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Heterologous expression and characterization of amine oxidases from *Saccharopolyspora* to reduce biogenic amines in *huangjiu*

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ABSTRACT

High content of biogenic amines in fermented food has adverse effects on the human body. Previous studies showed that biogenic amines could be reduced in some fermented foods using enzymes. However, this process was barely explored in alcoholic beverages. In this study, potential biogenic amine degarding genes were cloned from *Saccharopolyspora hirsuta* F1902 and *Saccharopolyspora hordei* F2002, and expressed in *E. coli* to study the enzymatic activities of copper-containing amine oxidases named as CaoA^{Shi} and CaoA^{Sho} in *huangjiu*. The degradation ratio of total biogenic amines by CaoA^{Shi} (31.72%) was higher than that of CaoA^{Sho} (24.37%). CaoA^{Shi} and CaoA^{Sho} had the highest degradation ratios of histamine, phenylethylamine, followed by tyramine, tryptamine or putrescine, and the smallest was cadaverine. The results indicated that CaoA^{Shi} had better biodegradability of biogenic amines than CaoA^{Sho} in *huangjiu* ($P < 0.05$), and the two enzymes had the same amine substrate characteristics. These results provide new insights for the reduction of biogenic amines using amine oxidases, which is useful not only for *huangjiu* brewing industry, but also for other fermented food industries.

1. Introduction

Biogenic amines (BAs) are low molecular weight nitrogenous organic bases with good thermal stability (Shalaby, 1996; ten Brink et al., 1990), which are frequently reported in a variety of foods, especially some fermented foods, such as cheeses (Herrero-Fresno et al., 2012), alcoholic beverages (Costantini et al., 2019; Hernandez-Orte et al., 2008; Zhong et al., 2012), and fermented sausages (Sun et al., 2016). Low dose intake of BAs is safe for the human body, while excessive intake may lead to a series of adverse physiological reactions or diseases, such as headache, respiratory disorders, and palpitations (Lorenzo et al., 2017; Niu et al., 2019; Santos, 1996; ten Brink et al., 1990). Therefore, considering their adverse effects, it is necessary to find appropriate strategies to prevent the accumulation of BAs in food.

The excessive presence of BAs in fermented food is generally considered to be the result of the introduction of substrate materials (i.e., excessive production of precursor amino acids), and the control of microbial malolactic fermentation, because of spontaneous and open fermentation (Lorenzo et al., 2017). Studies have reported a variety of methods to control the concentration of BAs in food, mainly by reducing the formation of BAs in the process of producing food (Guo et al., 2015; Liu et al., 2021; Xia et al., 2018), optimizing storage conditions of food (Palomino-Vasco et al., 2019), screening special microorganisms and enzymes degrading BAs (Callejon et al., 2014; Garcia-Ruiz et al., 2011) and so on.

Huangjiu, as a traditional fermented alcoholic beverage, is rich in a variety of amino acids and other functional components, which has been favored by consumers for millennia (Liu et al., 2016). Wheat *qu*, the major fermentation starter used in *huangjiu* brewing process, can provide abundant microorganisms and enzymes for *huangjiu* fermentation.

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Recent studies have reported the microbial community structure of wheat *qu* and fermentation mash in Shaoxing region, which suggested that *Saccharopolyspora* was the dominant genus in wheat *qu* and fermentation mash during the whole pre-fermentation process of *huangjiu* (Xie et al., 2013). Several *Saccharopolyspora* strains (Sun et al., 2021) have been reported to have the potential ability to degrade BAs in the fermentation process of *huangjiu*, while their reducing mechanism was not well understood.

The content of BAs in different types of *huangjiu* has been reported many times, with the content of 29.3–260 mg/L, which is higher than that of other alcoholic beverages (Lu et al., 2007; Zhang et al., 2013; Zhong et al., 2012). BAs can be produced at any stage of *huangjiu* brewing, especially during rice soaking and pre-fermentation (Xia et al., 2018; Zhang et al., 2013). It is well known that BAs are heat resistant. In other words, once these BAs are produced, they are difficult to be destroyed by subsequent processing (pasteurization, cooking, filtration, etc.) (Ruiz-Capillas & Herrero, 2019). At present, a variety of strategies for reducing BAs in *huangjiu* have been reported, such as knocking out the gene of *Saccharomyces cerevisiae* to reduce the production of BAs (Guo et al., 2015), screening lactic acid bacteria that degrade BAs (Niu et al., 2019) or not producing BAs (Liu et al., 2016), controlling recycling of seriflux inoculated with lactic acid bacteria to reduce the formation of BAs (Liu et al., 2021), and developing mixed starters to regulate the formation of BAs (Xia et al., 2018). However, due to the conditions that are not easy to change in *huangjiu* fermentation process, especially the microbial/chemical/physical conditions, current reduction strategies in alcoholic beverages are limited or failed in *huangjiu* (Sun et al., 2020). Therefore, it is necessary to further explore new and effective methods to reduce BAs in *huangjiu*.

Amine oxidases (AOs), a class of enzymes that catalyze the oxidative deamination of various small molecular amines to corresponding aldehydes, hydrogen peroxide and ammonia, can be produced by a variety of microorganisms (Foster et al., 2012; Sekiguchi et al., 2004). Although different authors have found that some microorganisms can degrade amines in several fermented foods, they all believe that abilities to degrade BAs are due to actions of AOs (Herrero-Fresno et al., 2012; Niu et al., 2019; Xiong et al., 2020). AOs from different strains may have different catalytic efficiency, reaction conditions and amine substrate specificity (Foster et al., 2012; Roh et al., 1994; Sekiguchi et al., 2004; Yamashita et al., 1993). The strategy of using AO to degrade BAs has been used in some foods (Callejon et al., 2014), but has not been tried in *huangjiu*. Therefore, it is necessary to discuss the use of AO to eliminate BAs in *huangjiu*.

In this study, potential AO genes from *Saccharopolyspora* were cloned and expressed in *Escherichia coli* (*E. coli*) to study the mechanism of degrading BAs. The catalytic efficiency and biodegradation ability of AOs in *huangjiu* were optimized. This study makes up the blank of AOs from *Saccharopolyspora* reducing BAs and provides a theoretical basis for the use of *Saccharopolyspora* and its enzymes to control BAs in fermented foods.

2. Materials and methods

2.1. Materials

Saccharopolyspora hirsuta F1902 (*S. hirsuta* F1902) (the accession number, OP218498) and *Saccharopolyspora hordei* F2002 (*S. hordei* F2002) (the accession number, OP218503) strains were screened from 41 strains of *Saccharopolyspora* (Table S1) isolated from wheat *qu* and *huangjiu* fermentation mash (Shaoxing, Zhejiang). The strains were cultured for 2 days at 37 °C in a medium (1 g/L K_3NO_3, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.01 g/L $FeSO_4$, 0.5 g/L NaCl, and 20 g/L soluble starch). The relevant characteristics of *E. coli* JM109, *E. coli* BL21 (DE3) and plasmids used in this study were summarized in Table S2. Semi-dry and semi-sweet *huangjiu*, with total sugar contents (20 g/L and 45 g/L) and alcohol contents (14%, v/v), were from Shaoxing, China. Restriction enzymes (*Ned*I and *Eco*RI), DNA Marker, other genetic engineering-related enzymes and reagents were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). BA standards and other chemical reagents were provided by Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

2.2. Screening and expression of genes degrading BAs

2.2.1. Screening of potential genes reducing BAs in Saccharopolyspora

Based on the NCBI database (https://www.ncbi.nlm.nih.gov/), potential genes related to BA degradation were screened and obtained (Table S3). Furthermore, ten gene sequences were compared with known templates in the PBD database (https://www.rcsb.org/), and only templates with similarity higher than 30% were considered for further research. SWISS-MODEL (https://swissmodel.expasy.org/interactive) was used to build three-dimensional models of protein. Modeling results were shown in Table S4.

2.2.2. Cloning of AO genes

Standard methods were used for PCR amplification, plasmid construction, analysis of DNA fragments and electroporation (Green & Sambrook, 2001). Primers (Table S5) were designed using Primer Premier 6.0 software (Premier Biosoft, CA) and synthesized by Songong Biotech Co., Ltd. (Shanghai, China). Two fragments about 1800 bp from was *S. hirsuta* and *S. hordei* were amplified by primer SHI-5 and SHO-1 respectively. No sequence was amplified by other primers. The obtained PCR products were validated by gel electrophoresis and the sequences were compared using DNAMAN software with their corresponding amino acid sequences of PTX67440.1, WP_150069050.1, WP_179723446.1 (Figs. S1 and S2), and sequence similarities reached 88.7%, 95.92% and 99.84%, respectively.

2.2.3. Construction of recombinant plasmids

According to two gene fragments obtained above, primers F1902-R, F1902–F, F2002-R and F2002–F (Table S6) were used to amplified amine oxidase genes (*caoA*) from *S. hirsuta* and *S. hordei*. After digestion by *Nde*I and *Eco*RI, the PCR products were ligated with plasmid pET-28a (+) to construct recombinant plasmids of *S. hirsuta* (pET*caoA*^{Shi}) and *S. hordei* (pET*caoA*^{Sho}). These two recombinant plasmids were introduced into *E. coli* BL21(DE3), and then cultured on Luria–Bertani solid media (supplemented by kanamycin (50 mg/L)) at 37 °C for 12 h. They were verified by restriction enzymes *Ned*I and *Eco*RI, and sequence confirmation by Songong Biotech Co., Ltd. (Shanghai, China). Recombinant strains were preserved at −80 °C by glycerol preservation method until use.

2.2.4. Induced expression and purification of AOs

Recombinant strains were inoculated into LB liquid medium and cultured at 37 °C 170 rpm for 12 h. The above seed liquid was transformed into TB medium (peptone 12 g/L, yeast powder 24 g/L, disodium hydrogen phosphate 9.4 g/L, potassium dihydrogen phosphate 2.2 g/L, kanamycin 50 mg/L) with an inoculation amount of 2% (v/v) and cultured at 37 °C, 170 rpm until OD_{600} was 0.4. Then 1 mM isopropyl-beta-D-thiogalactoside (IPTG) was added to induce expression of AOs at 25 °C, 170 rpm for 12 h. The cells were obtained according to this reference (Liu et al., 2014), and then adjusted to 0.6 for OD_{600nm} value with 0.2 M PBS (pH 7.5). The culture was immediately lysed by sonication and centrifuged for 30 min at 16,000×*g* to obtain clarified cell extract. These crude enzyme solutions were further purified using an AKTA avant 25 instrument equipped with a HisTrap™ HP affinity column (17-5247-01, GE Healthcare Biosciences, Sweden), which were eluted using a linear imidazole gradient. SDS-PAGE was verified the production of AOs using Mini-PROTEAN® TETRA System (BioRad, USA). Protein concentrations were determined by the Bradford method with bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.3. Determination of AO activities

Activities of AOs were performed using SpectraMax 190 Microplate Reader (MD Electronics, USA) by colorimetric analyses (Foster et al., 2012; Moshides, 1988). The reaction was performed in 96 well plates containing 10 μL enzyme solution and 100 μL freshly prepared assay solution (200 mM PBS, pH 7.6, 1.5 mM 4-aminoantipyrine and 1 mM 2, 4,6-tribromo-3-hydroxybenzoic acid). In addition, 20 μL amine substrate solution and 70 μL 1.4 mg/mL peroxidase from horseradish were added to give a final volume of 200 μL (Foster et al., 2012). Absorbance was measured at 510 nm after incubation at 37 °C for 10 min, and the control was the initial reaction system. An enzyme activity unit (U) is defined as the amount of the enzyme required to produce 1 μmol H_2O_2 per minute. The specific activity of an enzyme (U/mg) is defined as the number of units of enzyme activity per milligram of protein.

2.4. Optimization of induction conditions of AOs

To obtain high concentration of AO protein expression, the induction temperature, induction time and IPTG addition were optimized. Firstly, when other conditions remained unchanged, induction temperatures were respectively set at 15, 20, 25, 30, 35 and 40 °C, and AO activities were compared after 12 h. Similarly, based on the optimal temperature, AO activities were measured after 12 h with different IPTG concentrations, which were set at 0.5, 1, 1.5, 2, 2.5 and 3 mM, respectively. In addition, when the induction time was respectively set at 4, 8, 12, 16, 20 and 24 h, AO activities were compared under the optimal temperature and IPTG concentration.

2.5. Application of AOs in huangjiu

Purified enzymes were applied to degrade BAs in different types of *huangjiu*. The application conditions, including enzyme amount (0, 100, 200, 300, 400, 500 and 600 mg/L), catalytic temperatures (20, 25, 30, 35, 40, 45 and 50 °C) and catalytic times (4, 8, 12, 16, 20, 24 and 28 h), were set. When different addition amounts were discussed, other catalytic conditions were set at 25 °C for 12 h. Based on the optimal enzyme addition conditions, the effects of catalytic temperature and time on BA content were discussed. Detection of the BA contents was carried out according to our previously described method (Liu et al., 2021). The degradation ratio of BAs is defined as the percentage of the reduction of BAs after optimization to the initial content of BAs (no enzyme was added) in *huangjiu*.

2.6. Statistical analysis

All experiments were performed in triplicate. Data was processed and analyzed by using statistical software Excel 2016 and Origin Pro 8.0 software. The results analyzed were expressed as mean ± standard deviation (SD), and significance of variance was tested by ANOVA at $P < 0.05$. Heat maps were made using the open online MORPHEUS software (https://software.broadinstitute.org/morpheus/), and Pearson correlation was carried out to determine relationships. The docking of enzymes with various BAs was completed by using AutoDockTools 1.5.6 software.

3. Results

3.1. Sequence characteristics and structure prediction of target proteins

The results of AO models predicted by SWISS-MODEL were shown in Table S4. The prototypes of protein sequence modeling of two items were both phenylethylamine oxidases from *Arthrobacter globiformis* (*A. globiformis*), which are homodimers containing two copper ions. The sequence identity of copper containing AO from *S. hirsute* ($CaoA^{Shi}$) to 2yx9.1.A (phenylethylamine oxidase from *A. globiformis*) (Moore et al., 2007) is 56.46%. The homodimer structure of $CaoA^{Shi}$ is shown in Fig. 1A. Each homodimer contains two active-sites, each of which contains a copper (II) ion and three amino acid residues (His430, His432 and His591) bound to a copper (II) ion (Fig. 1B). The sequence identity of copper containing AO from *S. hordei* ($CaoA^{Sho}$) to 2cwt.1.A (phenylethylamine oxidase from *A. globiformis*) (Chiu et al., 2006) is 55.52%. The homodimer structure of $CaoA^{Sho}$ is shown in Fig. 1C. Each homodimer also contains two active-sites, each of which contains a copper (II) ion and four amino acid residues (His426, His428, His587 and Tyr377) bound to a copper (II) ion (Fig. 1D). The OH^- of Tyr377 was close to metal complexes composed of copper ions and three histidine residues, which could be due to the stable ligand formed by OH^- and metal complexes.

3.2. Expression and purification of AOs

To verify the successful expression of recombinant AOs in *E. coli* BL21 (DE3) strains, recombinant plasmids were extracted and digested by *Nde*I and *Eco*RI (Fig. 2A). Obviously, about 5300 bp band (from pET-28a (+)) and 1800 bp band (*caoA* gene) were obtained by restriction enzyme double digestion, respectively. These results showed that pET-*caoA*^{Shi} and pET*caoA*^{Sho} were successfully built in *E. coli* BL21 (DE3), respectively.

Crude enzyme solutions of *E. coli* BL21 (DE3) containing pET*caoA* were purified with Ni-affinity chromatography and analyzed by SDS-PAGE taking unstained protein ladder as the standard (Fig. 2B). The molecular weights of $CaoA^{Shi}$ and $CaoA^{Sho}$ purified were about 80 kDa which were consistent with the predicted results of amino acid sequences.

3.3. Specific activities of AOs under different amine substrates

To explore the substrate specificity of AO, eight kinds of 10 mM amine substrate solutions including tryptamine (TRY), phenylethylamine (PHE), cadaverine (CAD), putrescine (PUT), histamine (HIS), tyramine (TYR), spermine (SPE) and spermidine (SPD) were separately added to enzyme reaction systems to incubate 10 min at 37 °C (Table 1). The specific activities of $CaoA^{Shi}$ under different amine substrates were HIS, PHE, TYR, PUT, TRY, and CAD in descending order, while those of $CaoA^{Sho}$ were PHE, HIS, TYR, PUT, TRY, and CAD. The results suggested that two enzymes had good affinity to HIS, PHE and TYR, followed by PUT, TRY and CAD, while there no specificities to SPE and SPD.

The degradation ratios of eight amine substrates were also analyzed after incubation to identify degradation abilities of these two enzymes to different amine substrates (Table 1). Concretely, $CaoA^{Shi}$ had the most significant degradation ability to PHE, HIS and TYR, with degradation ratios of 72.14%, 68.25% and 54.58% respectively, followed by TRY, PUT, and CAD. Similarly, degradation abilities of $CaoA^{Sho}$ to PHE and HIS were also the most significant, and degradation ratios were 57.27% and 61.15% respectively, followed by TRY, TYR, and PUT. These results suggested that PHE, HIS and TYR could be considered as their specific substrates, which was consistent with the results of specific activity.

Docking two enzymes with BA molecules based on binding energy using AutoDockTools 1.5.6 software. The binding ability of $CaoA^{Shi}$ with several BAs was SPE (−11.1) > SPD (−9.2) > TRY (−8.19) > CAD (−7.65) > PUT (−7.63) > PHE (−6.45) > TYR (−5.93) > HIS (−5.36), while that of $CaoA^{Sho}$ with several BAs was SPE (−11.44) > SPD (−9.93) > PUT (−9.38) > CAD (−9.08) > TRY (−8.76) > HIS (−7.01) > TYR (−6.54) > PHE (−6.46). These results suggested that the two enzymes might have certain degradation effects on several BAs, and the degradation ability might be different.

3.4. Optimization of induction conditions of AOs

To improve expressions and enzyme activities of target proteins, induction temperatures, IPTG concentrations and induction time were

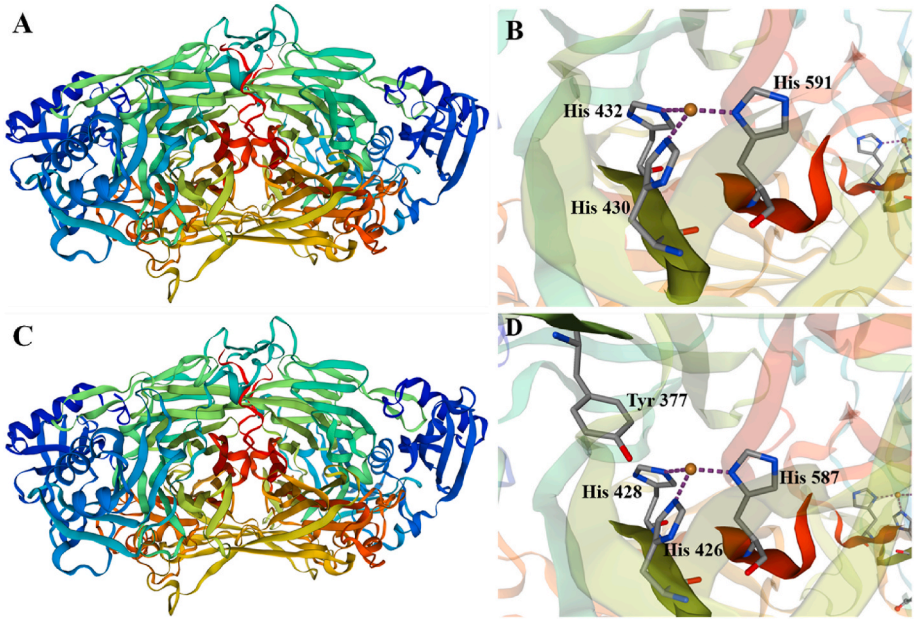

Fig. 1. Predicted model structures of CaoA^{Shi} and CaoA^{Sho} simulated by SWISS-MODEL. A and C are homodimers of CaoA^{Shi} and CaoA^{Sho}, respectively. B and D are the active sites of CaoA^{Shi} and CaoA^{Sho}, respectively.

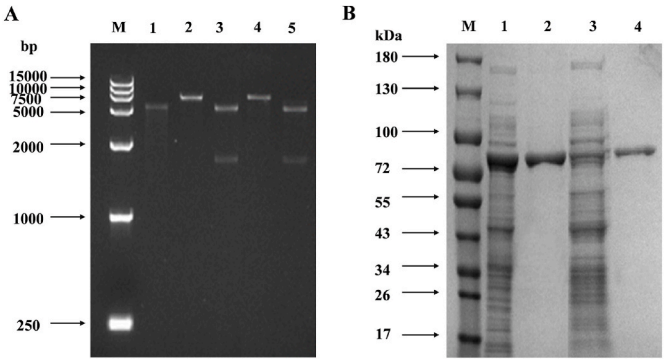

Fig. 2. Verification of recombinant plasmids by restriction enzyme digestion (A) and SDS-PAGE of purified recombinant enzyme proteins (B). In (A): M, DNA Maker; 1, pET-28a (+); 2, pET*caoA*^{Shi} digested with *Nde*I; 3, pET*caoA*^{Shi} digested with *Nde*I and *Eco*RI; 4, pET*caoA*^{Sho} digested with *Nde*I; 5, pET*caoA*^{Sho} digested with *Nde*I and *Eco*RI. In (B): M, unstained protein ladder; 1, crude protein of CaoA^{Shi}; 2, purified protein of CaoA^{Shi}; 3, crude protein of CaoA^{Sho}; 4, purified protein of CaoA^{Sho}.

Table 1
Specific activities and degradation ratios of AOs under amine substrates.

Substrate amines	CaoA^{Shi}		CaoA^{Sho}	
	Specific enzyme activity (U/mg)	Degradation ratio (%)	Specific enzyme activity (U/mg)	Degradation ratio (%)
HIS	0.55 ± 0.05^{a}	68.25 ± 6.25^{a}	0.44 ± 0.05^{a}	61.15 ± 4.62^{a}
PHE	0.51 ± 0.04^{a}	72.14 ± 5.79^{a}	0.48 ± 0.06^{a}	57.27 ± 4.64^{a}
TYR	0.47 ± 0.02^{ab}	54.58 ± 5.36^{b}	0.23 ± 0.03^{b}	36.58 ± 3.48^{b}
PUT	0.18 ± 0.01^{c}	27.05 ± 3.12^{c}	0.21 ± 0.02^{b}	24.92 ± 2.23^{c}
TRY	0.16 ± 0.01^{c}	33.28 ± 4.82^{c}	0.20 ± 0.03^{b}	39.04 ± 4.15^{b}
CAD	0.07 ± 0.01^{d}	16.55 ± 2.24^{d}	0.03 ± 0.01^{c}	9.89 ± 1.12^{d}
SPE	ND	ND	ND	ND
SPD	ND	ND	ND	ND

Mean values in the same column with different superscripted letters indicate that they are significantly different at $P < 0.05$. ND represented no enzyme activity was detected.

optimized with PHE, HIS and TYR as amine substrates. Under these induction conditions, the expression of AOs in *E. coli* increased at first and then decreased (Fig. 3). Whether PHE, HIS or TYR were used as substrates, these induction conditions had significant effects on the activities of the two enzymes ($P < 0.05$). As shown in Fig. 3A and B, CaoA^{Shi} and CaoA^{Sho} had the highest activity at 25 °C. As shown in Fig. 3C and D, the activities of CaoA^{Shi} and CaoA^{Sho} were the highest when 1.5 mM IPTG was used. As shown in Fig. 3E and F, the activities of CaoA^{Shi} and CaoA^{Sho} were the largest at 16 h. Although optimal induction conditions of CaoA^{Shi} and CaoA^{Sho} were consistent, these results showed that there were significant differences in their enzyme activity levels under the same induction condition ($P < 0.05$). Under the three optimal induction conditions, no matter which BA was used as the substrate, the maximum activity of CaoA^{Shi} was higher than that of CaoA^{Sho}.

3.5. Application of AOs in huangjiu

The main BAs in *huangjiu* are TRY, PHE, TYR, HIS, PUT and CAD (Zhang et al., 2013; Zhong et al., 2012). SPE and SPD have also been detected, but their contents are very low (Zhang et al., 2013). To reduce contents of BAs in *huangjiu*, taking changes of six main BAs as the index, application conditions of AOs in semi-dry and semi-sweet *huangjiu* were optimized, including enzyme addition, reaction temperature and reaction time (Fig. 4).

As shown in Fig. 4A and B, with the increase of addition amounts of CaoA^{Shi} and CaoA^{Sho} (from 0 to 400 mg/L), total BA contents in *huangjiu* decreased continuously. Above 400 mg/L, the decrease of total amounts of BAs was not significant with the increase of their addition. These results were applicable not only to semi-dry *huangjiu*, but also to semi-sweet *huangjiu*, which showed that the degradation of BAs by these two enzymes was not affected by the type of *huangjiu*. Furthermore, the specificity of CaoA^{Shi} and CaoA^{Sho} to different amine substrates was different (Fig. 4A and B), both CaoA^{Shi} and CaoA^{Sho} had high degradation amounts to PHE, HIS, TYR and PUT, followed by TRY and almost no effect on CAD. These results were consistent with that of protein activities determined for AOs as amine substrates (Table 1). Therefore, 400 mg/L CaoA^{Shi} and CaoA^{Sho} respectively added were further considered for the optimization of biodegradation conditions of BAs in *huangjiu*.

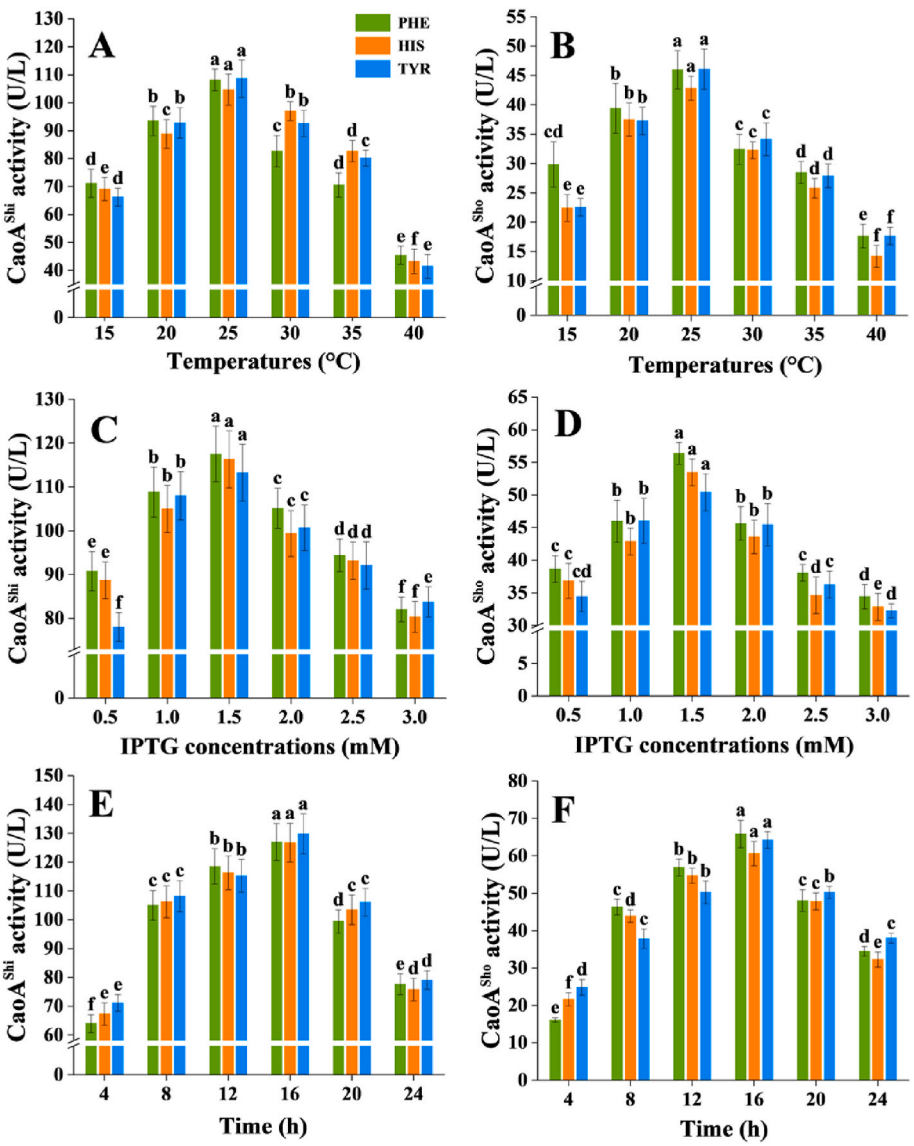

Fig. 3. Expression of CaoA^{shi} and CaoA^{sho} enzymes under different induction conditions. Activities of CaoA^{shi} and CaoA^{sho} were determined under different induction temperatures (A and B), IPTG concentrations (C and D), and induction time (E and F). Mean values in bar-type columns of the same color with different letters indicate that they are significantly different at $P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

As shown in Fig. 4C and D, with the increase of temperature, total BA contents in *huangjiu* decreased first and then increased. Specifically, total amounts of BAs decreased with the increase of temperatures (from 20 to 35 °C) and then remained almost unchanged (from 35 to 40 °C). With the increase of temperature (from 40 to 50 °C), the total amount of BAs increased, but lower than that in *huangjiu* without enzymes. Similarly, comparing Fig. 4C and D, the specificity of CaoA^{Shi} and CaoA^{Sho} for amine substrates at different temperatures was almost the same. CaoA^{Shi} and CaoA^{Sho} had obvious degradation ability on PHE, HIS, TYR and PUT in two kinds of *huangjiu*, followed by TRY, and almost no degradation ability on CAD. These results showed that temperature might not change the specificity of selective substrates for CaoA^{Shi} and CaoA^{Sho}, but only change the efficiency of degrading BAs, except CAD. Thus, the optimum reaction temperature of two enzymes in *huangjiu* was 35 °C, which was used for further condition optimization.

As shown in Fig. 4E and F, with the extension of reaction time, total BA contents in *huangjiu* showed downward trends, especially from 4 h to 20 h. After 20 h, with the extension of reaction time, total BA contents in *huangjiu* did not change significantly, although they also decreased. Similarly, determination results of six kinds of BAs showed that the amine substrate specificity of CaoA^{Shi} and CaoA^{Sho} under different reaction times was consistent. CaoA^{Shi} and CaoA^{Sho} at different reaction times had obvious degradation effects on PHE, HIS, TYR and PUT in *huangjiu*, followed by TRY, and almost no degradation effect on CAD (Fig. 4E and F).

Based on optimal reaction conditions, degradation ratios of six BAs in *huangjiu* were further analyzed (Table 2). As described in Table 2, the degradation ratios of total BA by CaoA^{Shi} in semi-dry *huangjiu* and semi-sweet *huangjiu* had reached 32.24% and 31.19%, respectively. The degradation ratios of total BAs by CaoA^{Sho} in semi-dry *huangjiu* and semi-sweet *huangjiu* had reached 24.40% and 24.34%, respectively. The degradation ratio of total BAs by CaoA^{Shi} (31.72%) is higher than that of total BAs by CaoA^{Sho} (24.37%) in *huangjiu*. Further, comparing the average degradation ratios of six kinds of BAs by the two enzymes, it was found that there were significant differences in the degradation of six BAs by CaoA^{Shi} and CaoA^{Sho}. For CaoA^{Shi}, the average degradation ratio of BAs in *hangjiu* was HIS, PHE and TYR, followed by TRY and PUT, and the smallest was CAD. For CaoA^{Sho}, the average degradation ratio of BAs in *hangjiu* was HIS, PHE and TRY, followed by TYR and PUT, and the smallest was CAD. The average degradation ratio of CaoA^{Shi} for each BAs was higher than that of CaoA^{Shi}.

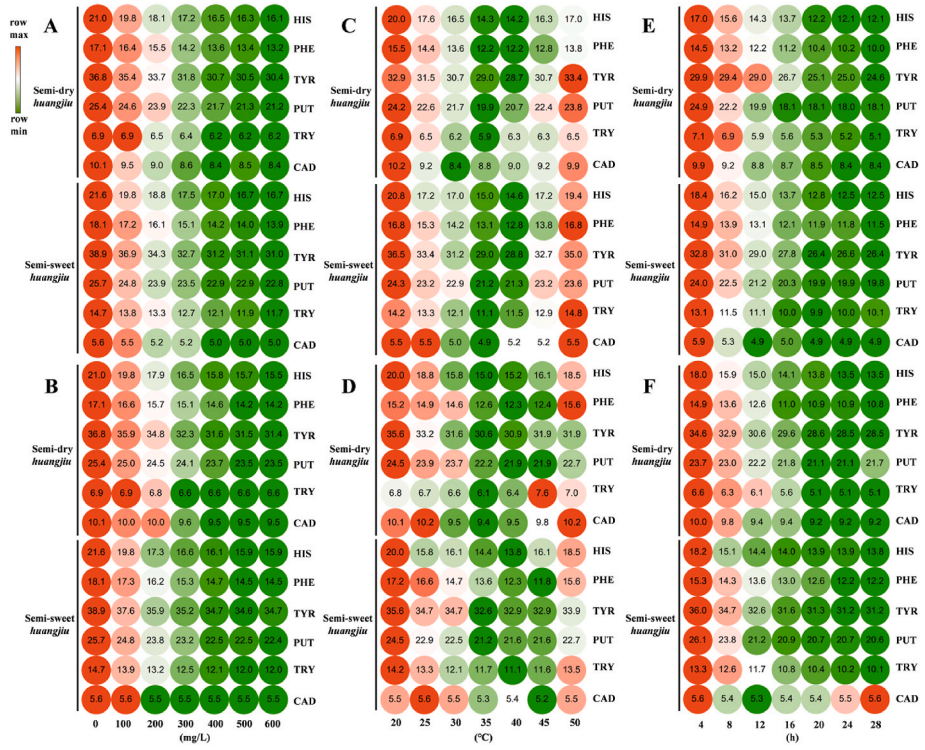

Fig. 4. Effects of enzyme amount, catalytic temperature and time on BA content in *huanjgiu*. 25 °C and 12 h were applied to optimize the addition of CaoA^{Shi} (A) and CaoA^{Sho} (B); the addition of 400 mg/L and 12 h were used to optimize catalytic temperatures of CaoA^{Shi} (C) and CaoA^{Sho} (D); the addition of 400 mg/L and 35 °C were used to optimize catalytic time of CaoA^{Shi} (E) and CaoA^{Sho} (F). The number in each circle means residual concentration (mg/L) of each BA.

Table 2
Comparison of two AOs (CaoA^{shi} and CaoA^{sho}) degrading BAs under optimal conditions.

BAs	Degradation ratio by CaoA^{Shi} (%)			Degradation ratio by CaoA^{Sho} (%)		
	Semi-dry *huangjiu*	Semi-sweet *huangjiu*	Average value	Semi-dry *huangjiu*	Semi-sweet *huangjiu*	Average value
TRY	24.39 ± 1.38^{c}	32.58 ± 2.85^{b}	28.46 ± 2.02^{bc}	26.12 ± 1.52^{b}	29.02 ± 1.87^{ab}	27.57 ± 1.70^{ab}
PHE	39.10 ± 3.81^{ab}	34.16 ± 3.41^{ab}	36.63 ± 3.61^{a}	33.05 ± 3.38^{a}	30.27 ± 3.94^{a}	31.66 ± 3.66^{a}
CAD	15.81 ± 1.16^{d}	12.36 ± 0.64^{d}	14.09 ± 0.91^{e}	8.60 ± 0.28^{d}	3.52 ± 0.36^{d}	6.06 ± 0.32^{d}
PUT	28.94 ± 3.24^{bc}	22.74 ± 2.45^{c}	25.84 ± 2.87^{dc}	16.83 ± 2.04^{c}	19.63 ± 2.18^{c}	18.23 ± 2.11^{c}
HIS	41.69 ± 4.25^{a}	40.87 ± 3.49^{a}	41.28 ± 3.87^{a}	37.11 ± 3.42^{a}	35.64 ± 3.72^{a}	36.38 ± 3.57^{a}
TYR	31.92 ± 3.93^{b}	32.18 ± 3.84^{b}	32.05 ± 3.89^{ab}	22.35 ± 3.69^{b}	19.63 ± 3.73^{c}	20.99 ± 3.71^{c}
Total BAs*	32.24 ± 3.26^{a}	31.19 ± 3.43^{a}	31.72 ± 3.35^{a}	24.40 ± 2.65^{b}	24.34 ± 2.58^{b}	24.37 ± 2.62^{b}

Different superscript letters in the same column indicate significant differences at $P < 0.05$, except for the row with '*'. *Different superscript letters in the same row indicate significant differences at $P < 0.05$.

4. Discussion

Previous studies have isolated and purified AOs from different strains many times (Foster et al., 2012; Roh et al., 1994; Yamashita et al., 1993). Molecular weights of AOs from different sources are about 70–95 kDa (Roh et al., 1994; Sekiguchi et al., 2004; Yamashita et al., 1993). In this paper, protein bands purified by us were about 80 kDa (Fig. 2B), and research results were basically consistent with predicted results. To evaluate characteristics of two kinds of AOs from *S. hirsuta* and *S. hordei*, their specific enzyme activities and substrate selection specificity were compared. The results indicated that AOs from two kinds of *Saccharopolyspora* had different catalytic properties even if expressed in the same *E. coli*, and the degradation effect of CaoA^{Shi} was better than that of CaoA^{Sho} for different amine substrates (Table 1). Interestingly, there were some differences in the selection of eight kinds of amine substrates for CaoA^{Shi} and CaoA^{Sho}. The results of specific activity and substrate specificity suggested that the specific activity of CaoA^{Shi} and CaoA^{Sho} and their abilities to degrade single BA are higher when one of three BAs (PHE, HIS and TYR) was used as substrates. In addition, the results of molecular docking also confirmed the selective specificity of the two enzymes for each BA.

Further, to improve protein expressions of AOs, IPTG concentrations, induction temperatures and time were explored. Results showed that expressions of two enzymes improved significantly ($P < 0.05$) with 1.5 mM IPTG concentrations, induction temperatures at 25 °C, and induction time of 16 h. The activities of CaoA^{Shi} and CaoA^{Sho} under different induction temperatures (Fig. 3A) showed that enzyme activities would be affected under low or high temperatures (Yamashita et al., 1993). It might be that under low induction temperatures, growth ratios of *E. coli*, as protein synthesis ratios, were low, and synthesis protein contents were also low. Under high induction temperatures, growth ratios of cells were faster and expression levels of soluble enzymes decreased. Similarly, results of adding IPTG indicated that the increase of IPTG concentration might lead to the increase of protein synthesis ratio, and further cause the lack of time for folding of many proteins, the increase of inclusion bodies and the decrease of soluble protein content. In addition, when induction time was short, microbial growth was slow and protein expression levels were low, but the longer induction time

might lead to cell aging and affect activities of AOs (Jiang et al., 2007).

It is found that the quantity order of BAs analyzed in wine from the highest to the lowest is as follows: TRY > CAD > TYR > PHE > SPE > SPD > HIS > PUT (Jiang et al., 2007). HIS and TYR are considered to be the most toxic in wine, PUT and CAD may enhance these effects (Niu et al., 2019). The analysis results of fourteen kinds of *huangjiu* from four brewing regions in China have shown that the total amount of BAs was 29.3–260 g/L, and the concentrations of HIS, TYR, CAD and SPE were higher than other BAs (Lu et al., 2007). The changes of BAs during *huangjiu* brewing process have showed that the concentrations of PUT, TYR, HIS and TRY were higher, while the concentrations of SPE and SPD were less (Zhang et al., 2013). And their results of total BA contents are consistent with that of semi-dry or semi-sweet *huangjiu* determined by Zhong et al. (2012). However, the main BAs in *huangjiu* are different according to the detected kinds and concentrations of various BAs, and the latter (Zhong et al., 2012) are PUT, HIS, PHE, CAD, TYR and TRY, which is consistent with our determination of six kinds of BAs in semi-dry or semi-sweet *huangjiu*.

To determine degradation ability of BAs from CaoA^{Shi} and CaoA^{Sho} and apply more appropriately them for industrial *huangjiu*, they were added to commercial *huangjiu*, and reaction conditions were further optimized. The results showed that 400 mg/L enzyme addition, catalytic temperature at 35 °C and catalytic time of 20 h were the optimal reaction conditions for the degradation of BAs by the two enzymes, whether in semi-dry or semi-sweet *huangjiu*. Under the optimal enzyme reaction conditions, the amount of CaoA^{Shi} to reduce BAs was higher than that of CaoA^{Sho} in *huangjiu* (Fig. 4), which indicated that CaoA^{Shi} might be more effective in the degradation of BAs in *huangjiu*, and this result could also be confirmed from the degradation ratio of total BAs (Table 2). In addition, the results of degradation ratios of six BAs by the two enzymes suggested that CaoA^{Shi} and CaoA^{Sho} had the best degradation effect on HIS and PHE, and the degradation effect of TYR, TRY or PUT was second only to HIS and PHE, while the degradation effect of CAD was the worst (Table 2).

Several studies have shown that different AOs have different specificity to amine substrates (Pistekova et al., 2020; Roh et al., 1994). CaoA^{Shi} had the highest degradation ratio of *huangjiu* among the two enzymes, up to 31.72%. The degradation effect was better than that of 25% BAs by eliminating the PEP4 gene in *Saccharomyces cerevisiae* during fermentation of *huangjiu* (Guo et al., 2015). However, it was lower than degradation ratios in smoked horse-meat sausage (degradation ratios of PHE, PUT, HIS and TYR were 54%, 52%, 70% and 40% respectively) (Li et al., 2021), which might be caused by inhibition of external environment, such as pH and ethanol concentration. The optimum pH of copper AO from *Arthrobacter aurescens* was about 7.0 (Lee & Kim, 2013), while the optimum pH of copper AO from *Arthrobacter crystallopoietes* was 9.0 (Sekiguchi et al., 2004). The activity of AO crude extract from lactic acid bacteria was inhibited by 12% alcohol (Garcia-Ruiz et al., 2011). *Huangjiu* is a system with low pH and high alcohol content, which has an obvious impact on the enzyme. Such a system is not conducive to the enzyme reaction. Our research confirmed that the degradation ratio of total BAs by the enzyme reached 31.72% in *huangjiu*, which was a good effect and could significantly reduce the reaction after drinking (Sun et al., 2020). To the best of our knowledge, it was the first report for using enzymatic method to reduce BA contents in *huangjiu*. It might be the most effective method in the future to reduce BA contents in industrial *huangjiu*.

5. Conclusion

To reduce the BAs content in *huangjiu*, AO genes of *S. hirsuta* F1902 and *S. hordei* F2002, which were two main microorganisms of Shaoxing rice wine, were obtained by virtual screening, and successfully expressed in *E. coli* to obtain CaoA^{Shi} and CaoA^{Sho} with amine substrate specificity. The results of molecular docking also confirmed that the two enzymes might have amine substrate specificity. Further, using three BAs (PHE, HIS and TYR) as substrates, the optimal conditions for inducing the expression of AO in *E. coli* were 1.5 mM IPTG concentration, induction temperature at 25 °C and induction time for 16 h. The optimum conditions for the application of CaoA^{Shi} and CaoA^{Sho} in *huangjiu* were as follows: 400 mg/L enzyme addition, catalytic temperature at 35 °C and catalytic time for 20 h, and the degradation effect of CaoA^{Shi} on BAs was better than that of CaoA^{Shi} in *huangjiu*. Under the optimal conditions, the degradation ratio of total BAs by CaoA^{Shi} was up to 31.72%. In addition, CaoA^{Shi} and CaoA^{Sho} had the best degradation effect on HIS and PHE, TYR, TRY or PUT were second only to HIS and PHE, while CAD was the worst. These results provide a new idea for the application of AOs in the degradation of BAs in *huangjiu*.

CRediT authorship contribution statement

Shuangping Liu: Conceptualization, Resources, Funding acquisition, Formal analysis. **Hongli Yao:** Methodology, Writing – original draft. **Mengfei Sun:** Data curation, Formal analysis, Validation. **Zhilei Zhou:** Supervision, Visualization, Resources. **Jian Mao:** Design, Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113963.

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