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Production of yeast hybrids for improvement of cider by protoplast electrofusion

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ABSTRACT

This study aimed to construct new yeast hybrid strains for introducing flavor and aroma diversity to ciders. The inactivated protoplasts of *Saccharomyces cerevisiae* and *Candida krusei* were electric-induced fused under the optimized electric condition of pulse field density 2200 V/cm, pulse time 20 μs, pulse number 2 times and pulse interval 1 s, and 69 fusants were initially obtained. By performing Durham's fermentation for ten generations, 9 stable hybrid strains were screened. The chemical analysis showed that the alcoholic degree of ciders fermented by R2, R4, R5, R6 and R8 achieved about 12% (v/v), which was statistically the same level as the one fermented by parental strain WF1. The GC–MS results showed different strain generated totally different aroma profiles. R4 produced significant higher concentration of 2-methyl-butanoic acid ethyl ester, 2-methyl-1-propanol, 3-methyl-butanol acetate, 1-butanol, acetic acid hexyl ester, 1-hexanol and 1-octanol. The 9 hybrid yeast strains and parental strains were further compared through fuzzy comprehensive evaluation combining sensory score and aroma components content. The results showed that the hybrid R4 scored highest and displayed desirable properties of both parents.

1. Introduction

Yeast fermentation of fruit juice not only produces ethanol and carbon dioxide, but also a range of minor yet sensorially important volatile metabolites, which give fruit wine specific character and style [1,2]. Yeasts play an important role in the major changes between juice and wine, such as modifying aroma, flavor, mouthfeel, color, chemical complexity and so on [1,3].

Traditional spontaneous fermentations involve the sequential contribution of different yeast species [4]. The genus *Saccharomyces* is usually predominant during alcoholic fermentation, exhibiting good fermentation performance. While the non-*Saccharomyces* genera, such as *Kloeckera*, *Candida*, *Pichia*, *Hansenula*, *Hanseniaspora* and *Metschnikowia* mainly grow during the first stages of the process and the metabolites mainly contribute to sensory complexity, characteristic aroma and flavor profiles [5,6]. Nevertheless, because of the unpredictable nature of spontaneous fermentation, at present, most of the fruit wines makers tend to inoculate with selected pure yeast cultures instead of spontaneous fermentation.

Man-made mixed fermentation experiments showed dynamic population fluctuations between strains [7], with unpredictable fermentation results. Using co-inoculum of *Saccharomyces cerevisiae* and non-*Saccharomyces* strains in fruit wine fermentation, typically, the non-*Saccharomyces* strains play only a minor impact on aroma and composition. Non-*Saccharomyces* strains cannot survive throughout the fermentation process because they are not alcohol-tolerant or restrained by the presence of viable *S. cerevisiae* cells at high concentrations [8,9]. But if only *S. cerevisiae* is incubated, ratio of total concentration of alcohols to esters would be abnormally high, inducing the aroma and flavor faint and monotonous [5,10].

Construction of intergeneric hybrid strains can solve the problems mentioned above and increase the impact of non-*Saccharomyces*, where the genomes of different species are contained within one cell. It will inherit the good character of *S. cerevisiae* and non-*Saccharomyces* strains. Previous works demonstrate that species of *S. cerevisiae* are able to mate with a number of genera to form intergeneric hybrids [11–13].

Protoplast fusion technology provides an approach to generate hybrid cells with novel combinations of both nuclear and cytoplasmic genes [14]. It has enabled the breeding of new yeasts with various properties in comparatively short time. Because protoplast fusion does not depend on ploidy and mating type and overcomes the limitations of conventional breeding (e.g. natural crossing barriers), it provides a more rapid alternative to traditional breeding methods and can be widely applied to yeasts. Some kinds of yeasts have been constructed with this method [3,15].

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As another fruit wine, cider share many similarities with grape wines. However, little literature has been reported about the hybrid yeasts special for cider making. The aim of this work is to construct new yeast hybrid strains for introducing flavor and aroma diversity to ciders. Intergeneric protoplast electric fusion was carried out between *Candida krusei* PF14 and *S. cerevisiae* WF1. Sensory evaluations and analytical determinations were performed on the ciders fermented by fusants. A model of fuzzy comprehensive evaluation was established combining chemical analysis with sensory evaluation to screen the best hybrid for cider fermentation.

2. Materials and methods

2.1. Yeast strains and medium

Parental strains, *C. krusei* PF14 and *S. cerevisiae* WF1 were preserved in Laboratory of Bioreactor in Northwest A&F University, which have been selected from a large number of cider yeasts for good aroma-producing ability and fermentability, respectively. All yeasts were grown in YPD medium (complete medium) (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) with shaking (150 rpm) at 28 °C. Protoplasts were grown in regeneration medium (YPD medium with 17% sucrose).

2.2. Protoplast preparation using ultrasonication

C. krusei PF14 and *S. cerevisiae* WF1 were pre-activated and then inoculated into 100 mL YPD medium in 250 mL Erlenmeyer flasks at 28 °C on a rotary shaker (150 rpm). The suspensions of the two strains at the mid-growth phase were centrifuged at 3000 × *g* for 5 min. The precipitates were suspended in 1 mL 0.05 M EDTA-Na_2 and 1 mL 0.1% mercaptoethanol at 28 °C for 10 min and then centrifuged and rinsed with PBS buffer twice, and the biomass sediments were suspended in PBS buffer. The suspension was treated using ultrasonic processor (Kunshan Co. Ltd., China) to get rid of cell walls to obtain protoplasts. The treated solutions were spread and cultured on the regeneration medium agar at 28 °C for 2 days. Protoplast formation rate and regeneration rate were calculated as follows:

$$X = \frac{A - B}{A} \times 100\% \quad (1)$$

$$Y = \frac{C - B}{A - B} \times 100\% \quad (2)$$

where X is protoplast formation rate; Y is protoplast regeneration rate. A is the number of colonies growing on the complete medium without any processing; B is the number of colonies growing on the complete medium after ultrasonication; C is the number of colonies growing on the regeneration medium after ultrasonication.

The optimization of the variables affecting the protoplast formation and regeneration was carried out using single factor experiments. The factors chosen were cell age, ultrasonic power, temperature and ultrasonic processing time. Factor levels were chosen by considering the operating limits of the experimental apparatus and the preliminary studies. Briefly, the levels of cell age of parental strains were 4, 8, 12 and 16 h; the levels of ultrasonic power were 240, 360, 480 and 600 W; the levels of temperature were 25, 30, 35 and 40 °C; the levels of ultrasonic processing time were 20, 40, 60 and 80 min for PF14, and 10, 20, 30, 40 min for WF1. By comparing formation and regeneration rate under different conditions, the optimal condition was determined.

2.3. Inactivation of protoplasts

Before fusion, the parental protoplasts of PF14 and WF1 were inactivated using optimized UV and heat method, respectively [16,17]. The protoplasts of PF14 and WF1 were UV-inactivated for 5 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min separately at the power of 30 W and the irradiation distance of 30 cm [16]. Furthermore, they were heat-inactivated for 5 min, 10 min, 15 min, 20 min, and 25 min separately at 60 °C [17]. By comparing the inactivation rate, different best inactivation condition was used for parental protoplasts.

2.4. Protoplast electrofusion

The inactivated protoplasts of PF14 and WF1, both 500 μL, were mixed and centrifuged at 3000 × *g* for 5 min. The precipitates were re-suspended in freshly prepared electrode buffer containing 0.8 M mannitol and 0.1 mM $CaCl_2$. The mixture was treated with BTX830 Cell electrofusion device (BTX Co. Ltd., America). The four parameters, pulse field density, pulse time, pulse number and pulse interval, were chosen as the variables tested in single factor experiment and orthogonal design experiment with protoplasts fusion rate as response, which was calculated as follows:

$$R = \frac{F}{A - B} \times 100\% \quad (3)$$

where R is fusion rate, F is the number of colonies growing on the regeneration medium after protoplasts electrofusion processing, A is the number of colonies growing on the complete medium without any processing, and B is the number of colonies growing on the complete medium after ultrasonication.

In the single factor experiments, the pulse field density was set at 1000, 1500, 2000, 2500 and 3000 V/cm successively; pulse time was 10, 30, 50, 70 and 90 μs separately, pulse number was 1, 3, 5, 7 and 9; pulse interval was 0.5, 1, 1.5, 2 and 2.5 s. By comparing the fusion rate under different conditions, the best condition for each factor was selected. Based on the results of the single factor experiments, an orthogonal test $L_9(3^4)$ was designed to study the optimum condition for protoplasts electrofusion. As shown in Table 1, the four factors were designated as A, B, C and D, and were prescribed into three levels, coded 1, 2 and 3 for low, intermediate and high value, respectively.

The electric-treated mixture were diluted properly with PBS buffer, then plated on regeneration medium and incubated at 28 °C for 2–3 days to calculate the fusion rate. The regenerated colonies were picked out and subcultured in apple juice placed in Durham's tube. To check the stability of these tentative hybrids, they were passaged for 10 generations, and the residual sugar content, fermentation rate, pH value, the biomass, ethanol production and the smell of every generation were evaluated. The unstable strains were eliminated gradually, while the strains showing similar characteristic for all the 10 generations were genetically stable and retained for further screening.

2.5. Fermentation of ciders

Apple juice was extracted from fresh Fuji apples with a mill and filtered using vacuum filtration system. It was modified as follows: the pH value was adjusted by malic acid to 3.4 and the sugar content was adjusted by sucrose to 200 g/L. Sulphur dioxide was added, up to 100 mg of $K_2S_2O_5$ per liter (50 mg/L of free SO_2), to inhibit bacterial growth. After equilibrating for 24 h at room temperature, the apple juice was inoculated separately with parental strains and different hybrids in triplicate. Fermentations were carried out in 600 mL of apple juice contained in 1000-mL Erlenmeyer flasks at 20 °C and were considered complete when no variation in weight