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ORIGINAL RESEARCH PAPER

Genetic modification of industrial yeast strains to obtain controllable NewFlo flocculation property and lower diacetyl production

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Abstract The expression cassette I10 containing the new-found flocculation gene, *FLONS*, was transformed into an industrial strain *Saccharomyces cerevisiae* YSF5. Upstream activating sequences of the *S. cerevisiae* alcohol dehydrogenase II (*ADH2*) gene promoter ($P_{U\text{-}ADH2}$) were used to regulate the expression of *FLONS*; α-acetolactate synthase gene *ILV2* was chosen for homologous recombination of I10 to the YSF5 chromosome; copper binding metallothionein (encoded by *CUP1*) was used for selection of transformants. Ten randomly selected transformants exhibited increased flocculation ability of 1.5 to 2.3 fold more than the original strain. Based on their sensitivity to glucose, maltose and sucrose, flocculation property of the transformants was supported to be NewFlo-type. After successive subculture, the introduced *CUP1* remained in the transformants. At the end of simulated fermentation test, diacetyl content of the culture media of 5I-1 was 0.45 g l^{-1}, lower than YSF5 (0.48 g l^{-1}).

Keywords ADH2 promoter · Diacetyl · NewFlo-type · Flocculation

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Introduction

Yeast flocculation is a reversible, asexual and calcium-dependent process in which yeast cells aggregate into clumps and sediment rapidly in the medium (lager yeasts) or rise to the medium's surface (ale yeasts) (Verstrepen et al. 2003). Mainly based on sugar sensitivity, flocculation phenotypes can be classified into two categories: the Flo1-type, which is only sensitive to mannose, and the NewFlo-type which can be inhibited by mannose, glucose, sucrose and maltose (Stratford and Assinder 1991).

Yeast flocculation involves flocculins—lectin-like cell wall proteins encoded by the *FLO* gene family—interacting with mannose residues on the surface of adjacent yeast cells. Flocculation allows the convenient separation of cells from the fermentation products, so yeast strains exhibiting strong flocculation toward the end of fermentation is desirable in industry. Genetic methods have been applied to improve flocculation behavior of yeasts (Cunha et al. 2006; Verstrepen et al. 2003). As NewFlo-type flocculation is sensitive to most fermentable sugars, yeast strains displaying this phenotype may possess

greater potential for practical application. *Lg-FLO1* encodes a flocculin which binds both mannose and glucose and is believed to be responsible for NewFlo phenotype of most lager yeasts, but only partial DNA sequences was determined due to the instability of *Lg-FLO1* in *E. coli* (Kobayashi et al. 1998). In our previous study, *FLONS* was cloned from a NewFlo-type strain *Saccharomyces cerevisiae* YN79 (a lager yeast) and was capable of bringing NewFlo-type flocculation property to nonflocculent laboratory strain *S. cerevisiae* YS58. Detailed analysis of nucleotide sequence of the intact *FLONS* (GenBank accession number: EF182714) revealed that, with a full length of 3,843 bp containing an ORF of 3,396 bp, *FLONS* was a derived form of *FLO1* and different from *Lg-FLO1* (Liu et al. 2007).

In this study, an expression cassette I10 harboring *FLONS* was introduced into an industrial strain *S. cerevisiae* YSF5; properties of transformants were then investigated.

Materials and methods

Strains and growth conditions

Escherichia coli DH5α was used as the host for development and characterization of recombinant plasmids. *E. coli* cells were grown at 37°C in LB medium. Ampicillin was added when necessary at 50 μg ml^{-1}.

Industrial strain *S. cerevisiae* YSF5 (Tsingtao Brewery Co., Ltd) was used as the transformation recipient of the expression cassette I10. *S. cerevisiae* YS59 (Flo1-type strain) and *S. cerevisiae* YN79 (NewFlo-type strain and donor of *FLONS*) were used as control in the analysis of transformants. Yeast strains were cultivated at 30°C in YPD medium. To repress or induce UAS of alcohol dehydrogenase II (*ADH2*)gene promoter ($P_{U\text{-}ADH2}$), yeast transformants were grown in YP medium containing 30 g glucose l^{-1}, or 10 g glucose l^{-1}, respectively (Price 1997).

Plasmids

The *E. coli*-yeast shuttle vector YEp352 (Amp^r, 2μ, *URA3*) was used for plasmid construction. pLZ-2 (Li et al. 2002) is the recombinant plasmid that α-acetolactate synthase gene *ILV2* (from positions −455 to +2476) was cloned into the *Bam*HI and *Sal*I sites of YEp352. YFp-S (Liu et al. 2007) is the recombinant plasmid that the 3.8 kb *Bam*HI–*Eco*RI *FLONS* fragment was cloned into the centromeric shuttle vector YCp50.

Expression cassette construction and yeast transformation

The YSF5 DNA was used as the PCR template of $P_{U\text{-}ADH2}$ and *CUP1*. $P_{U\text{-}ADH2}$ was amplified with the primers PA1 and PA2 (Table 1). Purified PCR product of $P_{U\text{-}ADH2}$ was digested by *Xba*I and *Bgl*II and then ligated to *Xba*I–*Bgl*II digested pLZ-2, generating pLZ-A. The intact *CUP1* was amplified with PC1 and PC2 (Table 1). PCR product of *CUP1* was purified and digested by *Bam*HI and *Eco*RI and then inserted into the *Bam*HI–*Eco*RI digested YEp352, generating YEp-CUP. The *Bam*HI–*Eco*RI *FLONS* fragment from YFp-S and the *Bam*HI–*Eco*RI *CUP1* fragment from YEp-CUP were inserted into the *Bgl*II-linearized pLZ-A, generating pIC10 (Fig. 1a).

The pIC10 was digested by *Bam*HI and *Pst*I. The fragment containing the expression cassette I10 (Fig. 1b) was purified and transformed into YSF5 using the lithium acetate method (Adams et al. 1997). Transformants were selected on YPD plates containing 5.5 mM copper sulfate.

Table 1 Primers used in this study

Primers	DNA sequences (5′–3′)	Restriction sites
PA1(−975)[a]	GACTCTAGAATCAACTGGCACCATCTC	*Xba*I
PA2(−137)	GACAGATCTTCGCTACTGGCACTCTAT	*Bgl*II
PC1(−454)	GTAGGATCCCGCTATACGTGCATATGTTC	*Bam*HI
PC2(+583)	CGAGAATTCATCTGTTGTACTATCCGCTT	*Eco*RI
PI2(+2467)	CCAAAGCTTAGAAAGAAGCGTCAGATC	

[a] The positions of the primers on their genes are indicated in the column

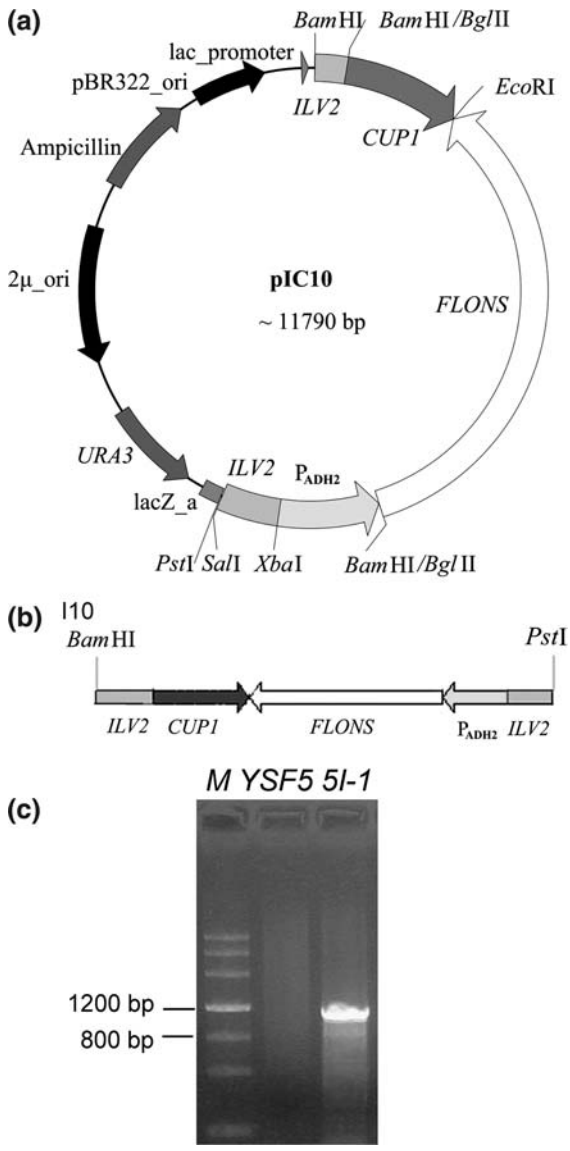

Fig. 1 Map of recombinant plasmid pIC10 (**a**) and expression cassette I10 (**b**). $P_{U\text{-}ADH2}$: upstream activating sequences of the *S. cerevisiae* alcohol dehydrogenase II (*ADH2*) gene promoter. (**c**) Confirmation of transformant 5I-1 by PCR

Flocculation assay

Flocculation assay was performed as described (Kobayashi et al. 1998) with modifications. After derepression of *ADH2* promoter for 12 h (Price 1997), yeast cells were harvested by centrifugation and washed in 0.01 M EDTA (pH 8.0) and sterile water twice and then suspended in 5 ml flocculation buffer (50 mM sodium acetate, pH 4.5) with or without 1 g $CaCl_2$ l^{-1}. After incubated in the water bath at 25°C for 30 min, OD_{600} of the upper 3 ml was measured. Flocculation ability was determined by the equation $F = 1 - B/A$, where F is the flocculation ability, B is the OD_{600} with $CaCl_2$ and A is the OD_{600} without $CaCl_2$. All the tests were repeated three times under the same conditions.

To evaluate sugar inhibition, 0.5 M sugars were added to the flocculation buffer. Yeast cells were killed by incubation for 5 min at 60°C when suspended in EDTA. This treatment did not affect the flocculating ability of yeasts (Stratford and Assinder 1991).

Glucose assay

Glucose concentration of the culture medium was determined by the dinitrosalicylic acid method (Bailey 1988).

Stability analysis

The transformants from YPD slants were cultivated in 5 ml YPD for 14 h at 30°C on a rotary shaker. Cultures were diluted and spread on YPD plates. After incubated for 48 h at 30°C, 100 colonies of each transformant were transferred to 0.5 ml sterile water and starved for 4 h at room temperature. A total of 10 μl of each starved suspension was inoculated on YPD plates containing 6 mM $CuSO_4$ and incubated for 48 h at 30°C (He et al. 2000). Flocculation ability of these colonies (with or without glucose in flocculation buffer) was also determined. The stability of the transformants was determined by their retained copper resistance and flocculation ability.

Fermentation test and diacetyl measurement

Wort (10°Bé) was used for fermentation. Yeast strains were incubated in 5 ml wort for 12 h at 30°C and then 1 ml was inoculated into 10 ml wort and grown for 36 h. The cells were collected and inoculated into 500 ml Erlenmeyer flask containing 270 ml wort. Initial cell density was adjusted to OD_{600} of 0.2. The flasks were cultivated for 16 days at 12°C (Wang et al. 2007).

Diacetyl concentration of the culture media was determined by the colorimetric method (Westerfeld 1945). A total of 1 ml 5% (w/w) creatine and 1 ml

α-naphthol solution (1 g α-naphthol dissolved in 20 ml 2.5 M NaOH, prepared before using) was added to 5 ml sample. The color was allowed to develop at room temperature for exactly 10 min and then A_{540} of the solution was measured.

Results and discussion

Construction of I10 and confirmation of transformants

The recombinant plasmid pIC10 and the expression cassette I10 are shown in Fig. 1a, b. Flocculation is a complex and strain-dependent characteristic of yeast and is influenced by genetic background, environmental factors (which can affect *FLO* gene expression and Flo protein activation), and factors act on the physical interactions between cells. Since *FLONS* possesses only 157 bp putative TATA element upstream of its ORF (Liu et al. 2007), an UAS is needed to regulate its expression to guarantee the proper flocculation behavior of the transformants. *ADH2* promoter is repressed in the presence of glucose and derepressed more than 200-fold when glucose is absent (Price 1997), so it is ideal for the regulation of *FLONS*. As YSF5 is a wild-type yeast strain and has no nutritional marker, the *CUP1* gene that encodes a metallothionein which binds copper and leads to increased resistance of yeast cells to copper ions was adopted for the selection of transformants (Karin et al. 1984). The flanking *ILV2* sequences are designed for homologous recombination of I10 to *ILV2* allele of the YSF5 chromosome. *ILV2* encodes α-acetolactate synthase which catalyze the conversion of pyruvate to acetolactate, a precursor of diacetyl (2,3-butanedione)—an off-flavor component of beer. Removal of diacetyl is crucia1 for beer maturation, and destruction of one *ILV2* gene could lower diacetyl production (Villa et al. 1995; Li et al. 2002). The deletion of *ILV2* and the integration of I10 were confirmed by PCR (Fig. 1c) with the primers PA2 and PI2 (primer of *ILV2*; see Table 1).

Flocculation property of transformants

Flocculation level of 10 randomly selected transformants 5I-1 to 5I-10 was examined (Fig. 2a). Their flocculation ability increased by 1.5 to 2.3 fold of

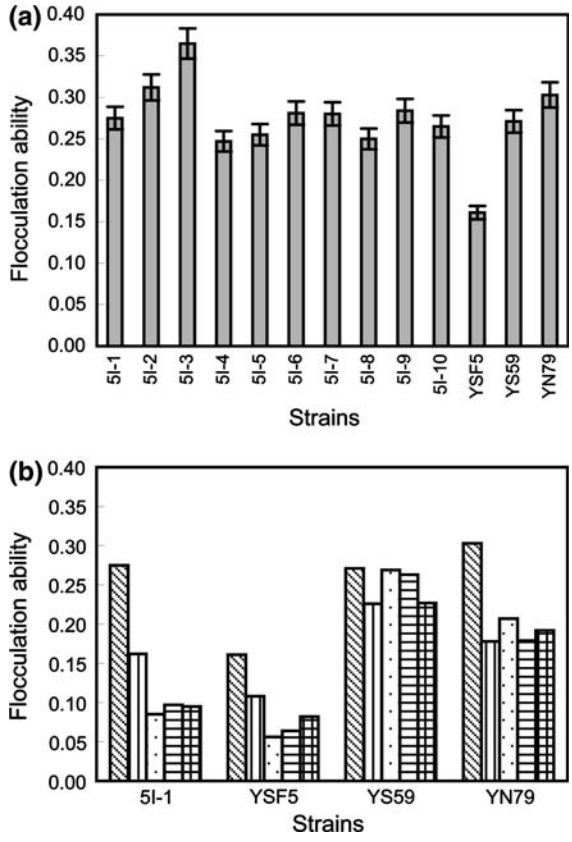

Fig. 2 Flocculation property of transformants. (**a**) Flocculation ability of transformants 5I-1 to 5I-10 compared with YSF5 (the original strain), YS59 (Flo1-type strain) and YN79 (NewFlo-type strain and donor of *FLONS*). (**b**) Effects of sugars (▨, No sugar; ▥, mannose; ⬚, glucose; ▤, sucrose; ▦, maltose) on flocculation ability of 5I-1, YN79, YS59 and YSF5

YSF5 and was equivalent to the positive control strains YS59 and YN79.

To infer the flocculation phenotype of the transformants, their flocculation behavior in the presence of mannose, glucose, sucrose and maltose was examined. Figure 2b shows the flocculation ability of transformant 5I-1 was inhibited by all the four kind of sugars. This was in accordance with the NewFlo-type strain YN79, but different from the Flo1-type strain YS59 which was only repressed by mannose.

In order to determine how long it would take to derepress *ADH2* promoter and get flocculation ability, change of flocculation level of the transformants in the induction process of *ADH2* promoter were recorded. Figure 3 shows that flocculation ability of the transformant 5I-1 displayed little increase comparing with YSF5 at the zero-hour point, but

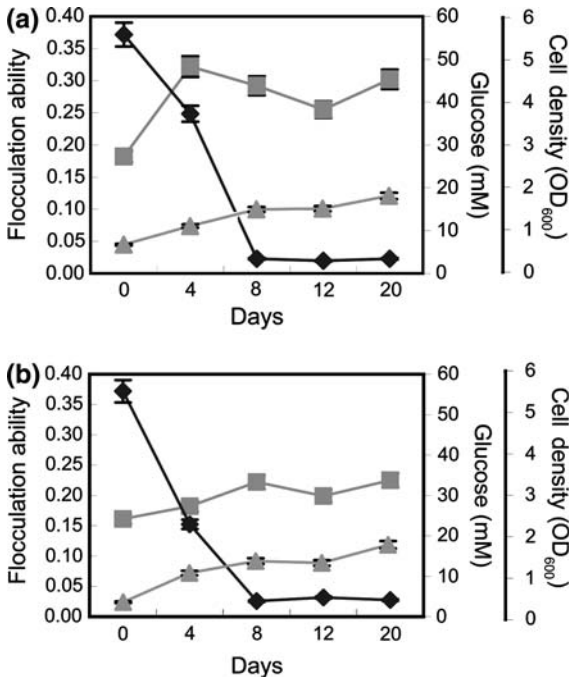

Fig. 3 Change of flocculation ability (■), glucose concentration (◆) and cell density (▲) of transformant 5I-1 (**a**) and the original strain YSF5 (**b**) during induction process of *ADH2* promoter

increased rapidly in the later 4 h as the glucose concentration of the media decreased. The regulation mechanism of *ADH2* promoter is typical carbon catabolite repression. Besides glucose, other sugars involved in metabolism are also able to affect the catabolite repression regulation (Gancedo 1998). Maltose and sucrose are the most wildly used sugars in the fermentation industry, so their effect on *ADH2* promoter was also measured. It was revealed that the effect of maltose and sucrose on the change of flocculation of 5I-1 was similar to that of glucose with peak values of 0.316 and 0.321, respectively.

Genetic stability analysis

Take 5I-1 as an example. All the transformed cells retained copper-resistant ability after successive subculture without selective pressure, indicating that *CUP1* was stable on the chromosome. However, compared with the original 5I-1 strain, slight fluctuation of flocculation ability of randomly selected colonies of 5I-1 after stability analysis was detected (Fig. 4), perhaps due to the fact that the *FLO* genes are very unstable.

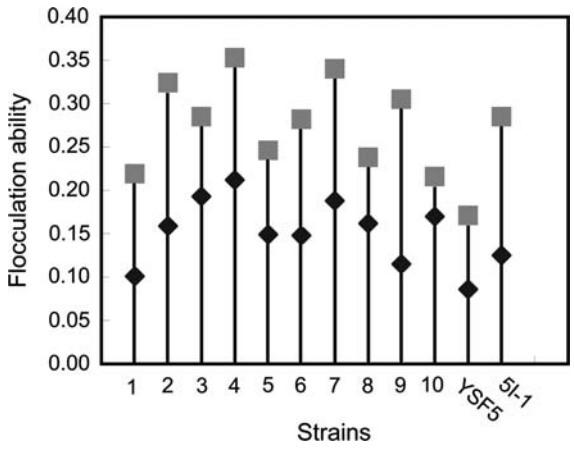

Fig. 4 Flocculation ability (■, no sugar; ◆, glucose added) of 10 randomly selected colonies of the transformant 5I-1 after stability analysis, compared with the host strain YSF5 and the original 5I-1 strain

Fermentation test

A fermentation test simulating beer fermentation was carried out to test the applicability of the transformants in industry (see Fig. 5). During the fermentation process, diacetyl content of the culture media of 5I-1 was lower than that of YSF5, and the peak value of 5I-1 was 83% of that of YSF5, but no obvious difference in fermentation rate (indicated by cell density) between 5I-1 and YSF5 was observed. At the end of simulated fermentation test, diacetyl content of the culture media of 5I-1 was 0.451 g l^{-1}, lower than YSF5 (0.483 g l^{-1}). This could provide a reference for later fermentation test.

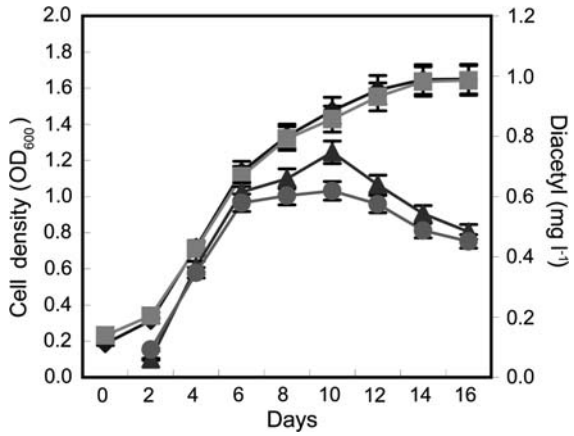

Fig. 5 Diacetyl production (●, 5I-1; ▲, YSF5) and cell density (■, 5I-1; ◆, YSF5) of the transformant 5I-1 and the original strain YSF5 in the fermentation test

Conclusion

The I10 harboring *FLONS*, $P_{U\text{-}ADH2}$, *CUP1* and *ILV2* were introduced into the industrial yeast strain YSF5. Since both the expression of *FLONS* under the control of *ADH2* promoter and the function of Flonsp were repressed by the presence of fermentable sugars, the transformants displayed NewFlo flocculation property toward the end of fermentation when sugar was almost exhausted. This may be desirable in industry. By disruption of *ILV2* allele, decreased diacetyl content were observed in the fermentation test of the transformants. In the stability test, the *CUP1* was stable on the YSF5 chromosome, although slight change of flocculation ability was detected. In addition, other sites of the chromosome can be used instead of *ILV2* to live up to different expectations. As nonyeast DNA was avoided in this study, the transformants were self-cloning strains which could bring down the risk of safety and are expected to provide an easier way to be accepted by the general public.

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