Tranasformation of Ferulic Acid to 4-Vinylguaiacol by Microbiological Method

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Abstract: 4-Vinylguaiacol (4-VG) is a kind of special flavor substance which widely exists in top-fermented beer, which has the characteristics of “smoky “and “clove “. It is usually produced by the decarboxylation of ferulic acid (FA) by thermal or decarboxylation of ferulic acid decarboxylase (Fdc1p). However, the activity of Fdc1p from yeast is low because of the restriction of beer fermentation conditions in china, so it is not enough to improve the overall taste of the top-fermented beer. To brew the top-fermented beer with unique flavor, smooth taste and good quality, we cloned a recombinant yeast strain with the best fermentation performance, named IYSBp. The content of 4-VG in the top-fermented beer brewed by IYSBp increased by 34%.

1. Introduction

Phenolic acid compounds contained FA, caffeic acid, p-coumaric acid and sinapic acid, which are a kind of phenolic ring organic acids widely existing in the grass, nut and plant [1]. There are many kinds of phenolic acid decarboxylases in nature, including Fdc1p from bacteria and fungi, p-coumaric acid decarboxylase, phenolic acid decarboxylase and phenylacrylic acid decarboxylase from fungi. Fdc1p can metabolize many phenolic acid compounds because of the poor substrate specificity, but the main metabolite is still 4-VG [2]. 4-VG has a high economic value and a wide range of applications. It is also a good antioxidant, food protector and aroma component of alcoholic beverages, so it plays a very important role in food, medicine, cosmetics and other industries [3].

In most bacteria and yeast strains, the structural characteristics of Fdc1p have a big difference in species. Fdc1p from bacteria is mainly encoded by *fdc1*, while Fdc1p from yeast is mainly encoded by *FDC1*. The Fdc1p encoded by them have similar physical and chemical mechanism. For example, the open reading frames (ORF) of Fdc1p from *B. pumilus* (*Bp fdc1*) and Fdc1p from *S. cerevisiae* (*Sc FDC1*) are 486 bp and 1512 bp [4-5], respectively. Compared with Fdc1p from bacteria, the activity of Fdc1p from yeast is nearly 1000 times lower, and the biggest reason for this phenomenon may be whether the assistance of factors is needed in the process. FA can also be metabolized into other compounds, such as vanillin, vanillic acid, vanillin alcohol and protocatechuic acid. The results show that the para hydroxyl group on FA is necessary in the decarboxylation mechanism, and it is one of the indispensable conditions for the formation of quinone intermediates.

In this study, *Bp fdc1* and *Sc FDC1* were successfully introduced into the genome of industrial yeast strain named IYS, achieved the heterologous expression of Fdc1p, and a recombinant yeast strain with the best fermentation performance was screened.

2. Materials and Methods

2.1 Materials, Strains and Growth Measurement

The top-fermenting yeast IYS (control strain) was stored in the China-Germany Brewing Technical Center, Qilu University of Technology. The *Escherichia coli* DH5α (Rockville, Maryland, USA) was used for sub-cloning. YPD medium used in cultivated the IYS and the recombinant strains supplemented with 400 mg L^{-1} G418 if necessary at 30 ℃. *E. coli* DH5α was grown in LB

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medium added with 100 mg L^{-1} of ampicillin if necessary at 37 ℃.

2.2 Construction of Recombinant Yeast Strains

Bp fdc1 was synthesized by gene combination method with biological company. The strong promoter and terminator of pJFE 3 plasmid was selected as the promoter and terminator of *Bp fdc1*, created the pJFE 3-BF used restriction enzyme with *Bgl* II and *Sal* I. IYS is a typical industrial yeast strain which is used for brewing beer, and the recombinant yeast strain after introducing plasmids is easy to lose plasmids in the nutrient rich medium. To prevent the loss of the target fragment, δ integration was selected to bring the target fragment into IYS genome. Using upstream and downstream primers of *Bgl* II-*TEF1p*-F (5'-GAAGATCTCCACACACCATAGCTTCAAAAT-3') and *Sal* I-*PGK1t*-R (5'- ACGCGTCGACAACGCAGAATTTTCGAGTTA-3') and pJFE 3-BF as the plasmid template, the fragment of *TEF1p-Bp fdc1-pGK1t* was cloned by PCR, resulted the recombinant plasmid pδKBF. *Pae* I and *EcoR* I were used for enzyme cutting, and the target fragment of δ1-*TEF1p-Bp fdc1-pGK1t-Kan MX4*- δ2 was introduced into the genome of IYS by lithium acetate transformation.

2.3 Enzyme Activity Determination Method of Fdc1p

The enzyme activity determination method of Fdc1p was determined by ultraviolet spectrophotometry. Crude enzyme solution was incubated with 1 mM FA at 30 ℃ for 20 min, and determination of the absorbance of FA at 310 nm before and after reaction to calculated the enzyme activity of Fdc1p.

2.4 Determination Method of Fa and 4-Vg

The concentration of FA and 4-VG were determined by HPLC using a method described by Zhu et al (2013) [6]. Briefly, the HPLC system consisted of a Waters model e2695 and 2998 UV detector. The analysis was performed on WondaSil C18 column (4.6 × 250 mm, 5 μm) eluted with $ddH_2O/CH_3OH/H_3PO_4$ (640:350:10, v/v) at 40 ℃, and the flow was 0.6 ml/min with injection volume of 10 μL. The standard solution of FA (Sigma, USA) and 4-VG (Sigma, USA) were dissolved in absolute methanol (Chromatographic grade, Fisher chemical, USA), and samples were filtered through 0.22 μm membrane filter of Nylon-66.

2.5 Laboratory-Scale Fermentation of Iys^{bp}

The IYSBp was inoculated into the wort medium containing 50 mg L^{-1} FA for two days of fermentation. The content of FA and 4-VG during the fermentation were monitored by HPLC.

3. Results and Discussion

3.1 Successful Construction of Iys^{bp}

To prove that ferulic acid decarboxylase gene expression frames have been inserted into the IYS, the IYS^{Bp} grown in the YPD solid plate contained the G418 was selected as temple of *TEF1p-Bp fdc1-pGK1t*, and the results were shown in Figure 1. The Figure 1 shown that the number 1 IYS^{Bp} and number 2 IYS^{Bp} were amplified the corresponding length fragment of *TEF1p-Bp fdc1-pGK1t*. Therefore, the ferulic acid decarboxylase gene expression frames successfully contained in the genome of IYS^{Bp}.

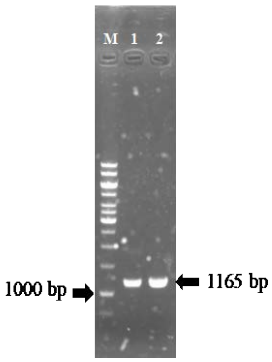

Figure 1. PCR results of IYS^{Bp} extracted genome. The swim lane from left to right, M: 1 KB DNA maker, 1: PCR verification result with number 1 IYS^{Bp}, 2: PCR verification result with number 2 IYS^{Bp}.

3.2 The Activity of Bp-Fdc1p is Higher Than Sc-Fdc1p

The activity of *Bp*-Fdc1p and *Sc*-Fdc1p from IYS^{Bp} and IYS were measured in cell lysate, culture supernatant and whole yeast cells respectively. The calculation results are shown in Figure 2. The results of Figure 2 shown that the total enzyme activity of phenolic acid decarboxylase of IYS and IYS^{Bp} are 0.59 and 24.7 U g^{-1} DW, respectively. The total enzyme activity of phenolic acid decarboxylase of IYS^{Bp} is higher than the control strain, so the heterologous expression Fdc1p improve the activity of Fdc1p.

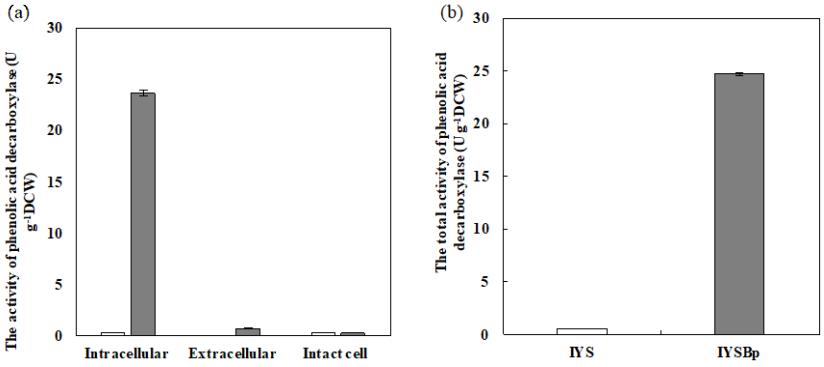

Figure 2. The activity results of phenolic acid decarboxylase. (a) Results of phenolic acid decarboxylase produced by IYS and IYSBp in cell lysate (black column), culture supernatant (white column) and whole yeast cell (gray column). (b) Total activity of phenolic acid decarboxylase of IYS and IYSBp. The error line represents the standard deviation of three parallel experiments.

3.3 The Good Effect of Heterologous Expression of Fdc1p in Recombinant Yeast

Bp-Fdc1p and *Sc*-Fdc1p are different in classification, catalytic mechanism, spatial structure and physical and chemical properties. To compare the ability of IYS and IYS^{Bp} to produce 4-VG, the *Bp fdc1* was introduced into the IYS genome by δ integration. The Figure 2 showed that the total enzyme activity of IYS^{Bp} was higher than IYS, which proved that this experiment achieved the expected effect. The figure 3 shown that the consumption time of FA with IYSBp was reduced from the original 24 hours to 12 hours, which further proves that heterologous expression of Fdc1p achieve the expected effect and increase the degradation rate of ferulic acid. Similar results were obtained in the production of 4-VG. At the end of fermentation, the content of 4-VG in IYS^{Bp} and IYS were 1.83 and 1.43 mg L^{-1}, respectively. Therefore, the fermentation performance of IYS^{Bp} was better than IYS in terms of total enzyme activity of phenolic acid decarboxylase, metabolism rate of FA and 4-VG production.

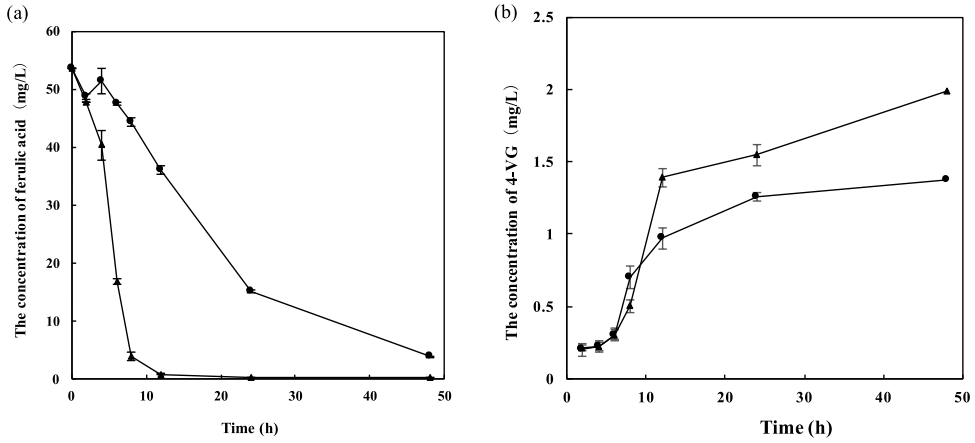

Figure 3. The effect of heterologous expression of Fdc1p on the fermentation of IYS (solid circle) and IYS^{Bp} (solid triangle). (a) The consumption of FA with IYS and IYS^{Bp} in wort medium containing 50 mg L^{-1} FA. (b) The content level of 4-VG with IYS and IYS^{Bp} in wort medium containing 50 mg L^{-1} FA. The error line represents the standard deviation of three parallel experiments.

4. Conclusion

In the analysis of heterologous expression of Fdc1p, the fermentation performance of IYSBp was better than IYS in total enzyme activity, the consumption time of FA and the content of 4-VG.

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