LWT - Food Science and Technology 154 (2022) 112705

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Analysis of genes from *Saccharomyces cerevisiae* HJ01 participating in aromatic alcohols biosynthesis during *huangjiu* fermentation

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ARTICLE INFO

Keywords:
Aromatic alcohols
Saccharomyces cerevisiae
Huangjiu
Acid protease
Ehrlich pathway

ABSTRACT

Aromatic alcohols (including β-phenylethanol, tyrosol and tryptophol) could contribute positively to the aroma flavor of *huangjiu* at an appropriate amount. In this study, it was found that the aromatic alcohols in *huangjiu* were predominantly synthesized by *Saccharomyces cerevisiae* HJ01. The Ehrlich pathway and shikimate pathway, including 31 genes, are responsible for aromatic alcohol biosynthesis in *S. cerevisiae*. During the first 120 h of *huangjiu* fermentation, the genes $GAP1^{HJ01}$ (permease), $BAT2^{HJ01}$ (transaminase), $PDC1^{HJ01}$ and $PDC5^{HJ01}$ (pyruvate decarboxylase), and $ADH1^{HJ01}$ (alcohol dehydrogenase) formed integrated Ehrlich pathway are responsible for aromatic alcohol biosynthesis. In *S. cerevisiae* HJ01, the mutations in the promoter region of genes $GAP1^{HJ01}$, $BAT2^{HJ01}$, $PDC1^{HJ01}$, and $PDC5^{HJ01}$ involved in the Ehrlich pathway increased the promoter strength and transcriptional levels of related genes. The addition of 20 U/g acid protease could substantially increase the expression levels of genes involved in the Ehrlich pathway, and therefore there were 34.7% increasing for the concentration of total aromatic alcohols.

1. Introduction

Huangjiu, also known as Chinese rice wine, is one of the most ancient alcoholic beverages around the globe (Mcgovern et al., 2004). It is also the raw material for cooking wine and grain vinegar production. Aromatic alcohols, including β-phenylethanol, tyrosol and tryptophol, are one cluster of higher alcohols (alcohols with more than three carbons) with a benzene ring (González et al., 2018). Although low abundance in *huangjiu*, aromatic alcohols could produce high flavor intensities at low thresholds, similar to volatile sulfur-containing compounds in *baijiu* (Sun, Wang, & Sun, 2021). Studies showed that aromatic alcohols (especially β-phenylethanol) could contribute positively to the sensory properties of fermentation food like *huangjiu*, cooking wine and grain vinegar (Fleet, 2003; Zhou et al., 2020).

Currently, *huangjiu* is predominantly produced through high-gravity fermentation using rice, wheat and water as raw material, which involves a complex ecological microbiological system (including fungi, yeast and bacteria) (Liu et al., 2019). *Saccharomyces cerevisiae* is the primary functional microorganism for ethanol production in various alcoholic beverages, including *huangjiu* (Hong et al., 2016). Studies showed that there were two pathways for aromatic alcohol synthesis in yeast, namely the shikimate pathway and the Ehrlich pathway (Fig. 1).

The shikimate pathway was the leading pathway for aromatic alcohol biosynthesis in the absence of aromatic amino acids in the medium, and it highly depended on the synthesis of amino acid precursors from glucose (i.e, *de novo* biosynthetic pathway) (Etschmann, Bluemke, Sell, & Schrader, 2002). The Ehrlich pathway was based on the catabolism of aromatic amino acids present in the medium, and the final concentration of aromatic alcohol was highly associated with the initial aromatic amino acid contents (Hazelwood, Daran, van Maris, Pronk, & Dickinson, 2008). Yeast cells could selectively use the wide range of assimilable nitrogen present in the environment (Liu et al., 2021), some

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https://doi.org/10.1016/j.lwt.2021.112705
Received 24 September 2021; Received in revised form 19 October 2021; Accepted 23 October 2021
Available online 30 October 2021

of these molecules could be directly used as ready-to-use metabolites (Chiva, Baiges, Mas, & Guillamon, 2009). There were 31 genes involved in aromatic alcohol biosynthetic pathway, 19 and 12 of which were related to the Ehrlich pathway and the shikimate pathway respectively. The existence of a large number of alleles, like *ARO10, PDC1, PDC5-6,* and *THI3* coding pyruvate decarboxylase, made it difficult to determine which genes participate in aromatic alcohol synthesis in *huangjiu* fermentation process (Fig. 1).

Many studies have shown a strong link between enzyme expression and aromatic alcohol production in beer and grape wine (Wang et al., 2019). The concentration of β-phenylethanol produced by *S. cerevisiae* increased 3 folds by over-expressing *ARO80*, *ARO9* and *ARO10* genes simultaneously (Kim, Cho, & Hahn, 2014). However, the biosynthetic pathway of aromatic alcohols and key genes related to this process are rarely explored for *huangjiu* fermentation. Additionally, the whole transcript behavior of the whole biosynthetic pathway should be considered, as the end product of a metabolic pathway is determined by a series of gene actions (Aminfar, Rabiei, Tohidfar, & Mirjalili, 2019).

Fermentation process optimization, such as incubation temperature optimization and low-intensity sonication, could also affect the content of aromatic alcohols and flavor of fermented foods (Yang et al., 2020; Gao, Liu, Zhang, et al., 2020). Recent studies showed that the concentration of higher alcohols could be diminished with the supplement of inorganic nitrogen nutrition (Liu et al., 2021). The raw material (i.e., rice) in *huangjiu* fermentation contained certain amounts of proteins, however, these organic nitrogen sources were relatively difficult to metabolism by yeast without heterogenous protease (Chiva et al., 2009).

In the present study, the aromatic alcohols' profiles of *S. cerevisiae* BY4743 (model organism) and *S. cerevisiae* HJ01 (*huangjiu* yeast) under different conditions were investigated to determine the contribution of aromatic alcohols produced by yeast in *huangjiu*. Biosynthetic genes related to aromatic alcohols production were then amplified and analyzed by qPCR to identify the key genes and their expression pattern in the aromatic alcohol biosynthetic pathway in *huangjiu* fermentation. Based on the physiological and metabolic analytical conclusion, medium optimization was conducted by adding acid protease to improve aromatic alcohols production in *huangjiu* fermentation. The results showed that adding acid protease could regulate the expression level of genes related to the aromatic alcohols biosynthesis.

2. Materials and methods

2.1. Strains and strain construction

All strains and plasmids used for strain construction in this study were shown in Table S1 of the supplementary material. *S. cerevisiae* BY4743 (baker's yeast, as a model strain) and *S. cerevisiae* HJ01 (*huangjiu* yeast) were used for this study (Baker et al., 1998). Yeast strain construction was carried out based on a previously described library screen transformation protocol for *S. cerevisiae* with minor modifications (Gietz & Schiestl, 2007). All primers used for strain construction are shown in Table S2 of the supplementary material. Briefly, oligonucleotides were used to amplify the reporter gene (enhanced green fluorescent protein gene, *eGFP*) from pEGFP-N1, and the obtained PCR-products were fused by ligation, reamplified and cloned into cloning vector (pMD19-T) using *Not*I and *Bam*HI restriction sites to generate plasmid pMD19-T-EGFP. The plasmid was then transformed into the expression vector, pY26TEF-GPD, and resulting plasmid generated (pY26-EGFP) was used as a new expression vector for later usage. Similarly, the adjacent region upstream genes of interest were amplified and cloned into pMD19-T using *Not*I and *Bam*HI restriction sites. The obtained plasmids were then transformed into expression vector pY26-EGFP, and generated plasmid pY26-promoter-EGFP. The promoter strength (i.e., the intensity of green fluorescence) was evaluated using flow cytometry and fluorescence microscopy (BD FACSArica III, USA).

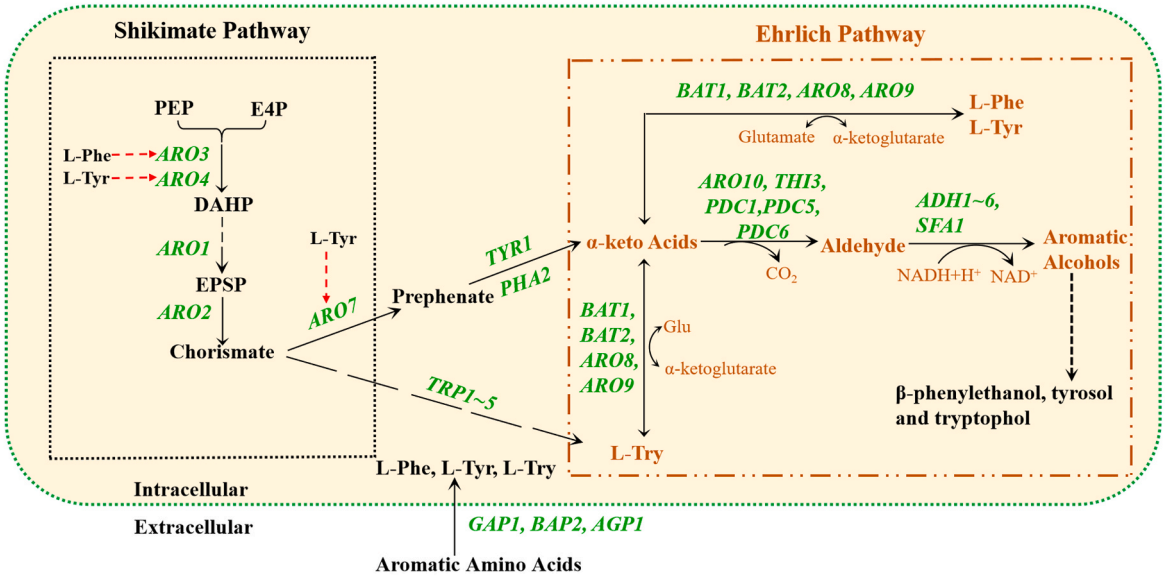

Fig. 1. The metabolic overview for aromatic alcohol biosynthesis in *Saccharomyces cerevisiae* via the shikimate pathway (left in black color) and Ehrlich pathway (right in orange color). Genes responsible for the enzymes are shown in green color beside each reaction step (black arrows), and red arrows represent feedback inhibition effect for the genes. Abbreviations: L-Phe (L-Phenylalanine), L-Tyr (L-Tyrosine), L-Try (L-Tryptophan), PEP (phosphoenolpyruvate), E4P (D-Erythrose 4-Phosphate), DAHP (3-deoxy-D-arabino-heptulosonate-7-phosphate), EPSP (3-Enolpyruvylshikimate 5-Phosphate), α-keto acids (Phenylpyruvic Acid, 3-(4-hydroxyphenyl)pyruvate, Indole Pyruvate), aldehyde (Phenylacetaldehyde, (4-hydroxyphenyl)acetaldehyde, Indole Acetaldehyde), DAHP synthase (*ARO3* and *ARO4*), Pentafunctional aromatic protein (*ARO1*), Chorismate synthase (*ARO2*), Chorismate mutase (*ARO7*), Prephenate dehydratase (*PHA2*), Prephenate dehydrogenase (*TYR1*), Tyrosinase-related protein (*TRP1-5*), permease (*GAP1*, *BAP2* and *AGP1*), transaminase (*BAT1*, *BAT2*, *ARO8* and *ARO9*), pyruvate decarboxylase (*ARO10*, *THI3*, *PDC1*, *PDC5* and *PDC6*), and alcohol dehydrogenase (*ADH1-6* and *SFA1*). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.2. Media and fermentation conditions

All mediums used in this study for cell activation, selection, and cultivation were shown in Table S3 of the supplementary material. Prior to each fermentation, cells were activated in yeast extract peptone dextrose (YPD) broth at 30 °C for 24 h with rotation (150 rpm), and 5% of the culture were passaged into 13° Brix rice hydrolysate medium (RHM) and incubated at 30 °C for 24 h. Then 11% of this culture were inoculated into RHM (simulated *huangjiu* fermentation condition, SHF) and *huangjiu* fermentation system (normal *huangjiu* fermentation condition, NHF), and the fermentation was carried out based on our previous report (Liu et al., 2016). During the early stage of fermentation (0 h–120 h post-inoculation), the incubation temperature was 25 °C for 120 h, with mixing once per day. During the late stage of fermentation (120 h–480 h post-inoculation), the incubation temperature was 15 °C, with mixing once every two days. Different concentrations (0 U/g, 6 U/g, 10 U/g, 15 U/g, 20 U/g, and 30 U/g) of acid protease (Solarbio, Beijing, China) were added at the time of inoculation. 100 ml samples were taken at different fermentation time when mixing the medium during the fermentation process. Samples were stored at −20 °C freezer until analyzed.

2.3. Analysis of physicochemical properties, aromatic alcohols and aromatic amino acids of huangjiu

Total acid and amino nitrogen content were determined by titration, residual reducing sugar content was determined by dinitro salicylic acid (DNS) method, ethanol content was determined by distillation (Liu et al., 2021; Gao et al., 2021). The concentration of aromatic alcohols was determined by high-performance liquid chromatography (HPLC, Waters e2695) according to a modified method described previously (Zupan et al., 2013). The analysis was conducted using an XBridge C18 column (250 mm × 4.6 mm, 5 μm) with methanol:water (50:50) as an isocratic elution and UV detection at 210 nm. The concentration of amino acids was also determined by HPLC (Agilent 1100) as reported previously (Wang et al., 2014). The analysis was conducted using ODS HYPERSIL column (250 mm × 4.6 mm, 5 μm) with UV detection set at 338 nm.

2.4. RNA isolation and qPCR analysis of genes involved in the biosynthesis of aromatic alcohols

Total RNA was extracted using RNAprep Pure Kit (Tiangen, Beijing, China), and the first-strand cDNA was synthesized from 4 μl of the isolated RNA templates by reverse transcriptase with Oligo-(dT)18 primers according to the instructions of the PrimerScript™ RT reagent Kit (Takara, Dalian, China). qPCR was done to investigate the expression levels of thirty-one genes encoding aromatic alcohols biosynthetic related enzymes in *S. cerevisiae* (Fig. 1). All the gene-specific primers were designed by Primer Premier 5.0 software (Table S4). The qPCR reactions were performed in a 20 μl volume including 10 μl TB Green® Premix ExTaq™ II kit (TaKaRa), 50 ng of cDNA and 300 nM of each primer (Li et al., 2019). PCR amplification was performed with three technical replications under the following conditions: 5 min at 95 °C, followed by 40 cycles of 95 °C for 5s and 60 °C for 30 s. Two housekeeping genes (*ACT1* and *TDH3* gene) were used to normalize the qPCR data as internal control (Vaudano, Noti, Costantini, & Garcia-Moruno, 2011; Gao, Liu, Yin, et al., 2020). The expression level was calculated according to the $2^{-\Delta\Delta Ct}$ method based on the mean of three independent determinations of the threshold cycle (Etschmann et al., 2002). In addition, the value of $2^{-\Delta\Delta Ct}$ was used to represent the relative expression of each gene.

2.5. Statistical analysis

Three biological replicates were performed for each experiment and the data were statistically analyzed using Fisher's least significant difference (LSD) method. Tukey's test was used to generate p-values, and the results are expressed as the mean ± standard deviation. R 3.5.1 software (freely available at http://www.r-project.org) was used to generate the heat map to analyze the relative expression levels of genes. In all the analyses, the $P < 0.05$ was used as the significance threshold.

3. Results and discussion

3.1. The aromatic alcohols in huangjiu were mainly produced by S. cerevisiae

Many studies showed that aromatic alcohols in alcoholic beverages and related food products were produced by yeast (Zhang et al., 2015). Since *huangjiu* fermentation was carried out by a large number of microorganisms, it was crucial to identify which microorganism was responsible for aromatic alcohols production. In the present study, we proposed that *S. cerevisiae* was the major microbe for aromatic alcohols biosynthesis in *huangjiu* fermentation as it was the major yeast present in the fermentation mash (Liu et al., 2019). The final results of fermentation were analyzed for *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743 under SHF and NHF conditions. (Table 1). The final ethanol content produced by *S. cerevisiae* HJ01 was considerably higher than that of *S. cerevisiae* BY4743 under both SHF and NHF conditions (25.9% and 11.5% increase, respectively), while no statistical difference was found in the concentration of final residual reducing sugar and amino nitrogen between *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743.

The aromatic alcohols were fully produced by *S. cerevisiae* under SHF condition since it was the only microorganism existing in the fermentation system. The total aromatic alcohols produced by *S. cerevisiae* HJ01 were significantly higher than that of *S. cerevisiae* BY4743 under both SHF and NHF conditions (42.9% and 26.4%, respectively), which indicated *S. cerevisiae* HJ01 had a higher capacity for aromatic alcohols production than *S. cerevisiae* BY4743. However, there were no statistical differences in the individual and total aromatic alcohol concentration per 1% ethanol content (v/v) between SHF and NHF conditions for both *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743. The concentration of aromatic alcohols were highly positively correlated with the ethanol concentration, as aromatic alcohols were the by-products of ethanol fermentation (Ma, Huang, Du, Tang, & Xiao, 2017). These results showed that the aromatic alcohols in *huangjiu* were predominantly

Table 1
Fermentation end results for *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743 under simulated *huangjiu* fermentation (SHF)[1] and normal *huangjiu* fermentation (NHF)[2] conditions.

Concentration	*S. cerevisiae* HJ01		*S. cerevisiae* BY4743	
	SHF	NHF	SHF	NHF
Residual Reducing Sugar (g/L)	43.60 ± 5.47	1.11 ± 0.04	48.84 ± 2.70	1.06 ± 0.09
Amino Nitrogen (g/L)	0.16 ± 0.00	0.67 ± 0.01	0.16 ± 0.00	0.65 ± 0.02
Ethanol (%v/v)	10.20 ± 0.24	17.4 ± 0.16	8.10 ± 0.22	15.6 ± 0.46
β-phenylethanol/Ethanol (mg/L/1%)	5.14 ± 0.22	5.04 ± 0.08	4.25 ± 0.24	4.18 ± 0.11
Tyrosol/Ethanol (mg/L/1%)	3.82 ± 0.19	3.88 ± 0.06	3.69 ± 0.07	3.72 ± 0.14
Tryptanol/Ethanol (μg/L/1%)	108.30 ± 5.37	107.8 ± 1.96	60.12 ± 3.02	58.50 ± 3.56
Total Aromatic Alcohols/Ethanol (mg/L/1%)	9.07 ± 0.34	9.03 ± 0.14	7.99 ± 0.22	7.96 ± 0.26
Total Aromatic Alcohols (mg/L)	92.52 ± 3.51	157.10 ± 2.46	64.74 ± 1.77	124.25 ± 4.00

1. Simulated *huangjiu* fermentation, yeast was the only microorganisms inside the fermentation system; 2. Normal *huangjiu* fermentation, the traditional *huangjiu* fermentation condition.

produced by *S. cerevisiae*, instead of other microorganisms present in the fermentation mash (i.e., fungi and bacterial).

The changes of aromatic alcohols and aromatic amino acids concentrations for both *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743 under SHF condition were shown in Fig. 2a–f. The aromatic alcohols were primarily synthesized during the early stage of fermentation, as there was a very limited increase in the concentration of aromatic alcohols during late stage of fermentation. During the first 72 h, the concentration of L-phenylalanine (L-Phe), L-tyrosine (L-Tyr) and L-tryptophan (L-Trp) decreased dramatically for both *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743. After 72 h, the concentration of L-Phe and L-Tyr fluctuated between 1 mg/L to 4 mg/L, and the concentration of L-Trp fluctuated between 5 mg/L to 7 mg/L. Studies showed that the shikimate pathway would be severely inhibited when a certain amount of L-Phe ($K_i = 75$ μM to Aro3p) or L-Tyr ($K_i = 75$ μM to Aro4p) existed (Gonzalez & Morales, 2017). Thus, we proposed that the biosynthesis of aromatic alcohols would be chiefly dependent on the degradation of extracellular aromatic amino acids by the Ehrlich pathway in the first 72 h.

3.2. Effect of mutations in the promoter regions on the expression level of genes related to the biosynthesis of aromatic alcohols

Promoter is a special nucleotide sequence which located at the upstream of a gene, and could affect the transcription level of the gene beyond the promoter (Danino, Even, Ideses, & Juven-Gershon, 2015). Compared to model strain *S. cerevisiae* BY4743, twenty-five genes' promoter regions (Fig. 2g) in *S. cerevisiae* HJ01 mutated among those thirty-one genes related to the biosynthesis of aromatic alcohols at the nucleotide level. The strength of the twenty-five promoters from *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743 were detected by measuring the fluorescence intensity of *eGFP* (Fig. 2g) from eGFP-expression vector (Table S1).

For *S. cerevisiae* HJ01, the promoter strength of genes related to the shikimate pathway was not remarkably different from that of *S. cerevisiae* BY4743 (*ARO4*, *TRP1* and *TRP5*). The promoters' strength of *ARO1*, *ARO7* and *TRP4* decreased to some extent while the promoters' strength of *ARO2* and *ARO3* was improved. Since Aro3p and Aro4p were involved in the first step of the shikimate pathway, the expression level of *ARO3/ARO4* would affect the metabolic flux of the entire shikimate pathway, while the activity of Aro3p or Aro4p was severely feedback inhibited by L-Phe or L-Tyr (Gonzalez & Morales, 2017).

The promoter strength of genes related to the Ehrlich pathway for *S. cerevisiae* HJ01, such as amino acid transporter genes (*GAP1* and *AGP1*), transaminase-related genes (*ARO10*, *BAT1* and *BAT2*), pyruvate decarboxylase genes (*PDC1* and *PDC5*) and alcohol dehydrogenase genes (*ADH5* and *ADH6*), were considerably higher than that of *S. cerevisiae* BY4743. However, the promoter strength of the amino acid transaminase (*ARO9*) for *S. cerevisiae* HJ01 was lower than that of *S. cerevisiae* BY4743. The increase in the promoters' strength of *BAT1*, *BAT2*, *GAP1*, *AGP1*, *PDC1*, and *PDC5* genes may explain the increased concentration of aromatic alcohols in *huangjiu* produced by *S. cerevisiae* HJ01 comparing with *S. cerevisiae* BY4743.

Although the promoter strength analysis showed a stronger Ehrlich pathway activity in *S. cerevisiae* HJ01, the key genes participating in aromatic alcohol production were still unclear, especially there were a bunch of alleles (Fig. 1). To investigate which genes were involved in the aromatic alcohol biosynthesis in *S. cerevisiae*, the expression profiles of related genes were analyzed by qPCR.

3.3. The key genes related to the biosynthesis of aromatic alcohols in S. cerevisiae

The expression levels of thirty-one related genes (Fig. 1) of the aromatic alcohol biosynthetic pathway were determined by qPCR for both *S. cerevisiae* BY4743 and *S. cerevisiae* HJ01 during the early stage of fermentation (Fig. 3). The gene expression levels were compared in two dimensions, cross-gene and cross-time. In the cross-gene comparison, the transcript level of a gene was compared with other genes at the same fermentation time for one strain, while in the cross-time comparison, the mRNA content of a gene for one strain was compared with its expression during the early stage of fermentation. Comparison of gene expression across genes was used to infer the link between elevated expression and biosynthesis of aromatic alcohol. The cross-gene comparison revealed that five genes related to the Ehrlich pathway were highly expressed during the early stage of fermentation, while expression levels of other genes were relatively low.

In the case of *S. cerevisiae* BY4743, the expression of $AGP1^{BY}$ (permease), $BAT1^{BY}$ (transaminase), $PDC1^{BY}$, $PDC5^{BY}$ (pyruvate decarboxylase), and $ADH1^{BY}$ (alcohol dehydrogenase) were significantly higher than that of other genes, which indicated that these five genes should be the key genes related to the aromatic alcohol biosynthesis for *S. cerevisiae* BY4743. The genes' expression pattern for *S. cerevisiae* HJ01 was similar compared with *S. cerevisiae* BY4743, however, there were differences in the key genes involved in the Ehrlich pathway. For permease and transaminase-related genes, the transcription level of $GAP1^{HJ01}$ and $BAT2^{HJ01}$ was more pronounced than that of other genes, which indicated that $GAP1^{HJ01}$ and $BAT2^{HJ01}$ should be the key genes for permease and transaminase. $PDC1^{HJ01}$, $PDC5^{HJ01}$ and $ADH1^{HJ01}$ were also key genes involved in the aromatic alcohol biosynthesis for *S. cerevisiae* HJ01.

Comparison of key genes expression across time suggested that permease and transaminase were mainly expressed in the first 72 h, while pyruvate decarboxylase and alcohol dehydrogenase were expressed during the entire process of the early stage of fermentation. Since the expression of permease (followed by transaminase) was induced by extracellular amino acids (Chiva et al., 2009), the expression levels of permease and transaminase-related genes would be highly associated with the extracellular amino acids concentration. Surprisingly, the expression level of $PDC1^{BY}$, $PDC5^{BY}$ and $ADH1^{BY}$ increased with the fermentation time going by, while the expression level of $PDC1^{HJ01}$, $PDC5^{HJ01}$ and $ADH1^{HJ01}$ decreased after 48 h of fermentation.

Both promoter strength and two-dimensional gene expression analysis suggested that aromatic alcohols biosynthesis by *S. cerevisiae* was chiefly based on the Ehrlich pathway. The concentration of total aromatic alcohols produced by *S. cerevisiae* HJ01 was higher than *S. cerevisiae* BY4743, which could be explained by the differences in the key genes between strains. *S. cerevisiae* HJ01 had a higher consumption rate of L-Phe, L-Tyr and L-Trp compared with *S. cerevisiae* BY4743 (Fig. 2a–f), which might be due to the difference in the permease between *S. cerevisiae* BY4743 and *S. cerevisiae* HJ01 (i.e., $AGP1^{BY}$ and $GAP1^{HJ01}$) (Sáenz, Chianelli, & Stella, 2014). Amino acids were then converted to the corresponding α-keto acids via transamination, which was catalyzed by branched-chain amino acid transferase encoded by *BAT1* and *BAT2* genes. Studies showed that the transamination was the rate-limiting step in the Ehrlich pathway, and Bat2p would be more efficient than Bat1p (Zhang et al., 2015), which explained the difference in the aromatic alcohols production between *S. cerevisiae* BY4743 and *S. cerevisiae* HJ01. *PDC1* and *PDC5* were both highly expressed by *S. cerevisiae* BY4743 and *S. cerevisiae* HJ01, which convert α-keto acid into aldehyde. Previous studies showed that *ADH1-6* and *SFA1* encoded a bifunctional enzyme with the activities of both alcohol dehydrogenase and formaldehyde dehydrogenase (Olga, Preez, & Jacobus, 2012). *ADH1* was highly expressed during the early stage of fermentation, which may be important to both aromatic alcohols biosynthesis and alcohol degradation at the same time.

3.4. Effect of acid protease on aromatic alcohol production of huangjiu

Since aromatic alcohols were synthesized through the Ehrlich pathway, the availability of free aromatic amino acids would be a keystone for elevating aromatic alcohol concentration. Previous studies showed that the crude protein accounted for 44.6% of the dry sake lees

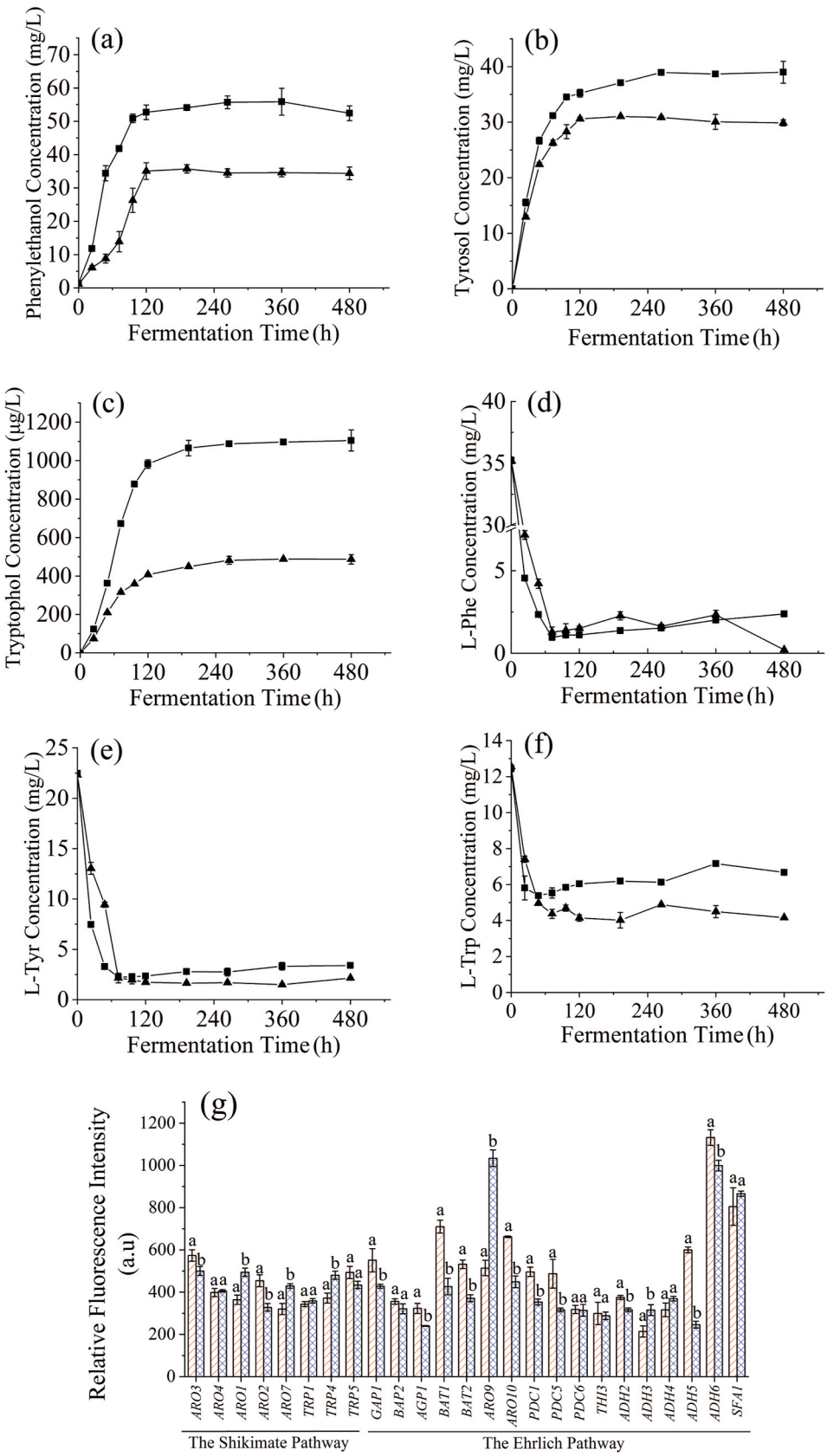

Fig. 2. Concentration changes of aromatic alcohols and aromatic amino acids during fermentation by *S. cerevisiae* HJ01 and *S. cerevisiae* BY4743 under SHF condition. Square represent concentration change of *S. cerevisiae* HJ01, while triangle represent concentration change of *S. cerevisiae* BY4743. (a), Change of β-phenylethanol concentration during *huangjiu* fermentation. (b), Change of tyrosol concentration during *huangjiu* fermentation. (c), Change of tryptanol concentration during *huangjiu* fermentation. (d), Change of L-Phe concentration during *huangjiu* fermentation. (e), Change of L-Tyr concentration during *huangjiu* fermentation. (f), Change of L-Trp concentration during *huangjiu* fermentation. Error bar represent standard deviations from the means of three biological replicates. (g), Comparison of the relative promoter strength between *S. cerevisiae* HJ01 (bar with orange slash) and *S. cerevisiae* BY4743 (bar with blue cross wire) by relative fluorescence intensity. Error bars represent the statistical variations between three biological replicates. Different letters of the same gene indicate significant differences ($P < 0.05$).

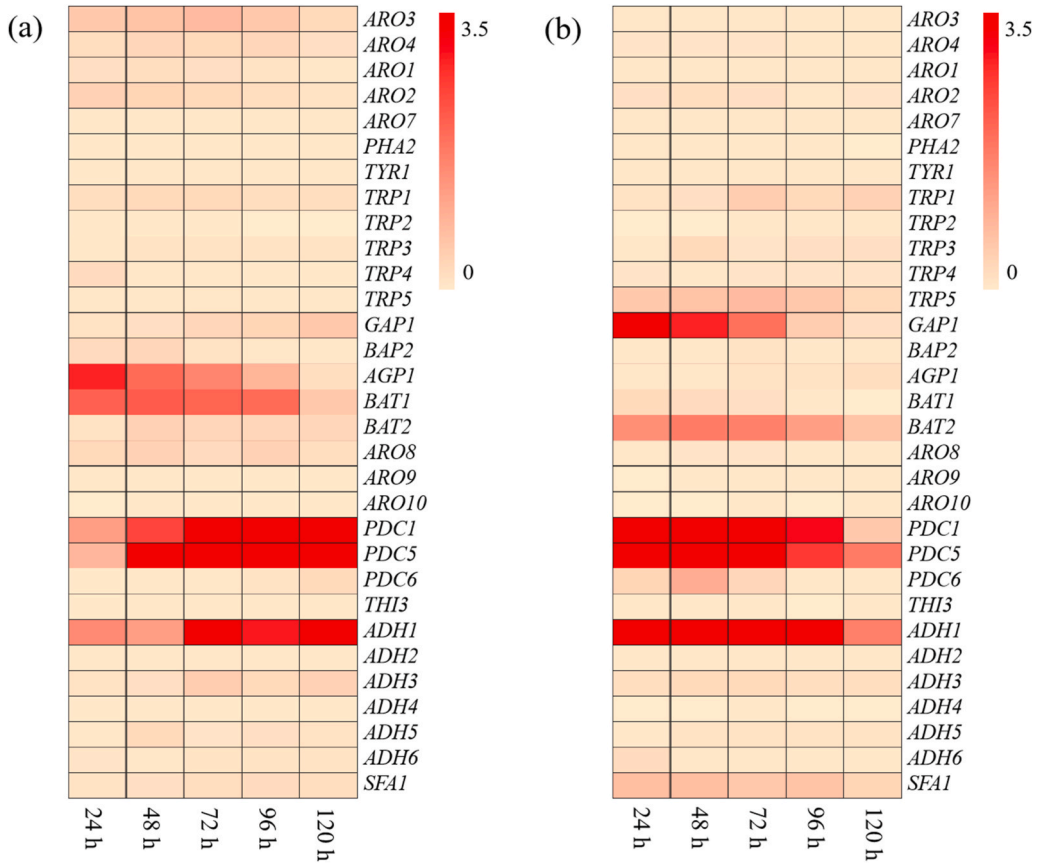

Fig. 3. The expression levels of 31 genes related to the Ehrlich pathway and the shikimate pathway for (a) *S. cerevisiae* BY4743 and (b) *S. cerevisiae* HJ01 under simulated *huangjiu* fermentation condition for 120 h post inoculation. The expression levels of genes were normalized based on two housekeeping genes (*ACT1* and *TDH3* gene). Gene names were listed on the right of the heat maps, and fermentation time were shown at the bottom.

obtained by brewing from liquefied rice (Tsutsui, Yamamoto, & Iwami, 1998), which suggested that amino acids present in the raw material had not been fully consumed by the yeast. Recent study showed that alkaline proteinases were added to accelerate *Jiuzao* protein hydrolysates, a waste product from *baijiu* production, to optimize the production of *Jiuzao* tetrapeptide Asp-Arg-Glu-Leu *in vivo* (Jiang et al., 2021). Since the concentration of aromatic alcohols in *huangjiu* was highly depended on the availability of extracellular aromatic amino acids (Section 3.1), acid protease was added to the fermentation mash at the time of inoculation to increase the content of free aromatic amino acids under NHF condition (Table 2). With the concentration of acid protease increasing, the concentration of total free aromatic amino acids and aromatic alcohols present in *huangjiu* augmented accordingly. Under conditions of 20 U/g of acid protease addition, the concentration of β-phenylethanol, tyrosol and tryptophol increased by 23.9%, 44.8% and 154.7% compared with the control group, respectively. When the concentration of acid protease was greater than 20 U/g, there were no significant further increase in the concentration of aromatic alcohols.

To study the effect of acid protease addition on the expression levels of genes involved in the aromatic alcohol biosynthesis pathway, 0 and 20 U/g of acid protease were added under SHF condition. The transcription levels of genes related to the shikimate pathway had no significant difference compared with the control group at 24 h post-inoculation, except the expression of *ARO3*, *ARO4* and *ARO7* decreasing slightly (Fig. 4a). The expression of *ARO3* was suppressed by L-Phe, and the expression of *ARO4* and *ARO7* were suppressed by L-Tyr (Braus, 1991). The addition of acid protease would increase free amino acid content in the fermentation mash (including L-Phe and L-Tyr), which would suppress the expression of *ARO3*, *ARO4* and *ARO7*. While the expression levels of genes related to the Ehrlich pathway (such as *ARO8*, *ARO9*, *BAT1*, *ARO10*, *PDC5*, *ADH1*, *ADH6*, *GAP1* and *BAP2*) were remarkably enhanced compared to the control group. The gene

Table 2
The effect of acid protease addition on the concentration of free aromatic amino acids and aromatic alcohols at 120 h post-inoculation under normal *huangjiu* fermentation condition[1] (mg/L).

	0 U/g	6 U/g	10 U/g	15 U/g	20 U/g	30 U/g
L-Phe	53.29 ± 0.91	53.95 ± 2.95	82.48 ± 0.83	83.03 ± 1.35	99.71 ± 1.20	172.40 ± 2.24
L- Tyr	64.66 ± 2.92	76.94 ± 3.28	109.4 ± 2.09	109.84 ± 2.48	128.27 ± 2.95	192.28 ± 2.58
L-Trp	22.87 ± 1.58	28.89 ± 0.37	33.20 ± 0.86	45.45 ± 0.67	57.20 ± 1.76	82.64 ± 2.69
Total Free Amino Acids	140.82 ± 4.27	159.78 ± 4.03	225.1 ± 2.79	238.32 ± 4.26	285.18 ± 2.39	447.31 ± 6.81
β-phenylethanol	104.01 ± 0.63	105.19 ± 0.61	113.9 ± 2.24	119.58 ± 0.71	128.92 ± 2.26	128.31 ± 1.16
Tyrosol	65.48 ± 0.86	75.40 ± 0.86	86.05 ± 1.35	91.45 ± 0.88	94.83 ± 1.63	104.94 ± 0.78
Tryptanol	3.85 ± 0.05	5.87 ± 0.03	5.95 ± 0.05	7.63 ± 0.15	9.81 ± 0.28	9.58 ± 0.15
Total Aromatic Alcohols	173.35 ± 0.94	186.46 ± 0.98	206.0 ± 3.35	218.66 ± 1.06	233.55 ± 3.39	242.8 ± 1.73

1. Normal *huangjiu* fermentation, the traditional *huangjiu* fermentation condition.

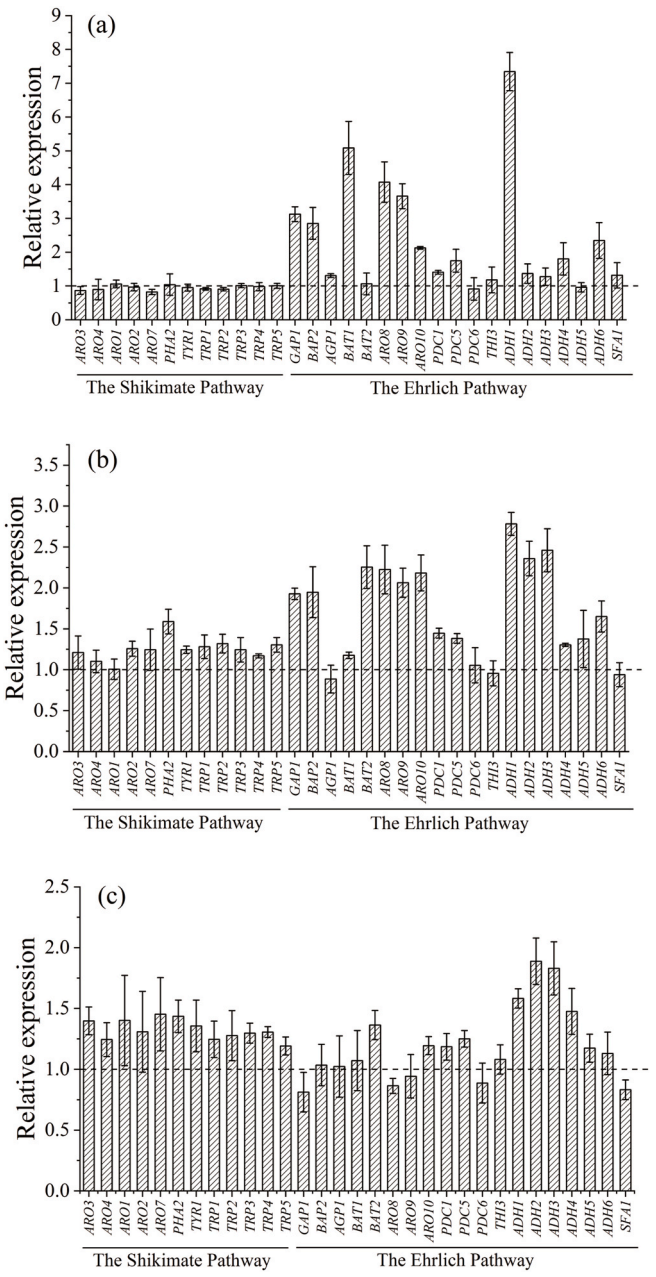

Fig. 4. Effect of adding 20 U/g acidic protease at the time of inoculation on the relative expression levels of genes related to the biosynthesis of aromatic alcohols by *S. cerevisiae* HJ01 under simulated huangjiu fermentation condition. The gene expression level was normalized using the gene expression value of the control group. (a), The relative expression levels of thirty-one genes at 24 h post-inoculation. (b), The relative expression levels of thirty-one genes at 72 h post-inoculation. (c), The relative expression level of thirty-one genes at 120 h post-inoculation. Error bars represent the statistical variations between three biological replicates.

expression profiles showed that *GAP1*^{HJ01}, *BAT2*^{HJ01}, *PDC1*^{HJ01}, *PDC5*^{HJ01} and *PDC6*^{HJ01} were key genes for aromatic alcohol synthesis (Section 3.3), and the Ehrlich pathway was enhanced for aromatic alcohol formation in *huangjiu* after acid protease addition.

At 72 h, the expression level of most genes related to the shikimate pathway was up-regulated to certain extend (10%–50%) compared with the control group, except for *ARO1* (Fig. 4b). For genes related to the Ehrlich pathway, the relative transcription level of *GAP1* and *BAP2* (permeases), *BAT1-2*, and *ARO8-9* (transaminase), *ARO10*, *PDC1*, and *PDC5* (pyruvate decarboxylase) and *ADH1-6* (alcohol dehydrogenase) increased substantially, however, the expression level of these genes was lower than previous at 24 h. The expression level of *AGP1*, *PDC6* and *THI3* were similar or decreased compared with the control group. The expression of *BAT2* was significantly improved compared with that of 24 h, which was similar with previous study showed that *BAT2* was highly expressed during stationary phase and was repressed during exponential growth (while *BAT1* had the opposite expression pattern) (Hazelwood et al., 2008). At 120 h, the expression level of genes related to the shikimate pathway were all up-regulated compared with the control group (Fig. 4c), while the expression level of certain genes related to the Ehrlich pathway was similar to the control group. Only *BAT2* (transaminase), *ARO10*, *PDC1*, *PDC5* and *THI3* (pyruvate decarboxylase), and *ADH1-6* (alcohol dehydrogenase) were up-regulated at 120 h.

The present study suggests that the addition of acid protease at the time of inoculation could elevate the gene expression related to the aromatic alcohol biosynthesis in *huangjiu* fermentation. The transcription level of most genes related to the Ehrlich pathway was up-regulated, especially in the first 72 h, as the addition of acid protease increased the content of free amino acids in the fermentation mash, which in turn suppress the shikimate pathway. The expression level of genes related to the Ehrlich pathway was down-regulated after 120 h, as free aromatic amino acids in the fermentation mash were quite low (Fig. 2a–f), yeast cells need those amino acids for cell viability.

4. Conclusion

The aromatic alcohols in *huangjiu* were primarily produced by *S. cerevisiae* through the Ehrlich pathway at the early stage of *huangjiu* fermentation. The promoter strength of genes related to the Ehrlich pathway from *S. cerevisiae* HJ01 was substantially improved by a base mutation in the regulatory region, except for *ARO9*. The promoter strength of genes related to the shikimate pathway from *S. cerevisiae* HJ01 was not significantly different from that of *S. cerevisiae* BY4743, or the promoter strength was decreased to some extent. The expression levels of thirty-one related genes showed that *AGP1*^{BY} and *GAP1*^{HJ01} (permease), *BAT1*^{BY} and *BAT2*^{HJ01} (transaminase), *PDC1* and *PDC5* (pyruvate decarboxylase) and *ADH1* (alcohol dehydrogenase) are key genes related to the aromatic alcohol biosynthesis. The addition of acid protease could increase the expression levels of certain genes related to the Ehrlich pathway, as the addition of acid protease could increase the content of free aromatic amino acids in the medium, which up-regulated the amino acids catabolic pathway (i.e., the Ehrlich pathway). This study showed that the addition of 20 U/g acid protease could achieve the highest concentration of β-phenylethanol, tyrosol and tryptanol (130.31 mg/L, 111.38 mg/L and 9.78 mg/L, respectively), which were 1.27, 1.56 and 2.52 folds higher than the control group. This provided an economical and simple method to improve aromatic concentration in *huangjiu*, cooking wine and grain vinegar.

CRediT authorship contribution statement

Shuangping Liu: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Validation. **Mei Bai:** Methodology, Investigation, Software, Writing – original draft, Visualization. **Jiabing Zhou:** Methodology, Investigation, Software, Writing – original draft, Visualization. **Zimo Jin:** Investigation, Software. **Yuezheng Xu:** Methodology, Resources. **Qilin Yang:** Methodology, Resources. **Jiandi Zhou:** Methodology, Resources. **Songjing Zhang:** Investigation, Software. **Jian Mao:** Investigation, Software, Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

None.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (32072205, 31701593), the Special Project of Science and Technology Plan of Shaoxing (2019B11001), Lift Engineering for Young Outstanding Scientists of Jiangsu Association for Science, Sichuan Science and Technology Program (21ZDYF4133).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112705.

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