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Development of Industrial Brewing Yeast with Low Acetaldehyde Production and Improved Flavor Stability

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Abstract Higher acetaldehyde concentration in beer is one of the main concerns of current beer industry in China. Acetaldehyde is always synthesized during beer brewing by the metabolism of yeast. Here, using ethanol as the sole carbon source and 4-methylpyrazole as the selection marker, we constructed a new mutant strain with lower acetaldehyde production and improved ethanol tolerance via traditional mutagenesis strategy. European Brewery Convention tube fermentation tests comparing the fermentation broths of mutant strain and industrial brewing strain showed that the acetaldehyde concentration of mutant strain was 81.67 % lower, whereas its resistant staling value was 1.0-fold higher. Owing to the mutation, the alcohol dehydrogenase activity of the mutant strain decreased to about 30 % of the wild-type strain. In the meantime, the fermentation performance of the newly screened strain has little difference compared with the wild-type strain, and there are no safety problems regarding the industrial usage of the mutant strain. Therefore, we suggest that the newly screened strain could be directly applied to brewing industry.

Keywords Industrial brewing yeast · Acetaldehyde · Traditional mutagenesis · 4-Methylpyrazole

Introduction

Beer flavor is one of the most important factors in beer quality evaluation. The volatile compounds in beer are the main compounds that contribute to its overall flavor. Those compounds include alcohol, acetaldehyde, diacetyl, pentanedione, etc. [1, 2]. Acetaldehyde as one natural by-product of fermentation accounts for about 90 % of the carbonyl

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compounds in beer. It shows serious impacts on beer flavor. Exceeded amount of acetaldehyde always create a pungent aroma, while appropriate amount of acetaldehyde often leaves a pleasant green apple aroma. Besides being considered as one of the main off-flavor compounds, acetaldehyde affects beer staling, too. The aldol condensation reaction during beer storage negatively affects beer's shelf life. Thus, decreasing the content of acetaldehyde could improve the flavor as well as the resistant staling value (RSV) of beer [1, 3].

Alcohol dehydrogenases (ADH) are the key enzyme in acetaldehyde metabolism (Fig. 1). The ADH1p and ADH2p have a 90 % similarity in nucleotide sequence and 95 % similarity in amino acid sequence [4, 5]. However, the substrate-binding specificity of these two enzymes is about tenfold different due to the difference (Met294 of ADH1p is replaced for Leu294 in ADH2p) in the binding site. Furthermore, several amino acids differences in ADH2p could cause the change of alcohol dehydrogenase activity [6], which would affect the brewing process. Goihberg et al. [7] reported that the proline residue located at position 100 of ADH1p was crucial for maintaining its thermal stability. Rivera-Meza et al. [8] suggested that Arg-47-His would result in an increase in enzyme activity in animal mode studies. In previous studies, after disrupting the *ADH2* gene encoding alcohol dehydrogenase, we succeeded in reducing the acetaldehyde content in the final beer [1]. However, there were some unexpected problems when totally knocking out the *ADH2* gene such as cell viability and ethanol tolerance issues (not published).

4-Methylpyrazole (4-MP; fomepizole) is a competitive inhibitor of alcohol dehydrogenase 2 [9–11]. It has been widely used in ethanol metabolism studies as this compound can block the ethanol binding site in alcohol dehydrogenase 2, thereby inhibiting the enzyme activity in ethanol metabolism [10, 12, 13]. Daley et al. [11] reported that 4-MP could prevent or ameliorate ethanol intolerance and reduce the symptoms associated with acetaldehyde accumulation in order to reduce the risk of disease. Thus, in this study, 4-methylpyrazole was used as selection markers for lower acetaldehyde production of industrial brewing yeast.

Though lots of gene modifications have been done to the industrial brewing yeast in order to improve the fermentation capability of the yeast strains [1, 3, 14, 15]; however, in current brewing industry, there were hardly any genetic engineered strains used in brewing because of safety concerns, especially in China. Moreover, the evolution of the complex traits of a whole cell biocatalyst is not easy to achieve only by sequential multigene modification [2, 16–18] because

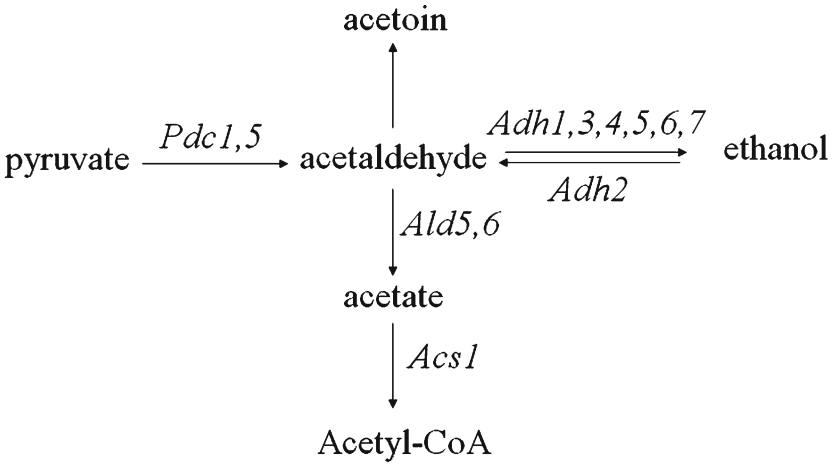

Fig. 1 Pathways for acetaldehyde metabolism in *S. cerevisiae* [5]

it always requires the simultaneous alteration of the expression levels of many genes. Taking all of the factors into consideration, traditional mutagenesis was applied in order to obtain a mutant strain with improved performance in fermentation ability and better flavor.

Materials and Methods

Strains and Cultivation Conditions

The sources and relevant genotypes of the strains used in this study are listed in Table 1. Industrial brewer's yeast M^I_4, which was obtained in our previous study, was used as the original strain. Yeast strains for ultraviolet mutation were cultured in 12 °P wort at 28 °C. Mutant strains were selected from the ethanol plate [2 % (*v*/*v*) ethanol, 1 % (*w*/*v*) yeast extract, 2 % (*w*/*w*) peptone, and 2 % agar] using 4-methypyrazole as a selection marker.

Isolation of Low Acetaldehyde Production Mutant Mutagenesis and Mutant Isolation

Strain M^I_4 grown in 5 ml 12 °P wort for 12 h was harvested by centrifugation and washed with 0.9 % (*w*/*v*) saline for twice. The cells were then resuspended in sterilized distilled water and adjusted to 108 colony-forming units (CFU)/ml. Cultivation (3 ml) was added to a 9-cm Petri dish, and mutagenesis of strain M^I_4 was performed by exposing the plates to UV light (15 W) while being stirred on a stirrer. The irradiation continued for 55 s to guarantee the survival rate of 5 % (Eq. 1). Afterwards, the cells were incubated in wort medium with shaking at 28 °C overnight in darkness and then washed, diluted, and resuspended in sterilized water. One hundred microliters of the cell suspension were spread on ethanol plate with 800 mg/l 4-methypyrazole as a selection marker and cultured for 3 days in dark. The plates were then transferred to anaerobic cultural incubator and incubated at 10 °C for 3 days. The colonies were stained with Schiff reagent, and all the colonies would be stained in purple. Schiff test provides a sensitive, fast, and simple method to test acetaldehyde production of yeast [19]. The decolorized Schiff reagent will develop a magenta or purple color when acetaldehyde is present. Thus, comparing the color of mutant strains and original strain, the colonies with lighter color were chosen for further screening.

$$\%\,\text{Survival rate} = \text{Colonies in the plate inoculated by the cells irradiated for certain time} / \text{Colonies in the plate inoculated by the cells without irradiation} \times 100\% \quad (1)$$

Analytical Methods

Assay of Alcohol Dehydrogenase Activity

The alcohol dehydrogenase activity was measured spectrophotometrically at $OD_{450\ nm}$ by using alcohol dehydrogenase activity assay kit (BioVision). Dry cell weight was calculated with the cells which were harvested, washed with distilled water, and dried at 80 °C. One

Table 1 Strains used in this study

Strain	Genotype	Source
M^I_4	Industrial brewer's yeast	Stored in authors' lab
MA12	Mutant strain	Stored in authors' lab

unit (U) of enzyme activity was defined as the amount of enzyme catalyzing the production of 1 μmol NADH per min under the specified conditions.

Genetic Stability Test of Mutant Strain

The mutant strain was transferred onto the 12 °P wort slant for 15 generations; each generation was cultivated for 36 h at 28 °C. Plate streaking of the 1st, 4th, 12th, and 15th generation strains was performed. After 48 h of cultivation at 28 °C, 100 single-grown colonies were chosen randomly and transferred to 0.5 ml of sterilized water and starved for 4 h at room temperature. A Grenier inoculation loop of the starved yeast suspension was used to inoculate wort plates containing 800 mg/l 4-methypyrazole and stored for 2 days.

At the same time, the 1st, 4th, 12th, and 15th generation mutant strains were processed for conical flask fermentation. The alcohol dehydrogenase activity of the 1st, 4th, 12th, and 15th generation strains were analyzed as described above.

Acetaldehyde Concentration Measurement

Acetaldehyde from final beer was measured using the automatic headspace gas chromatography (Shimadzu, Japan). The headspace injector equilibrium time was 30 min at 70 °C. The column temperature was initiated at 40 °C and programmed to 180 °C at a rate of 10 °C/min. The flow rate was 1.2 ml/min, and 1.8 g NaCl was added into the vials to enhance the volatility of samples.

Assay of Biomass and Preservative Qualities

Biomass was determined by the method described by Wang et al. [3]. Yeast cells were harvested, washed with distilled water, and then dried at 80 °C to constant weight (24 h).

The preservative qualities of the fermentative broths, and the corresponding antiaging characters of the various strains were determined by measuring the thiobarbituric acid (TBA) value and resistant staling value [20], which are two significant indexes reflecting beer freshness. The RSV was taken as a reference of beer flavor refreshing period: RSV=1/4 (12/ΔTBA12+24/ΔTBA24+36/ΔTBA36+48/ΔTBA48). Stability index (SI), which counted as 100× diphenylpicrylhydrazyl scavenging activity/TBA value, was introduced to measure the flavor stability of beer [1].

Fermentation Test and Pilot Scale Brewing

The yeast cells were first activated in 5 ml 12 °P wort at 28 °C for 48 h, and 1 ml yeast culture was inoculated to 9 ml 12 °P wort and cultured at 28 °C for 48 h. After that, the yeast culture was transferred to a 2-l shaking flask with 900 ml 12 °P wort for intermediate culture at 16 °C for 48 h. The yeast cells were collected after 12-h natural sedimentation at 4 °C, resuspended in 200 ml sterilized wort, and transferred to a 6-l European Brewery Convention (EBC) tube with 5 l 12 °P wort for pilot-scale brewing. The initial cell density was 1.5×10^7 CFU/ml. The main fermentation was carried out at 9 °C for 1 day and 12 °C for 5 days. The post-fermentation was carried out at 8 °C for 7 days. The final beer was stored at 0 °C until flavor evaluation.

Real extract, attenuation degree, and ethanol concentration in the fermentation broth were measured by using the Alcolyzer Plus Beer machine WBA-505B (Kyoto Electronics Manufacturing Co., Ltd, Tokyo, Japan). Diacetyl and main residual sugars content were measured by using GC/MS as described by Landaud et al. [21]. TBA value and RSV were

determined as Parson et al. [20] described. SI was calculated to reflect the preservative qualities of beer. A comparative and qualitative test to evaluate the sensorial characteristics of the final beer was conducted on site by six tasting experts sent in from the division of the Tsingtao Brewery Co., per company quality control procedures.

Result and Discussion

Ultraviolet Mutation of Industrial Brewer's Yeast

In order to obtain a strain with lower acetaldehyde production, industrial brewer's yeast M^I_4 was treated with UV rays and inoculated on ethanol plate with 800 mg/l 4-MP for colony development. ADH serves as the key enzyme in the ethanol metabolism in *Saccharomyces cerevisiae*. 4-MP, a recognized ADH2p inhibitor, has been proved effective in several pharmaceutical studies in treating the symptoms of increased aldehyde accumulation in ethanol intolerant people [8, 22]. The 4-MP would block the binding site of ADH2p so as to inhibit the enzyme activity. Besides, the strains producing acetaldehyde would develop visible colors with Schiff's reagent. The lighter color reflected the lower acetaldehyde concentration [19]. Thus, the media containing 4-MP could be used to select the mutant strains, and 31 mutant strains were selected on the selection plate (ethanol plate with 800 mg/l 4-MP). Afterwards, the alcohol dehydrogenase activity of each mutant and the original strain were measured. Most mutants showed lower enzyme activities than the industrial brewing yeast strain. The alcohol dehydrogenase inhibitor 4-MP in the selection plate blocked the binding site of such enzyme, resulting in the decrease of alcohol dehydrogenase activity (mutant strains exhibited 40.02–60.67 % lower activities than those of industrial brewing yeast strain). Twenty strains were selected and processed for conical flask fermentation to test the acetaldehyde production.

The acetaldehyde concentration of M^I_4 measured by headspace gas chromatography was 28.13 mg/l, while all the 20 mutant strains produced lower amount of acetaldehyde (10.21–16.53 mg/l) (Fig. 2a). As the wild-type strain exhibited an alcohol dehydrogenase activity of 7.86 U/mg, the mutant strains showed 44.66–61.96 % lower activities during the conical flask fermentation test (2.99–4.35 U/mg). Alcohol dehydrogenase 2 catalyzes the reaction of ethanol oxidization to produce acetaldehyde. In this study, the key enzyme in the reaction was weakened due to the mutation; accordingly, the acetaldehyde concentration in the supernatant decreased as a result. The mutant strain MA12 (10.21 mg/l acetaldehyde concentration) was selected for further study due to its lowest acetaldehyde concentration and relatively lower alcohol dehydrogenase activity.

Genetic Stability Analysis

It is important to ensure that the strain used in industrial is genetically stable throughout the long-term production. After continuously passage for 15 generations, plate streaking was employed to test the stability of the strain, and all 100 single colonies of the 1st, 4th, 12th, and 15th generations of mutant strain MA12 grew on the ethanol plate in the presence of the selection marker (800 mg/l 4-MP), whereas the industrial brewer's strain M^I_4 did not. Such long-term stable growth in 4-MP indicated that the mutant strain is genetically stable. Likewise, the 1st, 4th, 12th, and 15th generation mutant strains exhibited a similar tendency

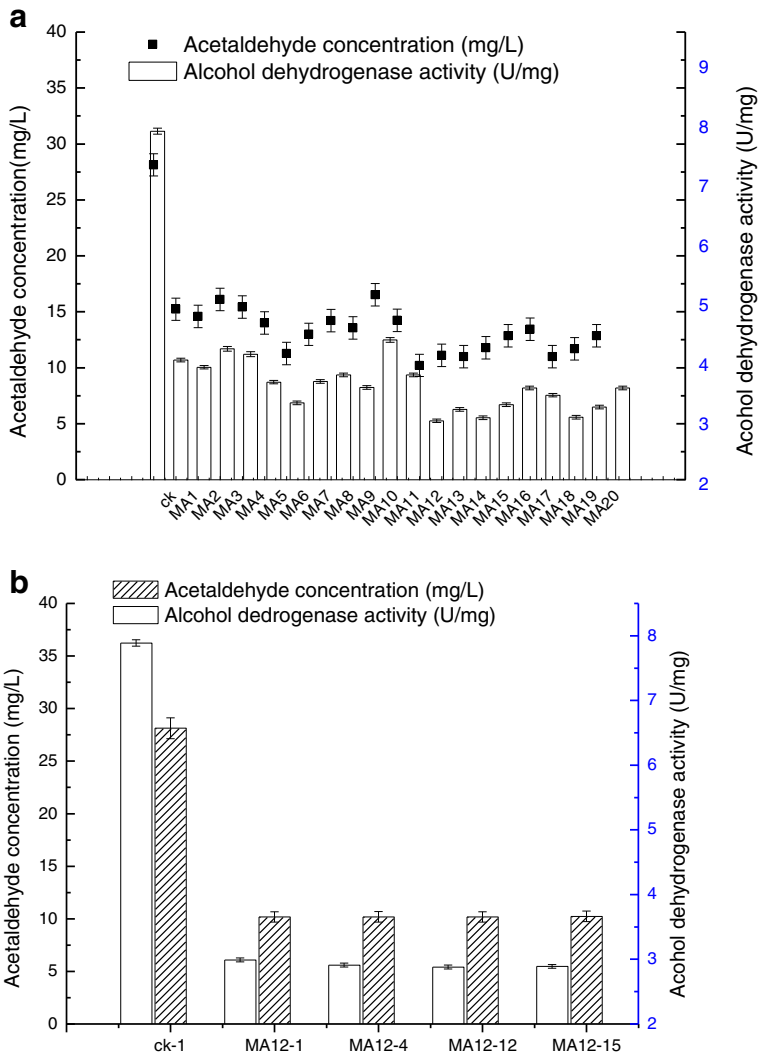

Fig. 2 **a** Determination of acetaldehyde concentration and alcohol dehydrogenase activity of mutants and wild-type strain. **b** The acetaldehyde concentration and alcohol dehydrogenase activity of wild-type strain (CK) and different generations of mutant MA12. The data are means of three independent experiments with the *error bars* indicating standard deviations

in acetaldehyde concentrations and alcohol dehydrogenase activities in conical flask fermentation (Fig. 2b). All the above results showed that MA12 was genetically stable.

Fermentation Test and Pilot-Scale Brewing

The mutant strain MA12 and industrial brewing strain M^I_4 were tested for their fermentation capability and flavor generation in a pilot-scale brewing. The CO_2 reduction and attenuation degree (data not shown) of the mutant strain MA12 in conical flask fermentation were both similar to those of M^I_4, indicating that the mutagenesis to M^I_4 did not make any difference in

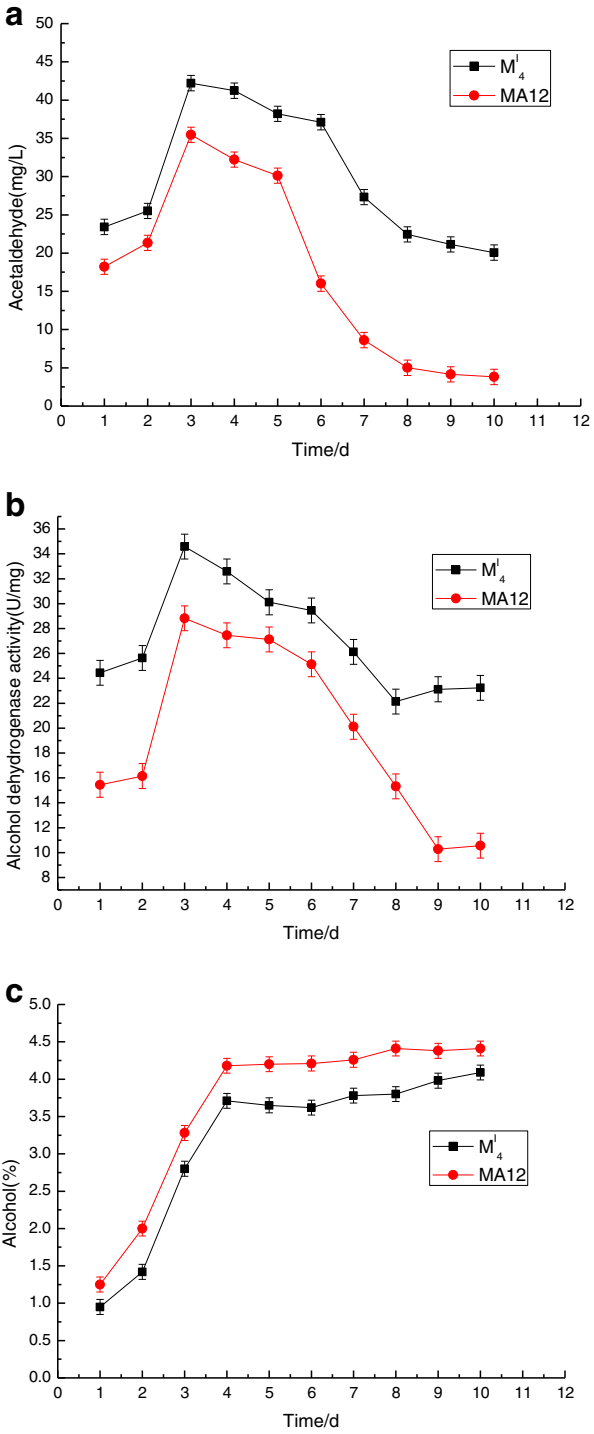

Fig. 3 Measurements of acetaldehyde concentration (**a**), alcohol dehydrogenase activity (**b**), and alcohol concentration (**c**) of M^{I}_{4} and MA12 during fermentation

fermentation ability. At the end of conical flask fermentation, the acetaldehyde content of MA12 fermentation broth was 60.27 % (10.55±0.34 mg/l) lower than that from M^I_4 fermentation broth (26.56±1.15 mg/l).

During the 10-day EBC tube fermentation, the MA12 strain always showed lower acetaldehyde production than that of M^I_4 strain (Fig. 3a). By the end of EBC tube fermentation, the acetaldehyde content from MA12 fermentation broth was 81.67 % (3.07± 0.01 mg/l) lower than that of M^I_4 fermentation broth (16.75±0.07 mg/l). This result is even lower than the acetaldehyde content produced by genetically engineered strains [1, 18]. At the same time, similar results were observed in the alcohol dehydrogenase activity measurement (Fig. 3b). The MA12 exhibited an obvious lower alcohol dehydrogenase activity than M^I_4 during the EBC fermentation. Lower enzyme activity directly led to less amount of acetaldehyde production. Therefore, we detected less amount of acetaldehyde in the MA12 fermentation broth. The alcohol concentration of both fermentation lines was tracked during EBC tube fermentation, too. As shown in Fig. 3c, it seemed that there are no obvious differences between MA12 and M^I_4. However, the alcohol concentration of MA12 fermentation broth was always higher than that of M^I_4 for about 0.5–1 %. This indicated that the mutation not only did not affect the fermentation ability of MA12 but also increased the alcohol production of mutant strain. Due to the inhibition of alcohol dehydrogenase activity by 4-MP, less acetaldehyde was generated during fermentation process, resulting in the accumulation of ethanol. So, it is not surprising to find higher ethanol concentration from MA12 fermentation broth [23]. It might be because of the changes in the secondary structure of alcohol dehydrogenase 2 that caused the decrease of alcohol dehydrogenase activity [24–26].

At the end of fermentation, several parameters related to beer quality were measured and listed in Table 2. The real attenuation of MA12 was similar to industrial brewing strain M^I_4, indicating the similar capability of fermentation ability. Diacetyl, one of the main off-flavor compounds, showed some decrease in MA12 compared with M^I_4. However, the amount of acetaldehyde showed a huge decrease in MA12 fermentation broth compared with M^I_4 fermentation broth (81.67 % lower amount). Acetaldehyde accounts for about 90 % of carbonyl compounds in beer, which are the main reason for the taste of staling. The TBA value reflects the amount of staling compounds such as carbonyl compounds. A smaller TBA value indicates a better anti-oxidizability, and RSV reflects the beer freshness. Higher RSV indicates longer flavor freshness period [1, 27]. As a result, MA12 showed a lower TBA value than that of M^I_4, indicating less staling compounds in fermentation broth. As a

Table 2 Parameters measured form the EBC tube fermentation (mean ± SD, n=3)

Parameter	M^I_4	MA12	F value	P value
Real attenuation (%)	67.7±0.02	68.5±0.06	22.15	0.009
Acetaldehyde (mg/l)	16.75±0.07	3.07±0.01	6,436.60	1.45e-7
Diacetyl (mg/l)	0.07±0.00	0.06±0.00	450	2.92e-5
Ethyl acetate	14.73±0.00	13.91±0.00	7.7086	1.49e-5
Isoamyl acetate	1.26±0.00	1.24±0.00	7.7086	0.1456
N-propanol	17.31±0.00	17.56±0.00	7.7086	9.54e-5
Isobutanol	8.22±0.00	7.97±0.00	7.7086	0.0011
Isoamylol	58.90±0.00	58.86±0.00	7.7086	0.001
TBA	0.213±0.00	0.133±0.00	191.06	0.0002
RSV	236.06±43.13	477.60±85.13	1,364.56	3.21e-6

result, the RSV which was based on the TBA result was higher. On the other hand, the parameters related to the aroma of beer including ethyl acetate, isoamyl acetate, *n*-propanol, isobutanol, and isoamylol were measured accordingly. As can be seen in Table 2, similar amount of aroma compounds was produced by MA12 compared with M^I_4, indicating that the mutation to MA12 had no effect on its production performance.

Nowadays, food safety problems have drawn more and more attentions. Most of the recombinant strains were limited in industrial use, especially in food industry, because of the residues of heterogeneous DNA and drug-resistant genes. In this study, the mutant strain MA12 was obtained using UV mutagenesis strategy; thus, there are no safety problems in terms of using this mutant strain in large-scale beer fermentation. Moreover, MA12 has a good hereditary stability and a good fermentation performance; therefore, the strain MA12 could be applied directly to brewery companies due to its lower acetaldehyde content and improved flavor characteristics.

Conclusion

Traditional UV mutagenesis strategy was introduced to screen a new mutant strain in this study. Ethanol and 4-MP were used as the sole carbon source and selection marker, respectively. As a result, lower acetaldehyde production and improved ethanol production were observed in mutant strain MA12 due to mutation. Alcohol dehydrogenase catalyzes the oxidation of ethanol. As the result of mutation, the alcohol dehydrogenase activity of the MA12 decreased to about 30 % of the industrial brewing strain M^I_4. During EBC tube fermentation, MA12 produced 81.67 % less amount of acetaldehyde than that of M^I_4. Moreover, its RSV was 1.0-fold higher. In the meantime, the UV mutagenesis did not affect other properties of the brewing strain. In addition, no safety problem would arise during the application of the newly constructed strain, making the MA12 strain a promising application prospect.

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