Iranian J Biotech. 2019 April; 17(2): e1990 DOI: 10.21859/ijb.1990
Research Article

Increased Acetate Ester Production of Polyploid Industrial Brewer's Yeast Strains via Precise and Seamless "Self-cloning" Integration Strategy

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Abstract

Background: Enhancing the industrial yeast strains ethyl acetate yield through a precise and seamless genetic manipulation strategy without any extraneous DNA sequences is an essential requisite and significant demand.

Objectives: For increasing the ethyl acetate yield of industrial brewer's yeast strain, all the *ATF1* alleles were overexpressed through "self-cloning" integration strategy.

Material and Methods: *Escherichia coli* strain DH5α was utilized for plasmid construction. *ATF1* alleles were overexpressed through a precise and seamless insertion of the *PGK1* promoter in industrial brewer's yeast strain S6. In addition, growth rates, *ATF1* mRNA levels, AATase activity, the fermentation performance of the engineered strains, and gas chromatography (GC) analysis was conducted.

Results: The two engineered strains (S6-P-12 and S6-P-30) overexpressed all *ATF1* alleles but unaffected normal growth. The *ATF1* mRNA levels of the S6-P-12 and S6-P-30 were all 4-fold higher than that of S6. The AATase (Alcohol acetyl transferases, encoded by *ATF1* gene) activity of the two engineered strains was all 3-fold higher than that of the parent strain. In the beer fermentation at 10 °C, the concentrations of ethyl acetate produced by the engineered strains S6-P-12 and S6-P-30 was increased to 23.98 and 24.00 mg L^{-1}, respectively, about 20.44% and 20.54% higher than that of S6.

Conclusions: These results verify that the ethyl acetate yield could be enhanced by the overexpressed of *ATF1* in the polyploid industrial brewer's yeast strains via "self-cloning" integration strategy. The present study provides a reference for target gene modification in the diploid or polyploid industrial yeast strains.

Keywords: Acetate Ester, *ATF1*, Polyploidy; *PGK1*

1. Background

During beer fermentation, the beer yeast strains produce higher alcohols and aromatic esters that affect the beer's organoleptic characteristics (1-3). These compounds are produced by yeast and have highly significant impacts on the smell and taste of the resulting beer. Beer quality is affected by the proportions of higher alcohols and esters in the fermentation production (4-6). However, the conventional genetic manipulation is difficult to regulate the synthesis of the higher alcohols and esters duo to polyploid industrial brewer's yeast strains cannot produce energetic spores. Thus, development of methods by which to modulate the proportions of the higher alcohols and esters is of great importance in the polyploid industrial brewer's yeast.

To achieve the maximum concentration of acetate ester, many attempts have been made to increase the activity of alcohol acetyl transferases (AATase). Previous reports have proved that transformants carrying multiple copies of the *ATF1* gene exhibited higher AATase activity and produced greater concentrations of the acetate esters than the control strain with one *ATF1* gene (7), but these transformants remained shuttle vectogene sequence. AATase activity was also increased

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through the overexpression the alcohol acetyltransferase gene (*ATF1*) by the *PGK1p-ATF1 -PGK1t* overexpression cassette (8, 9). However, the resulting strains could not be approved by human usage due to the introduction of the restriction site. The *BAT2* allelic genes were replaced by overexpression cassette *PGK1p-ATF1-PGK1t* for enhancing acetate ester synthesis in the industrial brewer's yeast strains (10). The method does affect an increase in the acetate ester content but the resultant yeast strains may be unsafe as the excision of the marker gene excision leaves behinds a single *loxP* site. Similarly, other target gene modifications using the recombinase-mediated marker excision system can result in unexpected deletions or chromosome rearrangements (11, 12). The "self-cloning" strategy was conducted as described by Dong, Walgate R, and Hirosawa (13, 14). In the recent study, this integration strategy has been used for overexpressing of the gene in the haploid yeast strains or to modify diploid strains (15, 16). Moreover, the method is useful in the introduction of the site-directed mutagenesis (17, 18). Therefore, we attempted to constructe polyploid industrial brewer's yeast strains with an increased the AATase I activity via "self-cloning" integration strategy.

2. Objective

In this study, *ATF1* alleles were overexpressed via "self-cloning" integration strategy in the S6 strain, the *URA3* gene was used as the selectable marker. A plasmid carrying a fusion fragment *ATF1p-PGK1p-ATF1* expression cassette was linearized and subjected to our integration protocol, resulting in strains without any extraneous DNA sequences. The mRNA levels of *ATF1* and the acetyltransferase activity in the transformant were investigated. The strains generated with this approach showed an increased ethyl acetate yield in the beer fermentation at 10 °C, demonstrating that our approach is an effective method for the development of the polyploid industrial brewer's yeast strains with an improved the taste of resulting beer.

3. Materials and Methods

3.1. Materials

The mediums used in experimental procedure were included of (LB medium: 10 $g.L^{-1}$ tryptone, 5 $g.L^{-1}$ yeast extract, and 5 $g.L^{-1}$ NaCl, pH 7.0) and ampicillin (100mg.L^{-1}) that was used for plasmid selection, YEPD medium (1 $g.L^{-1}$ yeast extract, 2 $g.L^{-1}$ peptone, and 2 $g.L^{-1}$ glucose), SC-ura3 medium (6.7 $g.L^{-1}$ yeast nitrogen base without amino acids, supplemented with all the auxotrophic requirements except uracil, and 20 $g.L^{-1}$ glucose), 5-Fluoroorotic acid (5-FOA) medium (6.7 $g.L^{-1}$ yeast nitrogen base without amino acids supplemented with all the auxotrophic requirements, 20 $g.L^{-1}$ glucose, and 2 $g.L^{-1}$ 5-fluoroorotic acid). All solid media used in this study have contained 2% agar. Fungal mRNA out kit, Quantscript RT kit and qRT-PCR SYBR green kit were obtained from Tiandz Biotech, Beijing, China. Ethyl acetate and isoamyl acetate were purchased from Merck (USA). *Bam*HI and *Kpn*I were purchased from Akara Biomedical Technology (Beijing) Co., Ltd. *Escherichia coli* strain DH5α was utilized for plasmid construction and propagation. 1.0 ml Tris–HCl (pH 7.5, 100 mmol.L-1), 20 ul ethanol (0.513 M), and 20 ul Acetyl-CoA (10 mg.mL-1) were used for enzyme activity assays. Acetyl-CoA was purchased from Solarbio.

3.2. Strains, Vectors, and Culture Conditions

The genetic properties of all strains and plasmids are listed in **Table 1**. *E. coil* was incubated in the Luria–Bertani medium at 37 °C, and ampicillin (100 mg.L^{-1}) was added for plasmid selection. The yeast strain was grown at 30 °C in YEPD medium. SC-ura3 mediumat 30 °C. 5-Fluoroorotic acid (5-FOA) medium was used only for the selection of uracil auxotrophic transformants.

3.3. Plasmid Construction

Plasmids YIplac211 was used for recombinant plasmids construction (19). DNA fragments were prepared as previously report (20). The primers are listed in **Table 2**.

Table 1. Strains and plasmids used in the current study

Strains or plasmids	Relevant characteristic	Reference or source
	Strains	
***E. coli.* DH5a**	supE44 DlacU169(u 80lacZDM15) hsdR17 recAl endAl gyrA96 thi-1 relA	Stratagene
W303-1A	*MATa*	wan k et al (30)
S6	Wild-type industrial brewer's yeast	Hao et al(29)
S6-ura3	Wild-type industrial brewer's yeast(mutant *URA3* gene)	This work
S6-P-10	Wild-type industrial brewer's yeast(partial *ATF1* allelic genes overexpressed)	This work
S6-P-12	Wild-type industrial brewer's yeast(all *ATF1* allelic genes overexpressed)	This work
S6-P-30	Wild-type industrial brewer's yeast(all *ATF1* allelic genes overexpressed)	This work
	Plasmids	
YIplac211	Amp^r *URA3*	(31.32)
YIplac211-UPD	Amp^r *URA3* containing *ATF1p-PGK1p-ATF1*	This work

Table 2. Primers used in this study

Primer name	sequence[a]	Restriction site
URA3-F	AGGAAGGAGCACAGACTTA	None
URA3-R	GTTACTTGGTTCTGGCGAG	None
pATF1-F	5'CGCGGATCCGCCATAAATATTCCTGTAATGAC3'	*Bam*HI
pATF1-R	5'ATCAAGAATGTAATTTTCAGTTTTGGATAGATCAGTTAGAGAGAGCTGATAAATTGATG3'	None
pPGK-F	5'GCACTTCATCAGTATCACAAATACCATCAATTTATCAGCTCTCTCTAACTGATCTATCC3'	None
pPGK-R	5'GCACGGGGGCCTGATTTTTCTCATCGATTTCATTCATGTTTTATATTTGTTGTAAAAAG3'	None
ATF1-F	5'TCAAGGAAGTAATTATCTACTTTTTACAACAAATATAAAACATGAATGAAATCGATGAG3'	None
ATF1-R	5'CGGGGTACCGCGGGGATAAAAATATCCG3'	*Kpn*I
YIPlac211-UPD-F	5'CGGCTAATTTGTCTTCCA3'	None
YIPlac211-UPD-R	5'CGTGCTGCTACTCATCCT 3'	None
YIPlac211-UD-F	5'CCCCAGGCTTTACACTTTAT3'	None
YIPlac211-UD-R	5'ATGCCCATTCTGAATCATCT3'	None
UD-F	5'CGGCACTTCATCAGTATCAC3'	None
UD-R	5'CAGGCAGATTTCCCTCAAAG3'	None
UP-F	5'GCACAGGTAGATATAAGCTC3'	None
UP-R	5'GTATGCGATAGTTCCTCACTC3'	None
PD-F	5'GACTTCAACTCAAGACGCACAG3'	None
PD-R	5'GAAGACCGACCATCAGACAT3'	None
RTATF1-F	5'GGGTTCAATATACAAGGCTTCG3'	None
RTATF1-R	5'GCATCGGGCTCCTCTAACTG3'	None
ACT1-F	5'TTATTGATAACGGTTCTGGTATG3'	None
ACT1-R	5'CCTTGGTGTCTTGGTCTAC3'	None

[a] Relevant restriction sites are underlined.

Table 3. The fermentation performance of the engineered strains and the parental strain

Yeast strains	Weight loss of CO_2, g	Ethanol, %, v/v, 20 °C	residual glucose, g/liter	Apparent degree of fermentation, %	Real degree of fermentation, %
S6	5.10 ± 0.05	4.64 ± 0.02	4.80 ± 0.05	74.68 ± 0.05	66.86 ± 0.02
S6-P-12	5.00 ± 0.05	4.63 ± 0.03	4.80 ± 0.10	75.23 ± 0.09	66.82 ± 0.04
S6-P-30	5.10 ± 0.05	4.62 ± 0.04	4.85 ± 0.15	73.29 ± 0.07	66.80 ± 0.02

The fragment of the upstream flank of *ATF1* （U） was amplified from the genomic DAN of S6 strain using primer pairs pATF1-F, containing the restriction site for *Bam*HI and nucleotides +1 to +23 of the U, and pATF1-R, nucleotides +1029 to +1047 of U (**Table 2**). The fragment of the *PGK1* promoter (P) and the downstream homologous fragment of *ATF1* (D) was similarly amplified using the pPGK1-F, pPGK1-R and ATF1-F, as well as ATF1-R primers. Primer ATF1-F containing the restriction site for *Kpn*I. In the fusion PCR, overlapping sequences served as primers to amplify the sequences. PCR, in which the purified *ATF1p* and *PGK1p* fragments were invoked as templates without primer addition was first conducted. Cycling parameters were included of annealing temperature 55 °C (45 s), the subsequent extension step at 72 °C (1.5 min), and 35 cycles in total. Then, PCR product was purified through gel extraction invoked as the template with primers U-F and P-R (**Table 2**) to generate gene sequence of the UP. Subsequently, these sequences were also purified and invoked as the template with primers U-F and D-R (**Table 2**) to generate gene sequence of the UPD. The resulting fusion PCR products were double digested by *Bam*HI–*Kpn*I, purified through gel extraction and were inserted into the same enzyme pair-digested plasmid YIplac211, resulting in plasmids YIplac211-UPD (**Table 3**). The homologous fragments of this plasmid were amplified using the designed primers YIPlac211-UPD-F, YIPlac211-UPD-R and YIPlac211-UD-F, YIPlac211-UD-R (**Table 2**).

3.4. Yeast Transformation and Screening

The mutant *URA3* gene was amplified from genomic DNA isolated from the industrial strain W303-1A using primer pairs URA3-F and URA3-R. Then, the mutant *URA3* gene fragment was transformed into the industrial brewer's yeast strain S6 using the LiAc method (21), creating the strain S6-ura3. Transformations were spread onto SC plates and verified by cultured on SC-5-FOA plates. The plasmid YIplac211-UPD was linearized with *Nru*I and transformed into industrial brewer's yeast strain S6-ura3, using the LiAc method described by Schiestl and Gietz (20). The first-step resulting strains S6-ΔU-U were verified by primer pairs of YIPlac211-UPD-F / YIPlac211-UPD-R, YIPlac211-UD-F / YIPlac211-UD-R (**Table 2**). Then, the transformant obtained from the first-step integration growth onto SC-5-FOA plates and verified by colony PCR, creating strains CLy12a-U-P (**Table 1**). To recovery the mutant *URA3* gene (*ura3*), the second-step integration was cultured and carried out as lithium acetate procedure reported previously (21). Then, the yeast strains were spread onto SC plates and

verified by culturing on SC-5-FOA plates. Standard molecular genetic techniques were used for nucleic acid manipulations (22).

3.5. Real-time Quantitative PCR (RT-qPCR) and Enzyme Activity Assays

Samples for total RNA extraction were prepared according to our previously work (16). The relative quantification of *ATF1* and *ACT1* mRNA was determined by qRT-PCRSYBR green kit using primer pairs RTATF-F, RTATF-R and ACT1-F, ACT1-R, respectively (**Table 2**). The quantitative real-time PCR was conducted using a Roche Light Cycler 480 Real-Time PCR machine, and the final data were calculated using the threshold cycle ($2^{-\Delta\Delta CT}$) method (23).

The *ATF1*-encoded AATase activity was measured using the method reported by Fujii et al. (24). AATase I assays were conducted for 150 xg at 30 °C for 6 h in a reaction medium containing certain weight of centrifuged yeast cell, 1.0 ml Tris–HCl (pH 7.5, 100 mmol.L^{-1}), 20 ul ethanol (0.513 M), and 20 ul Acetyl-CoA (10 mg.mL^{-1}). The produced ethyl acetate's concentration of was measured by gas chromatography–mass spectrometry (GC–MS). One unit of AATase I activity was defined as the amount of enzyme per 1 g of yeast cells obtained by centrifugation at definite g force (you should say what g force or rpm) that could produce 1 μmol of ethyl acetate per h at 25 °C.

3.6. Fermentation Test

3.6.1. Seed Culture

Control strain and engineered strains were cultured in 5 mL of 11°P wort medium at 30 °C for 12 h. Then transferred into 45 mL of the wort medium at 16 °C for 36 h.

3.6.2. Beer Fermentation

A volume of 15 mL of the primary culture was transferred into 135 mL of wort medium and incubated at 10 °C for 8–10 days. The fermentation was processed untill the weight loss of CO_2 after an interval period of 12 h was less than 0.1 g.

3.6.3. Fermentation Performance Analysis

The fermentation performance of the CO_2 weight loss, residual sugar, apparent degree of fermentation, real degree of fermentation and ethanol production were determined, respectively. Production of esters compounds was determined using gas chromatography (GC) analysis.

3.6.4. Gas Chromatography (GC) Analysis

Samples from the wort medium were filtered and distilled after fermentation and then were used for GC analysis. The analysis was performed according to our previously work (16).

4. Results

4.1. Construction of Engineered Brewer's Yeast Strains

URA3 gene was mutated in parent strain S6 via transformation, resulting strain S6-ura3 was verified with SC plate and SC-5-FOA plate (**Fig. 2** and **Fig. 4**). The two-step integration strategy is our previously work, which was performed to construct the engineered strains with overexpressing *ATF1* gene by the promoter *PGK1p*. The length of *ATF1p, PGK1p* and *ATF1* was 1,048-, 1,479- and 1,046-bp respectively (**Fig. 1**). The resulting recombinants were verified via PCR using the primer pairs of YIPlac211-UPD-F / YIPlac211-UPD-R, YIPlac211-UD-F / YIPlac211-UD-R (**Table 2**) with S6-ura3 (negative) and plasmid YIplac211-UPD (positive) as controls.

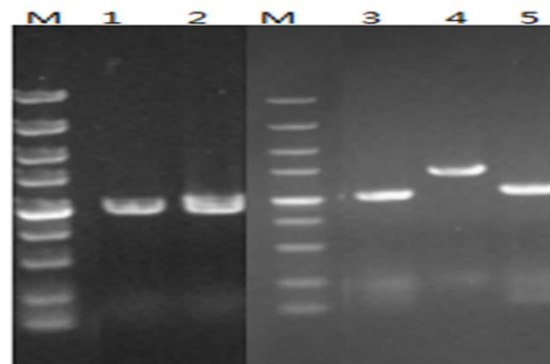

Figure 1: Agarose gel analysis and sequence analysis.
M, 5,000-bp DNA ladder marker; *lane 1* (mutant gene *ura3*) and *lane 2*(gene *URA3*), PCR amplification results from the W303-1A and S6 genome, respectively, using primers URA3-F and URA3-R; *lane 3, lane 4,* and *lane 5*, PCR amplification results from S6 genome, respectively, using primers pATF1-F / pATF1-R, pPGK-F / pPGK-R and ATF1-F/ATF1-R

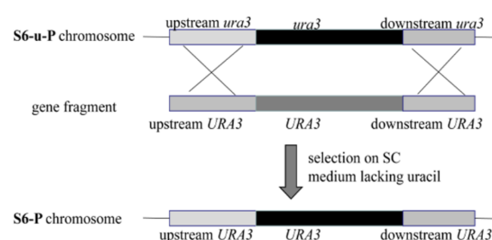

Figure 2. The procedure of recovery of the mutant gene *ura3*.

When UP-F and UP-R, PD-F and PD-R (**Table 2**) primer pairs were used to verify the recombinants, PCR products were amplified with S6-ura3 negative control. When UD-F and UD-R were used to verify the recombinants, S6-P-10 appeared two lanes, S6-P-12 and S6-P-30 appeared one lane. Results showed *PGK1p* was inserted into upstream of the ORF of the gene *ATF1*. Furthermore, at least one *ATF1* allelic gene of resulting strain S6-u-P-10 was inserted *PGK1p* and all *ATF1* allelic gene of the S6-u-P-12 and S6-u-P-30 were inserted *PGK1p*. Moreover, results of sequencing results indicated the precise insertion of *PGK1p* in the 5′-terminal of the target gene (*ATF1*) without any extraneous residual DNA. The mutant gene *(ura3)* and

the normal gene *(URA3)* were compared the results of which are shown in the **Figure 3** and **Figure 4**. The ura3 gene of S6-u-P was recovered via the method as described before.

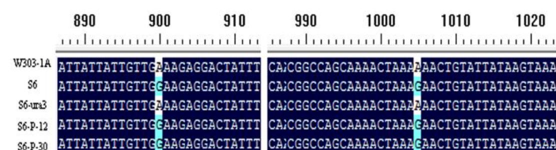

Figure 3. The genetic comparisons of *URA3* gene, controlled W303-1A, S6, S6-ura3, S6-P-12, and S6-P-30, using primers URA3-F and URA3-R.

In addition, we selected 36 colonies to verify *URA3* marker elimination. The frequency of pop-up is approximately 10^{-4}, which is similar to the *hisG* repeats reported previously (25). As well, the desired insertion occurred with a frequency of approximately 10^{-5}, which are consistent with our previous studies (15, 16).

4.2. Analysis of ATF1 mRNA Levels and Measurement of AATase Activity

The *ATF1* mRNA levels and measurement of AATase activity of parental strain and engineered strains were performed, respectively. The RT-qPCR results and AATase activity in the engineered strain were 4- and 3-fold higher than that of parental strain, respectively (**Fig. 5A** and **Fig. 5B**). These results confirmed that the *ATF1* gene was overexpressing by *PGK1p* insertion with an increase of the gene expression and the enzyme activity.

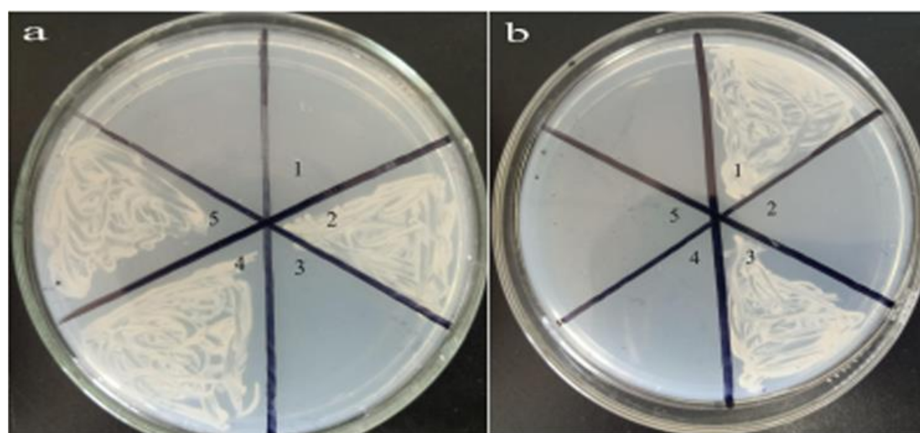

Figure 4. Growth of W303-1A, transformants, and the parent S6 on SC plates (a) and SC-5-FOA plates (b) at 30 °C for 24 h. *1* W303-1A, *2* parent strain S6, 3 S6-ura3, *4,* and *5* show the transformants

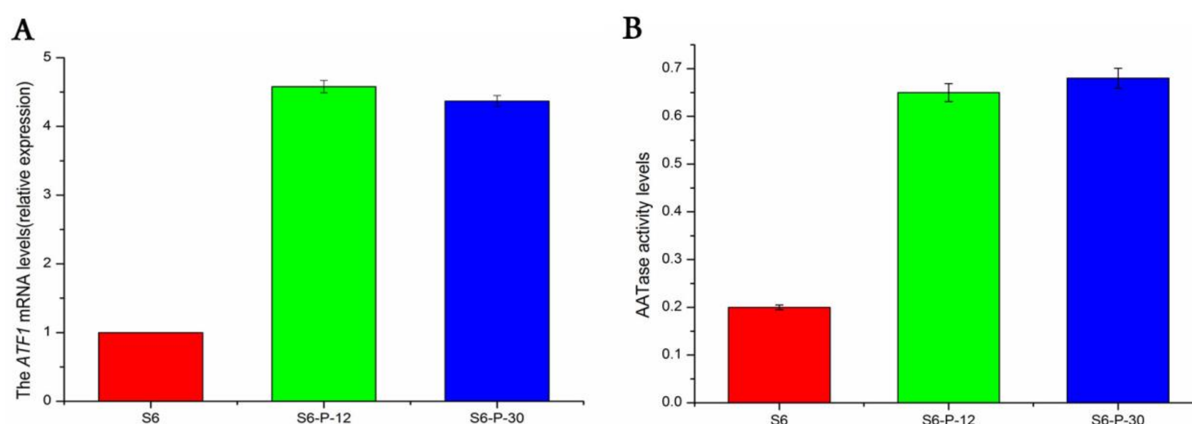

Figure 5. (A) The *ATF1* mRNA levels (relative expression) and (B) AATase activity levels of the strains S6, S6-P-12, and S6-P-30. The experiments were repeated three times. Data are the average of THE three independent experiments. *Error bars* represent ±SD.

4.3. Fermentation Performance of Engineered Strains

The growth performances of the engineered strains and parent strain were tasted, respectively, and there are no any significant distinctions (**Fig. 6**). Then, the fermentation performance of the engineered strains were performed, and results were compared with those obtained from the parent S6. The results (**Table 3**) show no obvious distinction among the tested strains.

4.4. Effects of ATF1 Overexpression on the Production of Volatile Flavor Compounds

After beer fermentation, the concentrations of the ester components were determined by GC analysis. As shown in **Figure 7** and **Table 4**, the concentration of ethyl acetate produced by the engineered strains S6-P-12 and S6-P-30 increased to 23.98 and 24.00 mg L^{-1}, respectively, or 20.44% and 20.54% higher than that produced by S6.

There was not an obvious distinctions in the isoamyl acetate content were observed in the fermentation samples of the tested strains. These results confirmed that the two-step integration strategy can overexpress *ATF1* gene via *PGK1* promoter seamless insertion and led to an increase in the ethyl acetate synthesis.

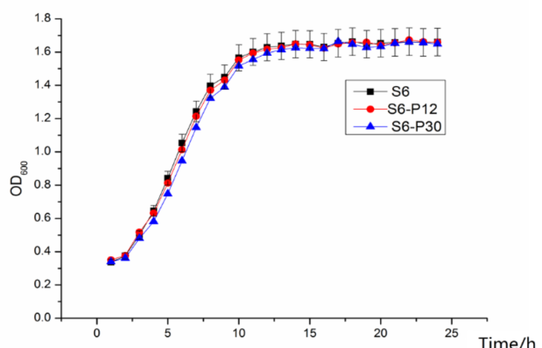

Figure 6. The growth curve of the parent strain and the engineering strains. Data are the average of the three independent experiments. *Error bars* represent ± SD.

Table 4. The volatile flavor compound productions of the engineered strains and the parental strain

Yeast strains	Ethyl acetate (mg /L)	Isoamyl acetate (mg /L)
S6	19.91±0.15	2.98±0.12
S6-P-12	23.98±0.07	2.98±0.06
S6-P-30	24.00±0.13	3.34±0.24

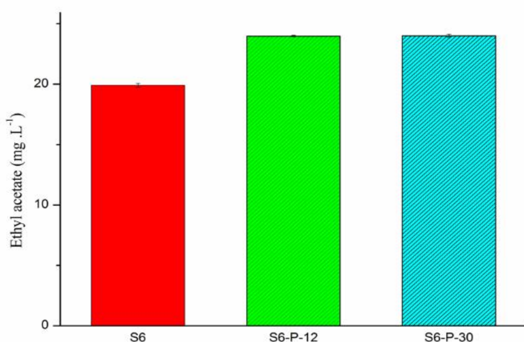

Figure 7. The production of ethyl esters in the recombinant strain: S6-P-12, S6-P-30, and S6. Data are the average of the three independent experiments. *Error bars* represent ±SD.

5. Discussion

In our work, we used polyploid industrial brewer's yeast; S6, as the parent strain. Meanwhile, we have overexpressed all *ATF1* alleles in the industrial brewer's yeast through insertion of the *PGK1p*. The method is also effective for the polyploid yeast strain with an equal frequency of *hisG* repeats (25), and is less time-consuming compared to the site-specific recombinase and the delitto perfetto system (26) because only a single transformation is required in this case. Importantly, "self-cloning" integration strategy can overexpress all *ATF1* alleles gene without any further genetic manipulations (13, 15, 16, 27).

The conventional genetic manipulation is easy to regulate the higher alcohols and esters synthesis of the haploid yeast strains, however, ineffective for the industrial brewer's yeast due to the fact that it cannot produce energetic spores. Thus, the introduction of the methods which could modulate the proportions of the higher alcohols and esters content is of great importance in the polyploid industrial brewer's yeast. This method provide an useful method to realize the site-directed mutagenesis (28) and genes overexpression (14) in the industrial strains.

6. Conclusions

The harmonious complexity of the perceived flavor in the industrial brewer's yeast is significantly important to ensure the best test in the end product (29). In the work, *ATF1* gene was overexpressed in polyploid industrial brewer's yeast through the insertion of the *PGK1* promoter. Brewing with all *ATF1* allelic gene overexpression recombinant industrial brewer's yeast strains increased ethyl acetate yield. Our investigations show that the *ATF1* gene expression levels and AATase activity of the beer brewed with the strains with all overexpressed *ATF1* copies (S6-P-12 and S6-P-30) were increased compared with that of parent strain S6. The engineered strain with stable fermentation property provided a new dimension of the optimized strains research in the industrial brewer's yeast.

The industrial brewer's yeast strains cannot produce energetic spores as the strains are usually diploid and polyploid. Therefore, the conventional genetic manipulation is difficult to obtain ideal brewer's yeast strains. Our work demonstrates that two-step integration protocol may be helpful to get good industrial brewer's yeast strains. Moreover, the resulting mutant, S6-P, in which the *PGK1p* derived from the starting strain, was seamlessly inserted into the upstream of the *ATF1* without an introduction of the restriction sites (13, 15, 16). As well, our method is effective as marker excision occurred with an equal frequency of the *hisG* repeats and can avoid unexpected deletions or chromosome rearrangements due to the none foreign sequences (a single *loxP* site) remaining after yeast genetic modification (GM) (11, 12, 30). Therefore, the engineered strain would be securely applied and accepted by the consumers.

In summary, we have constructed high ester productivity brewer's yeast strains S6-P-12 and S6-P-30. Consequently, all *ATF1* allelic genes were overexpressed in the engineered strains with any heterologous sequences in their sequences. Therefore, the engineered strain would be easily accepted by the consumers. With a better understanding of and further research into the genetically modified organisms, these will likely be widely used in the modulation of the yield of volatile flavor in the industrial brewer's yeast strains, respectively.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31671838), National Natural Science Foundation of China (31471724), The National Key Research and Development Program of China (2016YFD0400505), the National High Technology Research and Development Program of China (Grant No. 2013AA102108) and Major Project of Research Program on Applied Fundamentals and Advanced Technologies of Tianjin (14JCZDJC32900).

Conflicts of Interest

There is no conflict of interest.

Author's Contribution

Jian Dong and Kun-Qiang Hong have contributed equally to the article.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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