, 24]. These conditions may decrease cell viability or even cause the autolysis of yeast cells, which will lead to a significant increase in the release of PrA into the beer [19, 25]. Yeast strains should be removed once the diacetyl concentration in the worts decreases below the taste threshold during beer fermentation for the reasons described above. Thus, shortening the time of diacetyl removal means the yeast can be cropped ahead of time. Once the yeast is removed, there is no way to release PrA into the beer. Hence, the timing of yeast cropping can be optimized to minimize PrA release into the beer if a brewer's yeast strain with lower diacetyl production is used. In this study, the yeast cropping time was performed 2 days earlier using the *ILV5* overexpression strain (S-CSIK12) instead of the *ILV5* wild-type strains (S-CSK10 and S-6). The assay results show that the PrA activity of the wort brewed with S-CSIK12 was lower than that of S-CSK10 on day 15 and after cold conditioning (Fig. 5). The S-CSIK12 yeast was cropped on day 13 such that it could not release PrA into the wort during subsequent brewing. Hence, the yeast should be cropped immediately

once the diacetyl level in the wort is below the taste threshold. Optimized yeast handling decreases the PrA content of beer. Longer yeast bleed-off times do not contribute to diacetyl removal, the higher the probabilities of high PrA release into the finished beer.

The beer head retention of S-6 and the recombinant strains S-CSIK12 and S-CSK10 was determined after cold conditioning. As shown in Table 3, the head retention of beer brewed with S-CSIK12 (260 ± 2 s) and S-CSK10 (244 ± 3 s) was better than that with the host strain S-6 (212 ± 3 s). Based on the PrA activity assay, the PrA activity of beer is closely related to beer head retention. The head retention of the beers brewed with S-CSIK12 and S-CSK10 indicates that brewing using strains with low diacetyl production positively affects beer foam stability. This result shows that disruption of two *PEP4* alleles

reduces the PrA activity of the finished beer by 35 % and increases its head longevity by 32 s. By contrast, *ILV5* gene overexpression reduces the PrA activity of the finished beer by 44 % and increases its head longevity by 48 s.

The main beer performance indices

The flavor component profiles assayed after cold conditioning show that the transformant with two disrupted *PEP4* copies (S-CSK10) did not observably affect the aromatic compound content of beer compared with the host strain (S-6) (Table 3). However, the *Ilv5p* (acetohydroxyacid reductoisomerase) overexpression transformant (S-CSIK12) had increased fusel alcohols, esters, and acetaldehyde (Table 3). Considering fusel alcohols and esters are the products of amino acid catabolism in the ILV biosynthetic pathway, their increased amounts may have been caused by increased flux in the ILV biosynthetic pathway [41–43]. Although the fusel alcohols and acetaldehyde, which negatively affect beer quality, were increased when S-CSIK12 was used, their concentrations in the beer were within the normal sensory thresholds.

The sensorial analysis results indicated that the beer brewed with the host strain (S-6) and that brewed with the two *PEP4* allele disruption strain (S-CSK10) did not differ in flavor or taste. The tasting experts considered the beers brewed with the two *PEP4* allele disruption and *ILV5* overexpression strain (S-CSIK12) better tasting than those brewed with S-6 and S-CSK10. The flavor component profiles of the finished beer products also showed the values determined for the beers brewed with S-6, S-CSIK12 and S-CSK10, as shown in Table 4. In addition, the resulting beers produced by microscale fermentation were comparable with the pilot brews of beers.

The wort apparent extract was detected, and a slight reduction in wort sugar consumption during fermentation was observed in the two *PEP4* allele disruption strains

Table 3 The head retention and flavor component profiles of the finished beer products

	S-6	S-CSK10	S-CSIK12
Beer head retention(s)	212 ± 3	244 ± 3^a	260 ± 2^a
Acetaldehyde (mg l^{-1})	7.23 ± 0.04	6.95 ± 0.02^a	8.82 ± 0.04^a
Ethyl acetate (mg l^{-1})	14.37 ± 0.2	15.63 ± 0.2^a	19.06 ± 0.5^a
Propyl alcohol (mg l^{-1})	14.78 ± 0.1	14.33 ± 0.6	17.87 ± 0.5^a
Isobutyl alcohol (mg l^{-1})	9.35 ± 0.4	8.82 ± 0.6	11.7 ± 0.3^a
Isoamyl acetate (mg l^{-1})	1.09 ± 0.05	1.14 ± 0.03	1.99 ± 0.05^a
Isoamyl alcohol (mg l^{-1})	56.87 ± 2.8	55.84 ± 1.9	63.54 ± 3.0^a

All the results shown are mean values of three independent experiments including standard deviations

^a Values of the recombinant yeast strains are significantly (Student's *t* test $P \leq 0.05$) different from those for the wild-type strain

Table 4 The flavor component profiles of the finished beer produced by microscale fermentation and pilot scale fermentation

Parameter (mg l^{-1})	Microscale fermentation			Pilot scale fermentation		
	S-6	S-CSK10	S-CSIK12	S-6	S-CSK10	S-CSIK12
Diacetyl	0.72 ± 0.03	0.75 ± 0.03	0.71 ± 0.02	0.69 ± 0.04	0.73 ± 0.03	0.65 ± 0.04
Acetaldehyde	7.05 ± 0.08	6.53 ± 0.05^a	7.79 ± 0.09^a	6.82 ± 0.06	6.25 ± 0.08^a	7.46 ± 0.08^a
Ethyl acetate	12.98 ± 0.4	13.56 ± 0.6	19.12 ± 0.6^a	14.35 ± 0.6	14.95 ± 0.4	21.12 ± 0.8^a
Propyl alcohol	12.24 ± 0.6	12.82 ± 0.4	16.02 ± 0.2^a	13.66 ± 0.6	14.02 ± 0.36	17.85 ± 0.9^a
Isobutyl alcohol	9.96 ± 0.2	9.12 ± 0.5	12.8 ± 0.6^a	8.48 ± 0.6	8.98 ± 0.3	10.98 ± 0.4^a
Isoamyl acetate	1.22 ± 0.08	1.16 ± 0.04	1.36 ± 0.06	1.16 ± 0.02	1.28 ± 0.04^a	1.42 ± 0.04^a
Isoamyl alcohol	50.82 ± 3.2	51.62 ± 2.6	58.88 ± 3.7^a	46.6 ± 2.4	48.2 ± 2.8	54.66 ± 4.4^a

All the results shown are mean values of two independent experiments including standard deviations

^a Values of the recombinant yeast strains are significantly (Student's *t* test $P \leq 0.05$) different from those of the wild-type strain

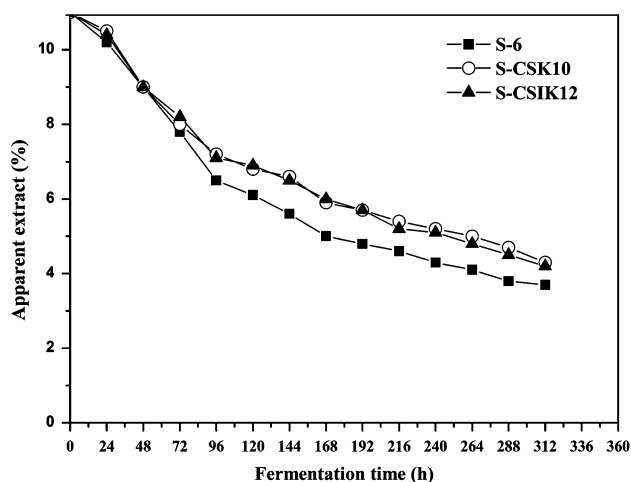


Fig. 6 Determination of wort sugar consumption by the recombinant strains S-CSIK12 and S-CSK10, and the host strain S-6. The values are the means of triplicate experiments with standard deviations lower than 5 %

(S-CSK10 and S-CSIK12) compared with the host strain (S-6) (Fig. 6). This phenomenon should be interpreted as follows: First, PrA is essential to the *Saccharomyces cerevisiae* vacuolar proteolytic system during nutritional stress and vegetative growth [44], especially during beer fermentation. Second, Chen et al. [39] reported that in industrial *S. cerevisiae*, PrA may control glycolytic enzyme expression (HK: hexokinase, Pfk: phosphofructokinase, and PYKi: pyruvate kinase) directly or indirectly, which delays cell metabolism in PrA-modified strains, and that glycolytic flux direction and rate may be regulated by vacuolar PrA in industrial *S. cerevisiae* [39]. Mutations at the *PEP4* locus may exhibit a dosage effect on sugar consumption. PrA deficiency in the yeast may affect the physiological and metabolic functions of cells, including sugar consumption.

Conclusions

Brewing with two *PEP4* allele disruption and *ILV5* overexpression recombinant industrial brewing yeast strains significantly decreased the PrA activity of beer, improves foam stability, and decreases diacetyl production in beer. Our investigations show that the PrA activity of the beer brewed with strains with two disrupted *PEP4* copies (S-CSIK12 and S-CSK10) was decreased compared with that using the host strain. Head retention was also improved. In addition, the diacetyl production of *ILV5* overexpression strain (S-CSIK12) was always lower than that of the host strain at all stages of beer fermentation. Brewing with lower diacetyl production strain optimizes the timing of yeast cropping, decreases the PrA activity of beer, and improves head retention. This discussed the

relationship between diacetyl production and beer head retention, two aspects of beer quality. The current study would help in maintaining beer foam performance, especially in stabilizing the foam in unpasteurized beer. The *KanMX* markers of all transformants were excised such that the beers brewed with the transformants were considered safe. However, genetically modified foods produced by the fermentation of recombinant microorganisms are not commercially available due to the lack of public acceptance. Thus, the recombinant yeast strains in this study could not be applied in the commercial brewing industry at present because of the controversy over genetically modified organisms (GMOs) in Europe. Nevertheless, the present study provides reference for the brewing industry as well as research on the diacetyl reduction and foam stability of beer. With stronger understanding of and further research into genetically modified organisms, these will likely be widely accepted in the future.

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