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

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

Keywords: Industrial brewing yeast, beer aging, amylolytic activity, flavor

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

Acetaldehyde is one of the main off-flavor compounds and is a natural by-product of fermentation. While creating a pungent aroma at a high concentration, it leaves a more pleasant green-apple aroma at a diluted concentration. Moreover, as the main aromatic aldehyde, acetaldehyde affects beer staling through an aldol condensation reaction during beer storage. Thus, decreasing the amount of acetaldehyde could improve the resistant staling value (RSV) of beer [28, 31]. Previous reports have shown that disrupting the *ADH2* gene encoding alcohol dehydrogenase can reduce the acetaldehyde content in the final beer [6, 28, 25, 31, 36].
Residual saccharides, such as maltose and maltooltriose, which result in a high caloric content and unusual flavor, are another problem for the brewery industry, as \textit{S. cerevisiae} is hardly able to hydrolyze starchy materials due to a lack of extracellular depolymerizing enzymes. Thus, in conventional beer fermentation, large amounts of exogenous enzymes are added to liberate fermentable sugars from polysaccharide-rich substrates, but this is costly and sometimes causes allergenic-related symptoms [3, 19]. It should be noted that traits for these characteristics [26, 27, 34] for use in wine. Meanwhile, \(\alpha\)-amylase, dextranase, and glucoamylase genes have been introduced to industrial brewing yeast strains to address the lack of amylolytic activity [8, 12, 15, 16, 28].

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

\textbf{MATERIALS AND METHODS}

\textbf{Strains and Culture Condition}

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

The yeasts were cultured in a YPD medium (1% yeast extract, 2% peptone, 2% glucose), whereas the recombinant strains were cultured and selected on YNBS media (0.67% yeast nitrogen base w/o amino acids with 1% soluble starch as sole carbon source) with copper sulfate (CuSO\(_4\)). All the solid media contained 1.5% agar. YPD or 12\(^{\circ}\)P wort was used for the assay of growth capability, fermentation ability, \(\alpha\)-amylase activity, alcohol dehydrogenase activity, \(\alpha\)-acetolactate synthase activity, and glutathione production.

\textbf{Construction of Recombinant Strains}

Plasmid pICG containing the \textit{GSH1} and \textit{CUP1} genes was constructed in a previous study [29]. The \textit{ALP1} gene encoding \(\alpha\)-amylase was amplified from the genomic DNA of ZF02 via PCR using primers ALP1-F/ALP1-R (Table 2) (Shanghai Sangon Biological Engineering Technology \& Services Co., Ltd., China). The strong promoter in \textit{S. cerevisiae}, PGK1 promoter (PGK1\_L) from pMPS [28], was introduced to promote the expression of the \textit{ALP1} gene, whereas the \(\alpha\) signal factor was introduced for the secretion of the \textit{ALP1} gene. All these fragments were then ligated into the SacI and Sph1 sites of pADH [31], generating pAPM (Fig. 1A).

The cassettes GC (\(\text{ilv2}\Delta:\text{GSH1}\)) and PM (\(\text{adh2}\Delta:\text{ALP1}\)) were prepared by PCR using primers ILV2-F/ILV2-R and ADH2-F/ADH2-R. The two fragments were then cotransformed to Y31 using the lithium acetate (LiAc) method, as previously described [24]. Thereafter, the recombinant strains were selected on YNBS plates with 8 mM CuSO\(_4\).

\textbf{Assay of Enzyme Activities of Concern}

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

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

The \(\alpha\)-acetolactate synthase activity was detected using the method described by Zhang \textit{et al.} [34].

\begin{table}[h]
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\caption{Microbial strains and plasmids used in this study.}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Strains or plasmids} & \textbf{Relevant genotype} & \textbf{Source} \\
\hline
\textit{Saccharomyces cerevisiae} Y31 & \textit{FrecA1 endA1 gyrA96 thi hsdR17 supE44 relA1 \(\Delta\) (argF-Lac-ZYA)} & Stratagene \\
\textit{Saccharomyces cerevisiae} Y31 & \textit{Industrial yeast strain} & Tsingtao Brewery Co., Ltd (Qingdao, China) \\
\textit{Saccharomyces fibuligera} ZF02 & \textit{Wild yeast strain} & Stored in author’s lab \\
\textit{Saccharomyces cerevisiae} Y31 & \textit{Recombined industrial yeast strain} & This study \\
\textbf{Plasmids} & & \\
\textbf{pICG} & \textit{Ap}\(^\beta\)\textit{ADH2GSH1CUP1URA3} & [25] \\
\textbf{pADH} & \textit{Cloning vector, \textit{amp}} & [27] \\
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\caption{Primers used in this research.}
\begin{tabular}{|l|l|}
\hline
\textbf{Primers} & \textbf{Sequences 5' to 3'} \\
\hline
\textit{ALP1-F} & GGTCTAGACATCTTCTTCAATACCCGCC (XbaI) \\
\textit{ALP1-R} & AAGAGCTCTGGTTTCCCCTGGATGACC (SacI) \\
\textit{ILV2-F} & CCCGACAATAAAGTAAATAG \\
\textit{ILV2-R} & AGAAGAAGGCTGAAGATC \\
\textit{ADH2-F} & GCTGTATGTTCACAGGTC \\
\textit{ADH2-R} & TCCAGGAGGACGGACAA \\
\textit{CUP-F} & CGCTATACGTCATATGTTTC \\
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