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Identification and functional study of a new *FLO10*-derivative gene from the industrial flocculating yeast SPSC01

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Abstract Yeast flocculation is an important property for the brewing industry as well as for ethanol fermentation to facilitate biomass recovery by sedimentation from the fermentation broth, which is cost-effective. In this study, a new flocculating gene $FLO10_{spsc}$ of 4,221 bp homologous to *FLO10* was identified in the industrial flocculating yeast SPSC01. Sequence analysis indicated that the N- and C-terminus of the deduced protein of this new *FLO* gene are 99 % identical to that of *FLO10*, but more intragenic repeats are included. The study on the function of $FLO10_{spsc}$ by its integrative expression in the non-flocculating industrial yeast indicated severe inhibition in the flocculation of the transformant by mannose and maltose, moderate inhibition by sucrose and glucose and no inhibition by xylose and galactose, and thus the NewFlo type was established. Meanwhile, the flocculation of the transformant was stable when the temperature was below 50 °C and the pH was in the range of 4.0–6.0. Furthermore, the medium containing 250 g/l glucose was completely fermented within 48 h by the transformant, with about 110 g/l ethanol and 5.5 g(DCW)/l biomass produced, and no significant difference in ethanol fermentation performance was observed compared to its wide-type strain. Therefore, the *FLO* gene and corresponding transformation strategy provide a platform for engineering yeast strains with the flocculation phenotype to facilitate biomass recovery.

Keywords Yeast flocculation · Biomass recovery · $FLO10_{spsc}$ · Integrative expression · Ethanol fermentation

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Introduction

The flocculation of *Saccharomyces cerevisiae* is important for the brewing industry, through which yeast cells aggregate and form flocs that can be recovered conveniently from the fermentation broth by cost-effective sedimentation [15]. Although yeast flocculation has been explored for a long time, the mechanism underlying this phenomenon is still not fully understood due to its intrinsic complexity, making its control in the brewing industry dependent to a large extent on expertise. Both medium composition and fermentation conditions affect yeast flocculation [2, 11], but the genetic background of yeast strains ultimately determines their flocculation phenotype, which was highlighted by the expression of the single gene *FLO1* in the non-flocculating laboratory yeast *S. cerevisiae* S288C for its flocculation [9].

Several *FLO* genes, including *FLO1*, Lg-*FLO1*, *FLO5*, *FLO9*, *FLO10*, and *FLO11* have been identified in *S. cerevisiae* and reviewed recently [10, 20]. While Flo1p, Lg-Flo1p, Flo5p, Flo9p, and Flo10p allow yeast cells to form macroscopic flocs [5, 14], Flo11p confers various phenotypes in *S. cerevisiae*, including adhesive and invasive growth, pseudohyphal formation and the formation of biofilm [3, 7, 19]. In addition to the above-mentioned flocculation genes, *FLO8* encodes a transcription factor that is capable of activating the transcription of *FLO1* and *FLO11* in *S. cerevisiae* [1, 4, 6]. Moreover, *FLO* genes contain conserved intragenic tandem repeats, which is responsible for the flocculating strength [16].

Compared to the unstable flocculation of yeast cells triggered by the depletion of fermentable sugars in the brewing industry, the flocculation of the yeast SPSC01 is constitutive and stable [18], which presents a valuable reservoir for exploring new *FLO* genes and their

functions. In this study, *FLO10*$_{spsc}$, a homologue of *FLO10* but with more intragenic repeats, was identified, and its function was explored by its integrative expression in a non-flocculating *S. cerevisiae* for the flocculation phenotype.

Materials and methods

Strains and plasmids

The self-flocculating yeast SPSC01, a protoplast fusant developed for ethanol production from the non-flocculating yeast *S. cerevisiae* K2 and the flocculating yeast *Schizosaccharomyces pombe* and deposited at Chinese General Microbiological Culture Collection Center (CGMCC 0587), was used to identify *FLO10*$_{spsc}$. The non-flocculating yeast *S. cerevisiae* 6525 was used to study the function of the *FLO10*$_{spsc}$. The integrative expression vector pHO10, derived from the sequence of *HO*-poly-*KanMX4-HO* [17], was constructed in this study.

Culture conditions

E. coli DH5α was used as cloning host and grown at 37 °C and 200 rpm in LB medium containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl supplemented with 50 μg/ml ampicillin. Yeast strains were cultured at 30 °C and 150 rpm in YPD medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose, and the resistant strains were selected on the YPD agar plate supplemented with 1 M sorbitol and 300 μg/ml G418, incubating for 48 h at 30 °C after electroporation transformation. The LB and YPD media were solidified by adding 1.5 % agar.

Construction of the integrative expression vector and yeast transformation

The constitutive *PGK1* promoter from *S. cerevisiae* S288C, *FLO10*$_{spsc}$ from SPSC01, and *CYC1* terminator from the plasmid p424GPD [8] were amplified by PCR using the primers P1–P6 (Table 1). The integrative expression vector pHO10 was constructed from the plasmid *HO*-poly-*KanMX4-HO*. The *PGK1* promoter, *FLO10*$_{spsc}$ and *CYC1* terminator were inserted sequentially (Fig. 1). Then, the pHO10 was cleaved by *Not* I for the fragment containing the expression cassette to transform *S. cerevisiae* 6525 at its *HO* locus by electroporation using the MicroPulser (Bio-Rad, Hercules, CA, USA). The transformant was incubated for 48 h at 30 °C on the YPD agar plate containing 1 M sorbitol and 300 μg/ml G418, and the positive ones were selected, correspondingly.

Table 1 Primers used in this work

Primer	Nucleotide sequence (5′ → 3′)
P1	GCACGTACGACTGTAATTGCTTTTAGTTGT
P2	CCGCCCGGGTGTTTTATATTTGTTGTAAAAAGT
P3	TATCCCGGGATGCCTGTGGCTGCTCGATAT
P4	GCCTTAATTAAACGATTGCCAGTAATAGGGA
P5	CCGTTAATTAATCATGTAATTAGTTATGTCA
P6	TTAGCGCGCAAATTAAAGCCTTCGAGC

P1 and P2 for *PGK1* promoter, P3 and P4 for *FLO10*$_{spsc}$, P5 and P6 for *CYC1* terminator, underlined regions of the primer sequences are restriction sites

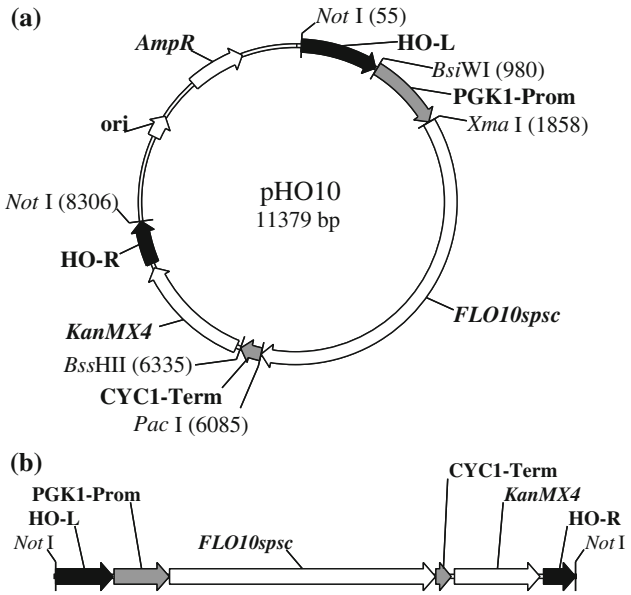

Fig. 1 Development of the recombinant plasmid (**a**) and integrative expression cassette (**b**)

Impact of medium composition on the flocculation of the transformant

The selected transformant was grown in a flask with the YPD medium for 24 h at 30 °C and 150 rpm, harvested through sedimentation and washed two times with the de-flocculating buffer (0.1 M sodium citrate, pH4.5) to de-flocculate the yeast flocs so that biomass concentration could be controlled accurately for further study.

The deflocculated yeast cells were adjusted to the OD_{620} of 2.0, and 20 ml suspension was sampled and centrifuged to collect cell pellets, which were washed two times with the flocculating buffer (50 mM sodium acetate, 0.1 % $CaCl_2$, pH 4.5) and suspended to 20 ml for yeast cells to re-flocculate. The impact of temperature, pH, sugar concentrations (0–0.6 M) including mannose, glucose, sucrose, maltose, galactose, and xylose and Ca^{2+} on the flocculation was evaluated with triplicate experiments.

Ethanol fermentation

The yeast was incubated in flask with the YPD medium for overnight at 30 °C, 150 rpm, and yeast flocs were collected and washed two times with the de-flocculating buffer. Then, 1 ml de-flocculated yeast cell suspension was adjusted to the OD_{620} of 1.0 and inoculated into a 250-ml flask containing 100 ml of fermentation medium composed of 5 g/l yeast extract, 10 g/l peptone, and 250 g/l glucose. The ethanol fermentation was carried out at 30 °C and 150 rpm.

Analytical methods

Glucose and ethanol were analyzed by the Biosensor (SBA-40C, China), and biomass (dry cell weight) was evaluated at the end of fermentation by collecting all yeast cells, washing three times with deionized water, and drying to a constant weight at 85 °C.

For yeast cell flocculation assay, the sedimentation performance was evaluated based on the method previously developed [5]. The de-flocculated yeast cell suspension (OD_{620} = 2.0) of 20 ml was centrifuged, and the cell pellets were collected, washed two times with deionized water, and suspended in the flocculating buffer to recover their flocculation. The re-flocculated yeast cells were rested for 5 min, and the OD_{620} of the supernatant was measured. The flocculation ability C was estimated by the following equation

$$C = (1-B/2) \times 100\%,$$

where B is the OD_{620} of the supernatant. Standard deviations were analyzed for all analytical data with three independent measurements.

Results and discussion

Sequence analysis of *FLO10*$_{spsc}$

The gene *FLO10*$_{spsc}$ was sequenced by the TaKaRa Company, Dalian, China, and the result was submitted to GenBank with an accession number HQ447085. The sequence analysis revealed a 4,221-bp open reading frame that encodes a protein of 1,406 amino acids. Moreover, both the DNA and amino acid sequences of the N- and C-terminal regions of the protein encoded by *FLO10*$_{spsc}$ were 99 % identity with those of the corresponding regions of the protein encoded by *FLO10* in *S. cerevisiae*. No homologous gene of *FLO10*$_{spsc}$ was found in *S. pombe* by BLAST search. Two repeat units A and B encoding for 27 and nine amino acids, respectively, were identified. The alignments of the 18 repeats in the unit A exhibit an identity of 56 %, while all five repeats in the unit B are identical (Fig. 2). In contrast, the numbers of the repeat units A and B in *FLO10* are ten and four, respectively [13]. The statistic analysis of the amino acid residues indicates that the Flo10p$_{spsc}$ is abundant with Ser and Thr (43 %), especially in the repeat regions (54 %).

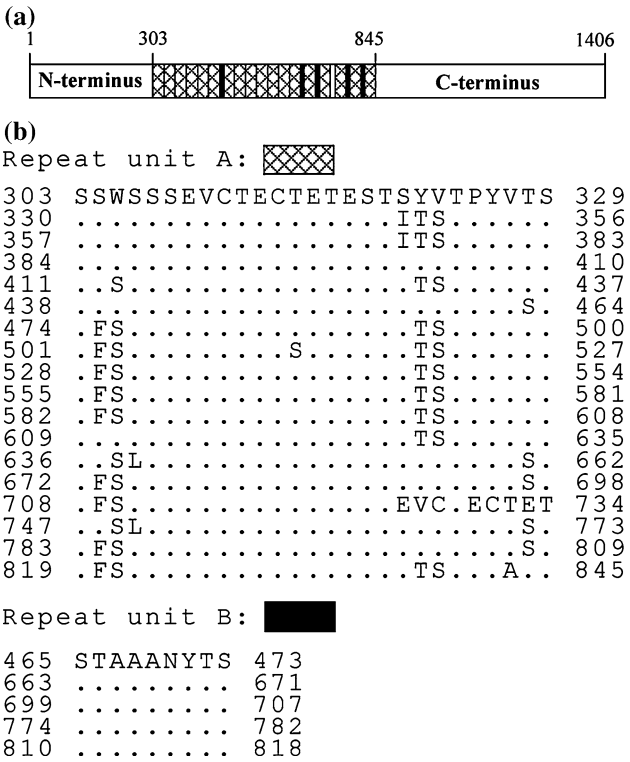

Fig. 2 Schematic diagram of Flo10p$_{spsc}$ (**a**) and the internal repeat regions (**b**). The *black dots* indicate identical amino acid residues to the first line sequence

Introduction of the *FLO10*$_{spsc}$ into the industrial yeast *S. cerevisiae* 6525

To assess the function of *FLO10*$_{spsc}$, its integrative expression in the non-flocculating industrial yeast *S. cerevisiae* 6525 was performed. The positive transformant exhibited the flocculation phenotype when grown in the YPD medium (Fig. 3). The PCR amplification of the genomic DNA confirmed the correct integration of *FLO10*$_{spsc}$ and its integration into the host strain under the transcriptional control of the *PGK1* promoter and the *CYC1* terminator, resulting in the acquisition of the flocculation phenotype, since no flocculation was observed for the transformant when the empty vector containing the *KanMX4* selective marker flanking by the *HO* fragments was integrated into the same strain, confirming that the acquired flocculation phenotype was contributed by *FLO10*$_{spsc}$. The stability of the flocculating phenotype was investigated by

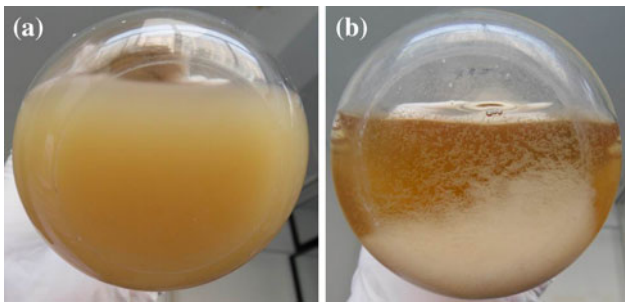

Fig. 3 Phenotypes of the wild-type yeast *S. cerevisiae* 6525 (**a**) and the transformant constitutively expressing $FLO10_{spsc}$ (**b**). Yeast cells were incubated in the YPD medium at 30 °C and 150 rpm for 24 h

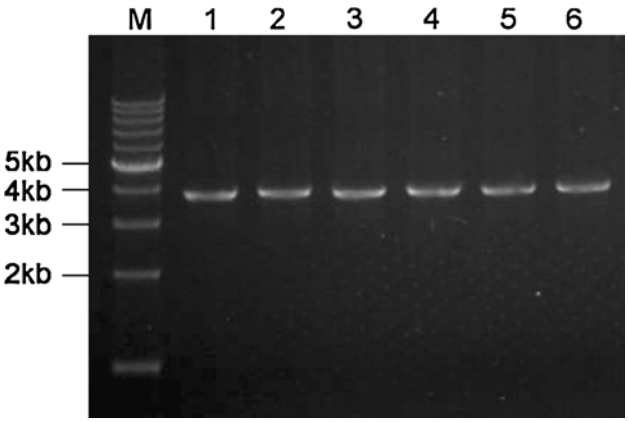

Fig. 4 PCR products of $FLO10_{spsc}$ from SPSC01 and 6525 transformants. M: 1-kb DNA marker; *Lanes 1* and *2*: SPSC01; *Lanes 3* and *4*: Original 6525 transformant; *Lanes 5* and *6*: 6525 transformant subcultured for 2 weeks

growing the transformant in the YPD medium without selective pressure and subculturing daily. After 2 weeks, the G418 resistance and flocculation of transformant were not changed significantly, compared to the original strain. In addition, we amplified the $FLO10_{spsc}$ gene by PCR with primers P3 and P4 from SPSC01 and 6525 transformants, and no significant difference was observed in the size of $FLO10_{spsc}$ PCR product (Fig. 4).

Effect of temperature, pH, sugars, and Ca^{2+} on the flocculation phenotype

The impact of temperature, pH, sugars, and Ca^{2+} on the flocculation of the transformant was further studied. Temperature in the range of 20–50 °C had no significant effect on the flocculation of the transformant (Fig. 5a), but the sedimentation of yeast flocs deteriorated rapidly when the temperature exceeded 50 °C. Free yeast cells were observed under microscope when the temperature increased to 70 °C, and the deflocculation was unrecoverable when the temperature was decreased, which might be due to the irreversible conformation alteration of the flocculating protein molecules under elevated temperature conditions, preventing them from binding with the cell wall mannose residues of neighboring cells to form flocs. The stable flocculation was observed for the transformant when pH changed from 4.0 to 6.0 (Fig. 5b), since the concentration of hydrogen ions affects surface charge of yeast cells, and the electrostatic repulsion among cells is weakened under acidic conditions, which is beneficial to the flocculation of yeast cells [11]. However, the flocculation decreased obviously when the pH was below 3.0, which maybe resulted from the denaturation of the flocculating protein at the lower pH [15].

The flocculation of the transformant was inhibited by sugars with the following sequence: mannose > maltose > sucrose > glucose > xylose > galactose, which confers different sensitivity of sugar inhibition: severe inhibition by mannose and maltose, moderate inhibition by sucrose and glucose, and no inhibition by xylose and galactose (Fig. 5c). When the sequence of the deduced product of $FLO10_{spsc}$ was analyzed, it was found that the N-terminus sugar recognition region is the same with that of *FLO10* [14]. However, the two sequences differ largely in the number of the repeated units. According to the difference in sugar sensitivity, yeast flocculation is classified into two main groups: Flo1 type is inhibited by mannose and presented in most laboratory strains; NewFlo type is inhibited by mannose and glucose, and is found in many brewing industrial strains [12, 13]. The experimental data showed that the transformant is the NewFlo phenotype.

Ca^{2+} is necessary for maintaining the active conformation of the flocculating protein [11], and the flocculation of the transformant was improved with the increase of Ca^{2+} concentration, but the saturation phenomenon was observed when Ca^{2+} was increased to about 10 mM under the designated biomass concentration (Fig. 5d).

Ethanol fermentation performance of the transformants

In order to compare the fermentation performance of the transformants with their wild-type strain, ethanol fermentation was carried out with initial glucose concentration of 250 g/l. The glucose consumption, ethanol production, biomass accumulation, and ethanol yield were shown in Table 2. As can been seen, glucose was consumed within 48 h, and about 110 g/l ethanol and 5.5 g(DCW)/l biomass were produced, correspondingly. No significant difference was observed in the growth and ethanol fermentation performance between the transformants and their wild-type strain.

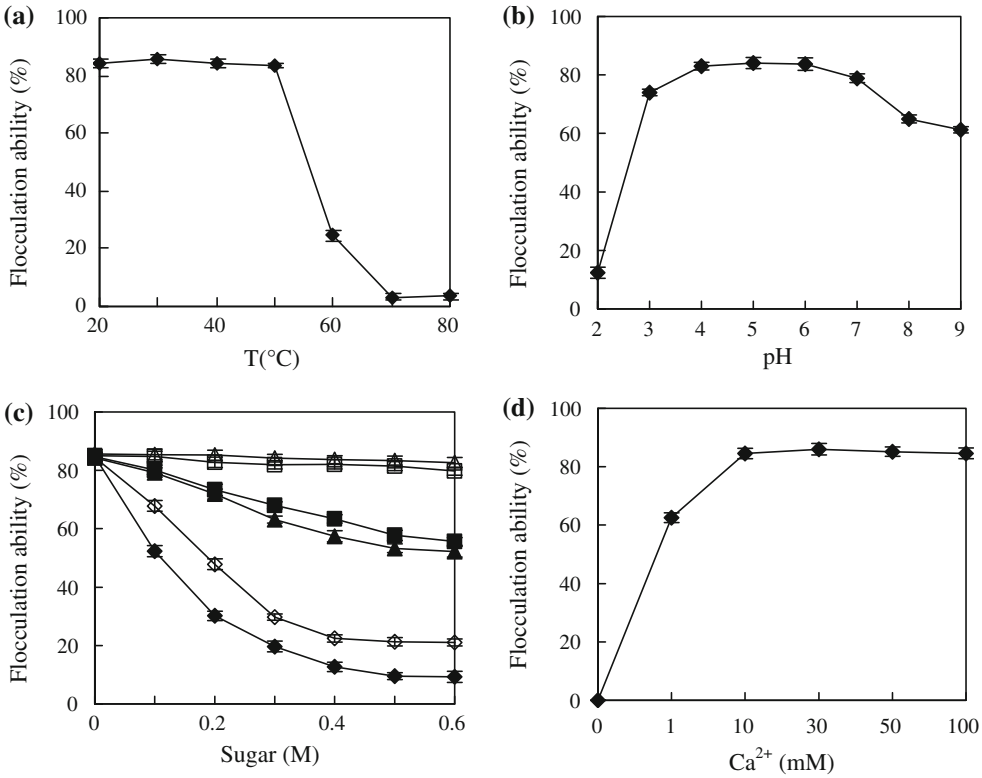

Fig. 5 Effect of temperature, pH, sugars, and Ca^{2+} on the flocculation of the transformant. *Filled diamond* mannose, *filled square* glucose, *filled triangle* sucrose, *open diamond* maltose, *open square* xylose, and *open triangle* galactose

Table 2 Ethanol fermentation performance of the transformants and their wild-type strain

Strains	Ethanol (g/l)	Residual glucose (g/l)	Biomass (g(DCW)/l)	Ethanol yield (%)
6525	109.3 ± 2.3	0.48 ± 0.20	5.53 ± 0.22	43.82 ± 0.93
6525HO[a]	111.3 ± 3.1	0.45 ± 0.18	5.49 ± 0.18	44.61 ± 1.22
6525FLO10[b]	110.0 ± 2.0	0.52 ± 0.13	5.50 ± 0.24	44.09 ± 0.80

[a] 6525HO: the empty vector *HO*-poly-*KanMX4-HO* integration in 6525

[b] 6525FLO10: 6525 carrying the $FLO10_{spsc}$

Conclusions

The new functional *FLO* gene was identified from the industrial flocculating yeast SPSC01, and the sequence analysis indicates that it is a derived form of *FLO10* with a full length of 4,221 bp encoding a protein of 1,406 amino acids. The transformant developed by the integrative expression of $FLO10_{spsc}$ into the non-flocculating industrial yeast displays the NewFlo flocculation phonotype, which is thermo-stable when the temperature is in the range of 20–50 °C, and not affected significantly by the pH change from 4.0 to 6.0. Moreover, the transformant exhibits similar growth and ethanol fermentation performance to its host strain, indicating that the *FLO* gene and corresponding transformation strategy could be a platform for engineering yeast strains with the flocculation phenotype to facilitate biomass recovery.

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