

Enhanced Heat Tolerance and Ethanol Production of Industrial *Saccharomyces cerevisiae* via Replacement of the CDC15 Gene

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Abstract

Background

CDC15 is an important gene involved in mitotic exit and cytokinesis in *S.cerevisiae*. However, its additional functions remain unclear. Comparative genomics analysis between the fast-growing strain MC15 and the high-ethanol-producing strain MF01 revealed *CDC15* as a significant sequence-divergent gene. To validate the hypothesis that replacing this gene could enhance MF01's growth rate, this study engineered the recombinant strain MF01-*CDC15* (MT) by substituting the *CDC15* in MF01 (WT) with that from MC15. Rapid growth is one of the desirable traits required for production strains. Achieving sufficient yeast cell density not only reduces contamination by contaminating microbes but also shortens the ethanol production cycle and improves production efficiency.

Results

The *CDC15* mutation significantly improved cell viability on solid medium at 50–54°C. In liquid culture, the MT entered the stationary phase 8 h earlier than WT. Furthermore, the maximum cell densities achieved by MT at 30°C, 37°C and 41°C increased by 37%, 57%, 155%, respectively, compared to WT. Unexpectedly, *CDC15* mutation caused ethanol yields to increase by 26.41% using sucrose and 29.56% using molasses as carbon sources at 30°C. Notably, MT cells maintained normal morphology at 41°C, whereas WT displayed cell adhesion. The intracellular trehalose content in MT was 4.8 times higher than that in WT at 41°C ($p = 0.014$). Homology modeling indicated that the Phe626→Leu626 mutation in Cdc15p of MT may contribute to structural stability. The combination of this structural change and the trehalose accumulation likely contributes to MT's superior heat resistance.

Conclusions

This study provides more detailed information about the function of *CDC15* and suggest some clues to efficiently improve the performance of industrial yeast strains.

1. Introduction

Although *Saccharomyces cerevisiae* is a famous cell factory for producing chemicals^[1, 2], it remains the most widely used microbial host for ethanol production and the dominant organism in fuel ethanol manufacturing^[3, 4]. Bioethanol is regarded as a promising renewable liquid fuel alternative to mitigate energy crises^[5, 6]. However, production costs are a major issue for the production of fuel ethanol compared with fossil fuels^[7]. Sugarcane molasses, a low-cost byproduct of sugar production containing approximately 45% (w/w)^[4, 8], even 50–65%^[9], fermentable sugars, has particular potential as feedstock in southern China's sugarcane-growing regions.

In Brazil, one of the largest ethanol biofuel producers and exporters in the world^[5, 10], fuel ethanol is produced mainly from sugarcane juice and, in some cases, from molasses^[7]. Molasses is diluted prior to fermentation^[5], since molasses's high osmotic pressure (derived from ~ 50% total sugars^[8, 9]) causes intracellular water loss in cells, leading to slow or even halted cell growth^[11]. Such hypertonic stress not only impedes yeast growth but also disrupts metabolic pathways, ultimately diminishing ethanol production efficiency and extending fermentation duration^[12]. Therefore, strains capable of withstanding high osmotic pressure for low-dilution or undiluted fermentation are particularly valuable.

Rapidly growing cells enable efficient acquisition of sufficient cell biomass to initiate ethanol production, and thereby reduce production costs. Alternatively, recycled cells can be employed^[5], which is not applicable in China. In Brazil, the fermentation process generally employs high cell densities to rapidly achieve sufficient cell numbers for efficient fermentation, with certain aspects depending on the growth performance of the strain under current manufacturing practices. The number of recycling cycles can be increased if the cells exhibit high ethanol tolerance, since ethanol is toxic to yeast at high concentrations. Ethanol accumulation in later stages inhibits cellular growth at 9% (v/v)^[4] or completely halts it at 12% (v/v)^[13], resulting in incomplete sugar utilization and reduced ethanol yield, thereby increasing production costs. Some pathways required for ethanol tolerance overlap with those for thermotolerance^[14].

Fermentation is an exothermic process, leading to an increase in fermentation temperature. While temperature control is essential for optimal fermentation, cooling systems significantly increase production costs^[15]. High-temperature fermentation using thermotolerant strains offers a cost-effective alternative, since a 5°C increase in the fermentation temperature could drastically reduce expenses^[7]. The United States produces fuel ethanol primarily from corn through saccharification using α -amylase and glucoamylase, followed by fermentation with *S.cerevisiae*. Under these conditions, employing thermotolerant yeasts can more effectively reduce costs^[7].

Besides, when producing fuel ethanol from corn through saccharification using α -amylase and glucoamylase, followed by fermentation with *S.cerevisiae*—a process primarily employed in the United States—employing thermotolerant yeasts can more effectively reduce costs^[7].

Unfortunately, *S. cerevisiae* strains face a critical limitation: their optimal growth range (25–30°C) and failure to proliferate above 40°C^[13]. Temperature exerts significant effects on cellular molecular composition, volume expansion, and protein folding^[16]. Heat shock induces the expression of genes associated with RNA processing/modification, protein degradation, protein folding/transport^[17], and also alters phosphorylation states of proteins involved in transcription, protein folding and degradation, cell cycle regulation and morphogenesis^[18]. Inappropriate temperature results in slower cell growth rates^[19], which affects fermentation rate during ethanol production. Additionally, temperature influences sugar utilization efficiency, and cell inactivation rate in ethanol-containing environments^[19]. Heat shock induces the expression of genes associated with respiration, and alternative carbon source utilization^[20].

Although thermotolerant mutants that could proliferate at 40°C^[21–23], 41°C^[13, 15] or 42°C^[14, 22, 24] have been obtained, their ethanol productivity remains unknown or low^[22]. Some existing thermotolerant mutants either rely on plasmid-borne gene responsible for the Htg⁺ phenotype, or derive from laboratory strains^[13, 24], rendering them unsuitable for industrial applications due to genetic instability, as plasmid carrying a resistance selection marker is easily lost in the absence of selective pressure^[25, 26]. Besides, laboratory strains lack the tolerance^[27], genetic, and phenotypic diversity^[28] of industrial strains, and do not exhibit the positive correlation between tolerance and fermentation capacity seen in industrial strains^[29].

We previously isolated three wild-type industrial strains, MF01, MC15, and ME13, from samples derived from the same environment (Table 1). Although MF01 exhibits the highest ethanol yield and has been adopted for industrial-scale ethanol production^[4, 30], its growth rate is lower than that of ME13 and MC15. In contrast, MC15, with the lowest ethanol yield, has the highest growth rate among them. Comparative genomics analysis of these strains identified the cell cycle key gene *CDC15* as a sequence-divergent gene, suggesting it may be a critical gene underlying differences in growth rate. Given *CDC15* is an important gene involved in cell cycle^[31]. In theory, replacing the *CDC15* gene in MF01 with *CDC15* from MC15 may enhance the growth rate of MF01, which could positively impact ethanol fermentation as biomass yield critically affects fermentation efficiency^[32]. In this study, the *CDC15* gene of MF01 was modified by substituting it with the homologous *CDC15* gene from MC15, generating the engineered strain MF01-*CDC15*. We hypothesize that homologous recombination of this cell cycle-associated differential gene derived from MC15 may accelerate the growth rate of MF01. As predicted, MF01-*CDC15* exhibited accelerated growth. Unexpectedly, *CDC15* replacement not only increased ethanol yield but also conferred enhanced thermotolerance—a critical trait for industrial fermentation where cooling costs and thermal stress are major bottlenecks. These findings first identifies *CDC15* as a previously uncharacterized key target for enhancing ethanol productivity in industrial yeast strains, thereby enabling the rational design of robust cell factories for scalable biofuel and bio-based chemical production.

Table 1
Strains used in this study

Strain	Genotype	Characteristics	Origin	Reference
MF01	Wild-type,MATa/MATa	Higher ethanol yield and exogenous gene integration rate compared with MC15 and ME13	Isolated by our lab from sugar mill waste in 2010	[4, 30]
MC15	Wild-type,MATa/MATa	Lower ethanol yield and higher growth rate compared with MF01 and ME13	Isolated by our lab from sugar mill waste in 2010	[4]
ME13	Wild-type,MATa/MATa	Intermediate ethanol production and growth rate among MF01, MC15 and ME13	Isolated by our lab from sugar mill waste in 2010	/
MF01-CDC15	Derived from MF01,MATa/MATa	Higher ethanol yield, growth rate and heat resistance compared with MF01	This work	/

2. Methods

2.1 Strain and Culture Conditions

Strain MF01 (MATa/MATa) is a wild-type (WT) diploid *S. cerevisiae*. Strain MF01-CDC15 (MATa/MATa), which is a diploid derived from MF01, was prepared by replacing the *CDC15* gene of MF01 with that from a fast-growing strain MC15. The yeast cells were incubated in YPD medium, as previously described ^[4], in 250 ml Erlenmeyer flasks, unless specified otherwise.

2.2 Construction and Screening of the Mutant Strain

2.2.1 Preparation and Transformation of *CDC15* Homologous Donor

The *CDC15* donor (Fig.SS1 in Miscellaneous File Not for Publication) used for homologous recombination was amplified by PCR with chromosomal DNA of the MC15 strain serving as the template. The donor was amplified with primers *CDC15*-F and *CDC15*-R described in Table 2, using the following PCR protocol: 94°C for 5 min; 94°C for 40 s, 56°C for 40 s, 72°C for 4 min for 35 cycles; and a final step of 72°C for 10 min. The PCR products were purified before transformation. Preparation of haploid strains was carried out as previously described^[4]. Both types a and α protoplasts of MF01 were subjected to electroporation to introduce the *CDC15* gene from MC15.

Table 2 Primers used in this study

Use	Primer name	Primer sequence (5'-3')	Notes
Tempate amplification for	<i>CDC15</i> -F	ACTCTATGCTATACTGTAGTGCTTT	Upstream of <i>CDC15</i>
homologous recombination	<i>CDC15</i> -R	TTATCATTACTGGCCTCTTT	Downstream of <i>CDC15</i>
Verification for homologous	8a—F	TCTGGCGTCGTGTATGGAAA	Upstream of <i>CDC15</i>
recombination	8a—R	ATTCTGACCTTGGTGGGTGTC	Downstream of <i>CDC15</i>

2.2.2 Screening for Recombinant Strains

The transformed mixed solution was transferred into 1 mL cold solution containing 18% sucrose and incubated for 1 h at 30°C without shaking. After addition of 1 mL YPD, the cells were cultivated again in a shaker and then plated onto YPD agar.

The endogenous *CDC15* locus in MF01 could be precisely replaced with the MC15-sourced *CDC15* sequence, owing to the high homologous recombination efficiency of *S. cerevisiae*^[26, 33, 34]. Selection of recombinant mutants was based on phenotypic variations in growth rate. The largest colony on the YPD plate was picked and cultured to exponential phase in liquid YPD. Equal numbers of cells were transferred to fresh YPD and cultured for 8 ~ 10 h followed by detection of OD₆₀₀. Cells with an OD₆₀₀ roughly 10% higher than that of the WT were selected, and then cultured and selected again as described above. Following serial screening, the potential mutant strains were obtained. After validation by PCR with the DNA of potential mutant as template and sequencing, haploid recombinant strains were obtained. Diploid recombinant strain, named MF01-*CDC15* (MT), was constructed from mating types a and α of recombinant strains by mating as previously described^[4]. To assess the genetic stability of MT, the strain was serially passaged for 30 generations, and the *CDC15* locus was PCR-amplified using chromosomal DNA from MT as template. The PCR product was verified by sequencing, and its sequence was identical to that in MC15 (data not shown), indicating successful replacement of *CDC15* and its genetic stability over generations.

2.3 Growth Curve Determination

A single colony was isolated and inoculated into 5 ml preculture overnight. Equal numbers of cells were harvested by centrifugation at 4°C, resuspended in sterile water and shifted to 50 ml fresh YPD, at a final concentration of roughly 1×10⁶ cells/ml, and cultured for 48 h at 30°C, 37°C, 41°C, respectively. Samples were taken at 8-h intervals and stored at 4°C until the number of cells in each sample was determined by Countstar BioFerm IY1200 (Shanghai Ruiyu Biotech Co.Ltd, shanghai, China).

2.4 Analysis of Yeast Cells for Trehalose Quantification and Enzyme Activity Assays

About 1× 10⁸ overnight cells were inoculated into 100 mL YPD and grown for 8-8.5 h. Cells from shake flask cultures were harvested by centrifugation (6000 rpm, 5 min, 4°C), washed once with sterile deionized water and twice with 0.1 M PBS. About 38 mg (wet weight) of the resulting pellet was transferred to a 1.5 ml sterile

EP tube for trehalose content determination, and the remaining pellet was resuspended in 2 ml 0.1 M PBS, and then quickly frozen (-80°C) until use for enzyme activities assayed.

2.4.1 Trehalose Content Determination

The above cell pellet that was washed twice with PBS was dried to a constant weight at 55°C , then the rehalose was extracted with trichloroacetic acid followed by anthrone-based detection as before^[4].

2.4.2 Enzyme Assays

After thawing the samples in room temperature, the crude enzyme solution prepared as the previous method^[4]. Protein determination was done using Bradford Protein Assay Kit(Order No. C503031, Sangon Biotech, shanghai,China).

2.4.2.1 Assay of SOD Activity

The intracellular total superoxide dismutase (SOD) activity was determined by hydroxylamine method as described previously^[35] with some modifications. (1) Preparation of solutions. The Xanthine oxidase (XOD) solution was prepared as follows: 0.5 mg of xanthine oxidase (50 U/mg, purchased from MACKLIN, China) was dissolved in 0.5 ml of buffer solution B (containing 104 mM KH_2PO_4 and 78 mM $\text{Na}_2\text{B}_4\text{O}_7$ in deionized water) to obtain a concentration of 50 U/ml (XOD solution I). The working XOD solution was then prepared by adding 125 μl of XOD solution I to 975 μl of buffer solution B. All reagents for the color developer were stored in bottles at $0-4^{\circ}\text{C}$ until use. (2) Assay Procedure. The procedure was as follows: 0.5 ml of water, 0.1 ml of crude enzyme solution, and 0.2 ml of reagent A (consisting of equal volumes of 1 mM hydroxylamine and 1 mM hypoxanthine, resulting in final concentrations of 0.2 mM for each) were mixed. The reaction was initiated by adding 0.2 ml of xanthine oxidase (XOD) solution (final concentration of 1.25 mU/ml XOD). This mixture (1.0 ml total) was incubated for 30 min at 37°C . Then, 1.25 ml of reagent III (prepared by mixing 1.5 volumes of 1.2 g/L sulfanilic acid with 1 volume of acetic acid just before use) was added, and the mixture was allowed to stand for 5 min at room temperature. Subsequently, 0.75 ml of reagent II (278.3 mg/L N-1-naphthylethylenediamine dihydrochloride dissolved in deionized water) was added. Reagents III and II were added in two separate steps (to final concentration of 300 $\mu\text{g}/\text{ml}$ sulfanilic acid, 5 $\mu\text{g}/\text{ml}$ N-1-naphthylethylenediamine, and 16.7% acetic acid), taking into account the color development process, which differed from the previous method. A control for each sample (the crude enzyme solution of each sample, boiled for 5 min to inactivate the enzyme) was prepared and added after the color developer to avoid incomplete enzyme inactivation leading to inaccurate data. The final mixture (3.0 ml) was allowed to stand for 20 min at room temperature, and the optical absorption was measured at 550 nm.

The principle of the assay is as follows: Xanthine oxidase catalyzes the reaction of hypoxanthine, generating superoxide anion radicals ($\text{O}_2^{\cdot-}$), which then oxidize hydroxylamine to form nitrite. The nitrite exhibits a purplish-red color in the presence of a chromogenic agent, and its absorbance is measured using a microplate reader. Due to the specific inhibitory effect of SOD in yeast cells on $\text{O}_2^{\cdot-}$, the formation of nitrite is reduced, resulting in a decrease in absorbance during colorimetric measurement.

2.4.2.2 Alcohol dehydrogenase assay

Alcohol dehydrogenase (ADH) activity was assayed by the NAD⁺ reduction method. ADH catalyzes the dehydrogenation of ethanol, and the removed hydrogen is received by oxidized coenzyme I (NAD⁺), converting it to reduced coenzyme I (NADH). The amount of NADH generated is measured by monitoring the absorbance change at 340 nm, since NAD⁺ has no absorption at 340 nm but NADH absorbs at this wavelength. The assay system contained 108.8 µl of 0.1 M PBS (prepared by dissolving 14.2 g Na₂HPO₄, 2.38 g KH₂PO₄, 80 g NaCl, 2 g KCl, in 1L distilled water), 40 µl of 5 mM NAD⁺ (prepared by dissolving 38.9 mg of NAD⁺ in 10 ml of distilled water), and 1.2µl of anhydrous ethanol as the substrate. The reaction was initiated by the addition of 50 µl of cell extract to the well of a 96-well microtiter plate. A control for each sample was included, prepared by replacing the cell extract with its inactivated enzyme (prepared by boiling for 5 min). After 20 min of incubation at 30°C, the activity was assayed by measuring the increase in absorbance at 340 nm. Activity is expressed as the increase in absorbance per milligram of protein.

2.5 Ethanol Fermentation

Sucrose fermentation

Fermentation with sucrose was performed as previously described^[4] with minor modification. Briefly, 0.4×10^8 overnight cells were inoculated into 40 ml yeast extract peptone sucrose medium (10 g/L yeast extract, 20 g/L peptone, 25 g/L sucrose, pH unadjusted) in a 250 mL Erlenmeyer flask and incubated with shaking for 38 h, followed by static incubation at 30 and 37 °C under separate conditions.

Molasses fermentation

Fermentation with molasses was conducted according to the prior method^[4] with slight modification. 0.4×10^8 overnight cells were grown in 30 ml molasses medium (containing 20.6 °Bx molasses) in a 250 mL Erlenmeyer flask and incubated for approximately 9 h. An additional molasses medium (replacing 20.6 °BX molasses with 50.3 °BX molasses) was supplemented, after which the cells were grown for another 30 h, followed by static conditions for 40 h at 30 and 37 °C in separate experiments.

2.6 Sugar Content Determination

The reducing sugars and total sugars in the fermented liquid were determined using the 3,5-dinitrosalicylic acid (DNS) reagent. Prior to analysis, the total sugars were hydrolyzed to reducing sugars following a previously described method^[4].

2.7 Statistical Analysis

Statistical analysis was performed with the Student's *t*-test using Microsoft Excel (Tails: two-tail, Type: paired *t*-test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ indicate statistical significance, and ns ($p > 0.05$) indicates non-significance.

3. Results

3.1 Sequence Comparison of Cdc15p among MT, WT and S288C Reveals Convergent Adaptation

Comparison of the predicted Cdc15p amino acid sequence in MT with the known sequences of WT and S288C revealed one residue difference between WT and MT (F⁶²⁶ L), two differences between WT and S288C (T⁸⁵¹ S, D⁹⁰² V), and three differences between MT and S288C (F⁶²⁶ L, T⁸⁵¹ S, D⁹⁰² V) (Fig. 1). The substitution at residue 626 (Phe→Leu) between WT and MT is caused by a single nucleotide polymorphism (SNP) at position 1878 of the ORF (T →A in MT, Fig.SS3 in Miscellaneous File Not for Publication). The higher protein sequence similarity between MT and WT may reflect their convergent adaptation to identical growth environments, as both strains were isolated from the same geographic site.

3.2 *CDC15* Mutation Does Not Alter Cellular Morphology at Normal Temperature but Causes Nonadherent Growth

Cdc15p plays a crucial and multifaceted role in the cell cycle, specifically in mitotic exit^[36]. While its role in cell cycle progression^[37] is well established and *CDC15* overexpression does not affect mitosis^[38], potential effects of *CDC15* mutations on cellular morphogenesis remain unexplored. To evaluate this phenotype, we compared the WT and MT strains under both solid and liquid culture conditions. On YPD agar plates, both strains formed colonies with white, viscous, normally smooth, and lustrous surfaces (Fig.SS5 in Miscellaneous File Not for Publication), indicating that their colony size and morphology were normal at 30 °C. These results indicate that *CDC15* mutation does not seem to have any adverse effects on morphology. Unexpectedly, some WT cells exhibited adherent growth at 37°C, with more pronounced effects observed at 41°C, but the MT did not (Fig. 2, Fig. S1).

3.3 The *CDC15* Mutation Improves Cell Viability under Thermal Stress (50°C to 54°C)

In practical ethanol production, localized temperatures within fermentation tanks may transiently exceed 50°C due to metabolic heat accumulation compounded by ambient thermal variations. Such transient thermal stresses, when exceeding the strain's tolerance threshold, invariably result in suboptimal fermentation performance ranging from diminished ethanol yield to complete process failure. Therefore, thermotolerant strains demonstrating high viability under production conditions is one of the desirable characteristics for strains. High cell viability is one of the critical criteria for strain selection in Brazil's industrial ethanol production^[5].

To evaluate the heat shock tolerance of WT and MT strains and investigate the impact of the *CDC15* mutation on thermotolerance, we analyzed the post-thermal stress viability of both strains. Following a 10-min heat shock at 42°C to 48°C, cell growth was delayed in both solid and liquid cultures, with no obvious distinction observed between the WT and MT. However, at temperatures between 50°C and 54°C, significant differences emerged between the WT and MT. On plates incubated at 50°C, the MT exhibited significantly higher colony counts than that of WT, and this difference became more pronounced at 52°C. A small number of mutant colonies survived at 54°C, whereas no surviving WT colonies were detected (Fig.SS7 in Miscellaneous File Not for Publication). These results indicate that after heat shock at 50°C to 54°C, the

mutant strain exhibited a higher cell viability than the WT strain, suggesting enhanced thermal tolerance in the mutants. No cell growth was observed at temperatures between 56°C and 62°C (data not shown).

It is reported that protein translation ceased after 10 min of exposure to 46°C [39], and that only rare thermotolerant strains survive following a 5-min heat shock at 54°C [13]. Strikingly, however, the MT strain in our study still exhibited survival after a 10-min heat shock at 54°C, but the WT did not. This not only demonstrates the superior thermotolerance of the MT strain, but also confirms that the *CDC15* mutant exhibits a significantly higher cell viability than the WT following heat shock.

3.4 The *CDC15* Mutation Promotes Cellular Proliferation under both Normal Conditions (30°C) and Thermal Stress (37°C, 41°C)

Previous studies suggest that although protein translation did not decrease significantly within the first 30 min of exposure to 42°C, the synthesis of de novo proteins was affected under prolonged thermal stress at 42°C [39]. To further investigate temperature-dependent phenotypic differences, we analyzed the proliferation capacity of the WT and MT under varying temperature conditions. The MT entered the stationary phase 8 h earlier (at 16 h) and achieved a higher maximum cell density (4.37×10^8 cells/mL) than the WT (3.18×10^8 cells/mL) at 30°C. As the temperature increases, both strains show decreased growth rates. However, the cell number of MT consistently exceeded that of the WT at all measured time points, and the fold difference increased with rising temperatures (Fig.S 2). The maximum cell densities of the MT at 30°C, 37°C and 41°C increased by 37%, 57%, 155%, respectively. Additionally, the cell number of MT cultured at 37°C was higher than that of WT at 30°C, and the cell number of MT at 41°C was comparable to that of WT at 37°C. In brief, the hierarchical distribution of cell counts is MT30 (MT at 30°C, similarly hereinafter) > MT37 > WT30 > WT37 ≈ MT41 > WT41 (Fig. 3). Collectively, this suggests that the *CDC15* mutation enhances the strain's ability to grow under both normal and heat stress conditions, consistent with observations in post-thermal stress viability.

3.5 *CDC15* Mutation Enhances Ethanol Production

The ability of MT to grow rapidly at 37°C and 41°C holds enormous potential for applications in ethanol production [23]. To evaluate the effect of the mutation on ethanol production, we tested its fermentation capability. Upon fermentation with sucrose as the sole carbon source, MT at 30°C showed the highest ethanol production among all samples after 35 h (Fig. 4A). When molasses was used as the sole carbon source, MT37 displayed the best ethanol productivity during the first 38 h, after which MT at 30°C showed higher productivity until 70 h when MT at 37°C again became highest (Fig. 4B). When sucrose was used as the carbon source, MT strain achieved maximum ethanol concentrations of $12.03\% \pm 0.01\%$ at 30°C and $11.14\% \pm 0.01\%$ at 37°C, representing 26.41% and 3.79% increases over WT, respectively. Similarly, with molasses, MT produced maximum concentrations of $11.02\% \pm 0.07\%$ at 30°C and $10.54\% \pm 0.06\%$ at 37°C, corresponding to 29.56% and 3.84% improvements over WT, respectively (Fig. 4). These results suggest that the *CDC15* mutation enhances ethanol production, and ethanol fermentation from molasses by *S. cerevisiae* is more difficult, resulting from the complex composition, compared with that from sucrose. Unexpectedly, both MT and WT strains showed higher ethanol production at 37°C than that of WT at 30°C, regardless of

carbon source, except with sucrose at 54–62 h and with molasses at 70 h (Fig. 4). These results may result from the genetic background of the strains and suggest that both strains, especially MT, hold potential for ethanol production under high temperatures (37°C) conditions.

3.6 Trehalose Content Explains Higher Heat Resistance of MT Compared to WT

High temperature induces the accumulation of trehalose in *S.cerevisiae* cells^[40, 41]. Trehalose accumulation accumulated in response to heat shock may maintain cell viability^[32] and promote growth under high-temperature conditions^[15] by preventing heat-induced damage to the cytoplasm^[40]. Cell viability declines dramatically when trehalose levels are reduced below 2mg/g dry cell weight (DCW)^[5]. High intracellular trehalose content serves as an indicator for ethanol-producing industrial strain screening in Brazil^[5]. Therefore, we investigated the trehalose content in MT and WT strains.

At 30°C and 37°C, MT showed slightly higher trehalose content than WT (67 vs. 34 mg/g DCW; 63 vs. 23 mg/g DCW) without statistical significance. However, at 41°C, both strains exhibited pronounced trehalose accumulation, consistent with the literature report by Hottiger^[40], with MT displaying significantly higher content compared with WT (MT: 972 mg/g DCW; WT: 202 mg/g DCW; $p = 0.014$). These results may suggest that the elevated trehalose content in MT contributes to its enhanced heat resistance relative to WT (Fig. 5).

Reported intracellular trehalose concentrations in *S. cerevisiae* vary widely across studies^[5, 40, 41], with limited datasets showing comparable values^[5, 41]. For instance, Hottiger et al. observed that *S. cerevisiae* accumulated 1 g trehalose per g protein under 40°C stress^[40], while the industrial strain PE-2 and a baker's yeast strain exhibited 12.5 mg/g and 6.3 mg/g trehalose, respectively^[5]. Notably, PE-2's elevated trehalose levels correlate with its industrial robustness. Although both the present study and Hottiger's work^[40] employed trichloroacetic acid extraction followed by anthrone-based detection of trehalose content, direct comparisons between these datasets are not feasible due to differences in strain cultivation conditions and normalization methods. Direct comparisons between MT (this study) and PE-2 are also confounded by divergent cultivation and extraction protocols: PE-2 was grown under harsh fermentation conditions (33°C, molasses medium, 9.1% ethanol) until the mid-fermentation phase^[5], whereas MT was cultured in YPD medium for 8–8.5 h. In this study, the trehalose content of WT cultured at 30°C was 34 mg/g DCW, which is close to the range of 31–40 mg/g DCW reported by Alexandre^[42]. However, Alexandre's protocol differed from both this study and existing literature^[5, 40, 41]. Specifically, after 1 h stress treatment at 40°C, cells were incubated in 0.25 M Na₂CO₃ for 2 h at 90°C, and the trehalose content was determined by measuring the glucose liberated by trehalase digestion^[42].

There are also discrepancies in the trends of trehalose content variation under temperature stress across different studies^[40, 41]. Asada^[41] reported trehalose yields of 14.88, 13.94, and 4.41 mg/g at 40°C, 45°C, and 50°C, respectively, which contrasts with Hottiger's observation of higher levels at 45°C versus 40°C and complete synthesis cessation at 50°C^[40]. Asada treated the cells by boiling and measured the trehalose

content using a detection kit ^[41]. It remains unclear whether different processing and assay methods influence observed trend.

Overall, variations in stress conditions, extraction methods, and quantification assays preclude direct cross-study comparisons. Nonetheless, MT demonstrated significantly higher trehalose content than WT at 41°C, which is consistent with MT's superior heat tolerance over WT, indicating better industrial adaptability compared to WT, despite the latter's current industrial application^[4].

3.7 Non-Dominant Roles of SOD and ADH in MT/WT Thermotolerance Divergence

It has been reported that superoxide dismutase (SOD) enhance the heat resistance of strains ^[43], while ADH activity can serve as a critical indicator of cellular ethanol tolerance ^[44]. *ADH1* overexpression can improve tolerance to glycolaldehyde ^[45] and co-expression of *ADH1* and *TAL1* improves ethanol production in the presence of furfural ^[46]. Hybrids of thermotolerant *Kluyveromyces marxianus* and thermosensitive *S. cerevisiae* produce more than 6% (v/v) ethanol at 45°C, and these hybrids contain ADH components derived from *S. cerevisiae*^[47]. To determine whether other factors apart from trehalose contribute to the improved thermotolerance of MT, we examined the activities of these tolerance-related enzymes.

As the temperature increased from 30°C to 37°C and then to 41°C, both SOD and ADH activities of both strains increased almost universally. At all temperatures, the SOD activity of MT was higher than that of WT, but the difference was not significant (Fig.S3). As the temperature increased, both strains exhibited an elevation in ADH activity. Nonetheless, the ADH activity of MT did not consistently surpass that of WT at identical temperatures, and no significant disparity was observed between them (Fig. S4). This suggests that SOD and ADH do not play a decisive role in the differential high-temperature tolerance between WT and MT under equivalent temperature stress conditions.

3.8 Effect of Trehalose Supplementation on Thermotolerance and Adherent Growth

Since strains with higher trehalose levels exhibit increased cell viability under heat stress ^[42] and exogenous trehalose supplementation enhances freeze tolerance of yeast ^[48], we added trehalose to the culture medium to evaluate growth differences between WT and MT under different temperatures with/without trehalose. At all temperatures, MT consistently exhibited higher cell counts than WT regardless of trehalose supplementation. At 37°C and 41°C, both strains generally showed increased cell density with trehalose supplementation compared to unsupplemented controls, except for MT at 48 h (37°C), WT at 40 h and 48 h (37°C), MT at 16 h and WT at 48 h (41°C). No significant differences were observed in cell growth between trehalose-supplemented and unsupplemented cultures at 30°C for either strain (Fig. 6). These results indicate that trehalose supplementation improves high-temperature (37°C and 41°C) tolerance in both strains. Without trehalose supplementation, MT cells exhibited higher numbers than WT at all tested temperatures and both strains showed a decline in cell counts with increasing stress intensity (Fig. 6),

aligning with prior observations (Fig. 3). Trehalose supplementation did not mitigate the adherent growth of WT cells under heat stress (Fig.S11 in Miscellaneous File Not for Publication).

4. Discussion

4.1 Mechanistic Hypothesis for Enhanced Thermotolerance in *CDC15* Mutant Strain

Our study identified a single residue difference between WT and MT (F⁶²⁶L, Fig. 1), and homology modeling of Cdc15p indicates that residue 626 is located within an α -helical region on the protein's outer surface, spatially proximate to an area enriched with charged residues (residues 281–305)^[37] (Fig. 7). The fragment encompassing residues 281–362, which contains this charged cluster, is able to interact individually with Tem1p, a protein known to interact with Cdc15p^[37]. Although both Leu and Phe are hydrophobic amino acids, the benzene ring of Phe displays significant rotational mobility, resulting in pronounced steric hindrance. In contrast, while Leu's side chain retains conformational flexibility, its spatial constraints are substantially reduced. The Phe626→Leu626 substitution may improve protein folding efficiency, stabilize the tertiary structure.

Since changes in amino acid sequences can alter the thermostability of proteins, thereby affecting the heat resistance of yeast strains^[49]. We hypothesize that the mutation of Cdc15p optimizes its interaction with protein partners such as Tem1p, thereby enhancing the heat resistance performance of the MT (Fig. 8). This is supported by the following evidence: First, *TEM1* is closely related to *CDC15*. For example, the lethality associated with *TEM1* deficiency can be rescued by overexpression of *CDC15*^[50], and other *CDC15* mutations (Leu356 and Phe357) weaken the interaction between Tem1p and Cdc15p, leading to temperature-sensitive growth, a defect that was efficiently suppressed by the overexpression of *TEM1*^[37]. Second, the mutated residue at position 626 is spatially adjacent to the region in Cdc15p that can independently interact with Tem1p (Fig. 7). However, these hypotheses require further validation.

4.2 Differences between the Aggregation of WT under Heat Stress and Classical Pseudohyphal Growth

It was observed that partial WT cells exhibited adhesion at 37°C and nearly all WT cells showed adhesion at 41°C, in contrast, MT cells remained dispersed (Fig. 2). To the best of our knowledge, this is the first reported adhesion growth trait associated with the cell cycle-related gene *CDC15*. Similarly, the deletion of another cell cycle-related gene *MBP1* leads to pseudohyphae formation^[4], whereas it was previously widely accepted that starvation is a prerequisite for pseudohyphae formation in *S. cerevisiae*^[51, 52]. However, in this study, under high temperature condition, WT cells formed clusters through adhesion aggregation, distinct from the chains of elongated cells aggregated together during typical pseudohyphae formation^[53, 54]. We speculated that the two different growth states associated with *CDC15* and *MBP1* arise from distinct mechanisms causing variations in "pseudohyphae" morphology and extent, which aligns with previous findings^[53].

The cell cycle-related genes *CDC15*^[36] and *MBP1*^[4] have been implicated in tolerance to heat stress (this study) and ethanol^[4], respectively, and *MBP1* is a known transcriptional regulator of numerous genes^[55]. Furthermore, the deletion of *MBP1* or mutation of *CDC15* results in cell elongation and aggregation^[4] and cell non-adhesion, different from WT, (this study), respectively, but their cooperative regulation of growth remains unknown.

4.3 Effects of Trehalose on Thermotolerance and Ethanol associated with *CDC15*

Intracellular accumulation of trehalose is believed to increase a yeast's tolerance to freezing, dehydration^[56]. Protection against freezing in yeast cells by trehalose requires its presence on both sides of the plasma membrane^[48]. Under high temperature conditions, the intracellular trehalose content of MT was higher than that of WT (Fig. 5), and MT exhibited superior growth capacity compared to WT (Fig. 3). Although the addition of trehalose increased cell counts of both strains, MT's count still exceeded WT's under stress (Fig. 6). The potential reasons for these differences may be found in the previous studies, which indicates that the degradation of intracellular trehalose facilitates cellular recovery from heat stress, and that intracellular trehalose is associated with cell cycle regulation, while extracellular trehalose can act as a carbon source^[57].

The higher intracellular trehalose content of MT may result from the mutation of the cell cycle-related gene *CDC15* affecting trehalose synthesis and/or degradation, since trehalose and its degradation are associated with cell cycle regulation^[57]. However, further verification is needed.

Trehalose can be hydrolyzed by trehalase into glucose, potentially providing more substrate for glycolysis^[57]. This means cells can mobilize more glucose to participate in glycolysis reactions, thereby generating more pyruvate, which is converted into ethanol under anaerobic conditions. This may result in the higher ethanol yield in MT, as MT shows higher trehalose content than WT (Fig. 5). Additionally, during the late stages of fermentation, cells utilize ethanol and/or glucose derived from trehalose hydrolysis to sustain growth due to the lack of available carbon sources. We speculate that WT likely consumes more ethanol because its trehalose content is lower than MT, which could also contribute to WT's lower ethanol yield compared to MT.

5. Conclusions

CDC15 mutation (Phe626 → Leu626) enhanced thermotolerance and suppressed heat-induced cell aggregation under high-temperature stress. This is likely mediated by trehalose-mediated stress protection and Cdc15p conformational flexibility. Furthermore, *CDC15* mutation improved ethanol production efficiency. Although the molecular mechanisms require further investigation, this unexpectedly discovery of enhanced thermotolerance and fermentation traits highlights *CDC15*'s multifaceted functionality in stress adaptation and metabolic regulation. This finding positions *CDC15* as a promising genomic editing target for developing robust industrial yeast strains, and fermentation efficiency are paramount.

Abbreviations

ADH
Alcohol dehydrogenase
SOD
superoxide dismutase
WT
MF01,wild-type
MT
MF01-*CDC15*
XOD
Xanthine oxidase
NAD⁺
oxidized coenzyme I
NADH
reduced coenzyme I
DNS
3,5-dinitrosalicylic acid
SNP
single nucleotide polymorphism
DCW
dry cell weight
MT30
MT grows at 30°C
MT37
MT grows at 37°C
MT41
MT grows at 41°C
WT30
WT grows at 30°C
WT37
WT grows at 37°C
WT41
WT grows at 41°C

Declarations

Authors' contributions

X.C.: Funding acquisition, Investigation, Validation, Writing – original draft, Writing – review & editing. D.C.: Methodology, Resources. Q.L.: Data curation, Investigation. Y. C.: Investigation, Funding acquisition. Z.L.: Validation. S.Z.: Methodology. All authors reviewed the manuscript.

Availability of data and materials

The original contributions presented in this study are included in the article/Supplementary Material/Miscellaneous File Not for Publication. Further inquiries can be directed to the corresponding authors.

Competing interests

The authors declare no competing interests.

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Figures

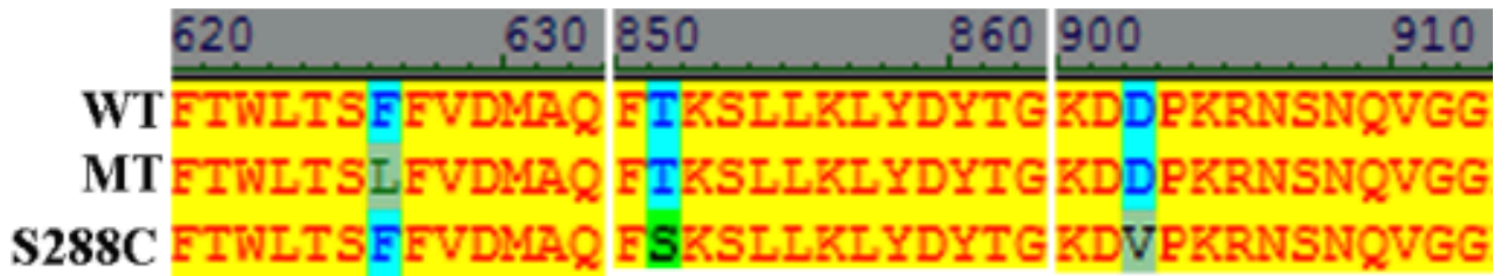


Figure 1

Cdc15p sequence alignment of MT, WT and S288C (NCBI Reference Sequence: NP_009411.2^[38], NC_001133.9)

Note: The differential amino acids were indicated in shadow.

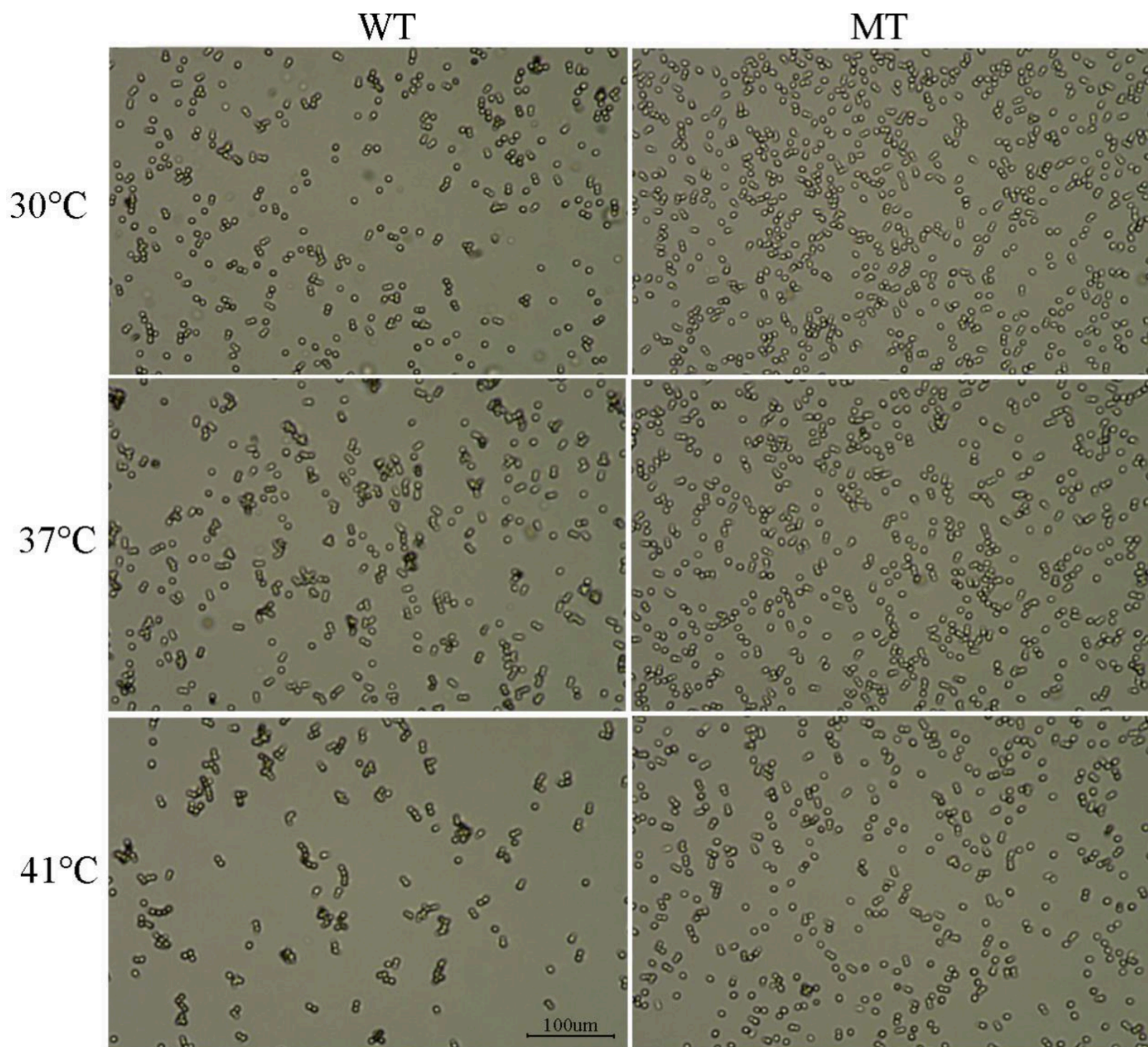


Figure 2

Morphological characteristics of cells cultured at varying temperatures. Representative micrographs were captured during growth curves determination using Countstar BioFerm IY1200 (one was randomly selected from two parallel cultures). Strain (WT, MT) and culture temperature (30 °C, 37 °C, 41°C) are labeled above and on the left of the figure, respectively. This image only displays cells at 8 h of cultivation. Further details are available in Fig. S1.

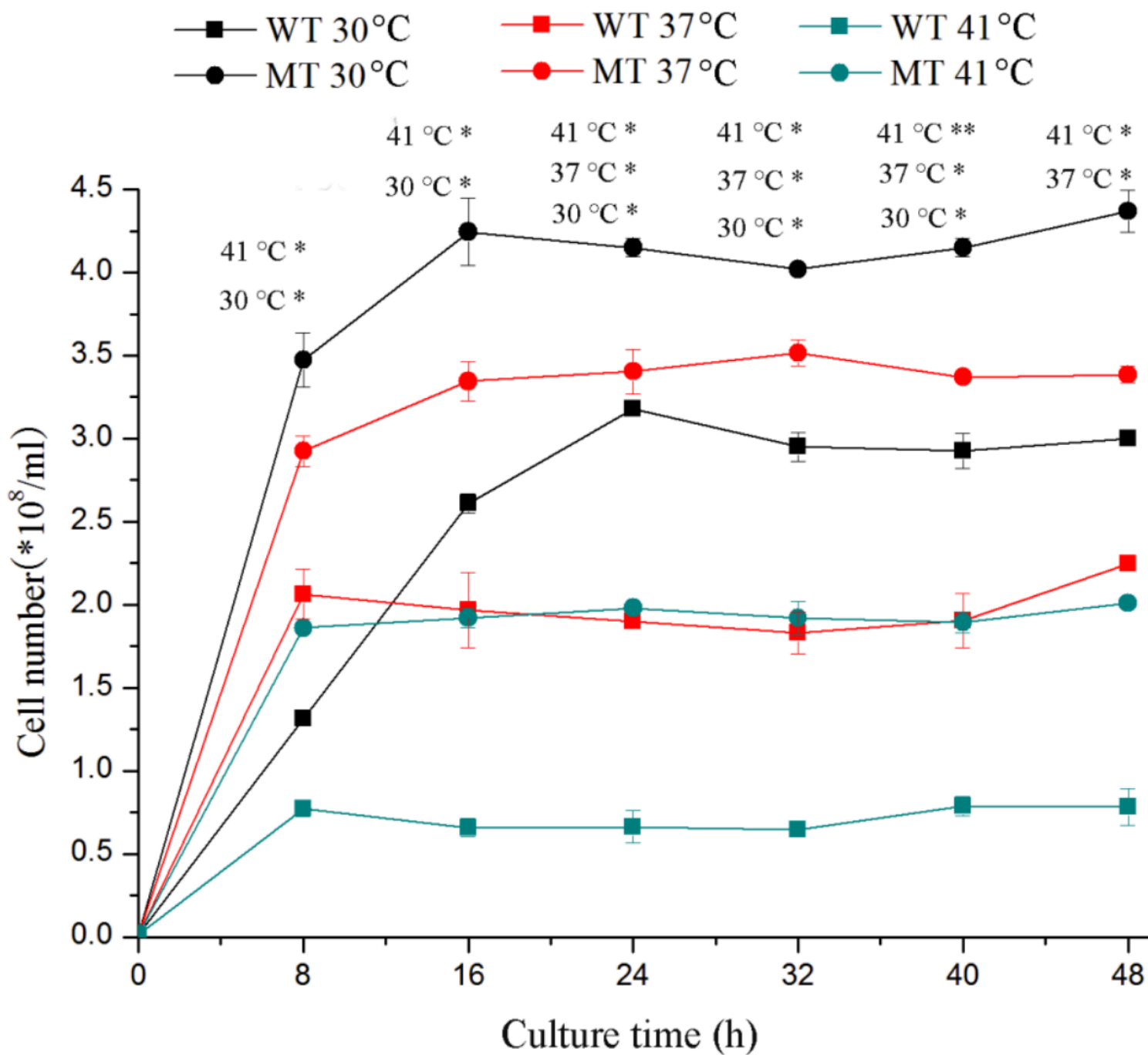


Figure 3

Growth curves of the strains under different temperature conditions.

The data obtained from the Countstar BioFerm IY1200 system represent the mean $\pm$ SD of two independent biological replicates. The asterisk adjacent to the temperature value denotes statistically significant differences observed at the corresponding time points under that specific temperature.

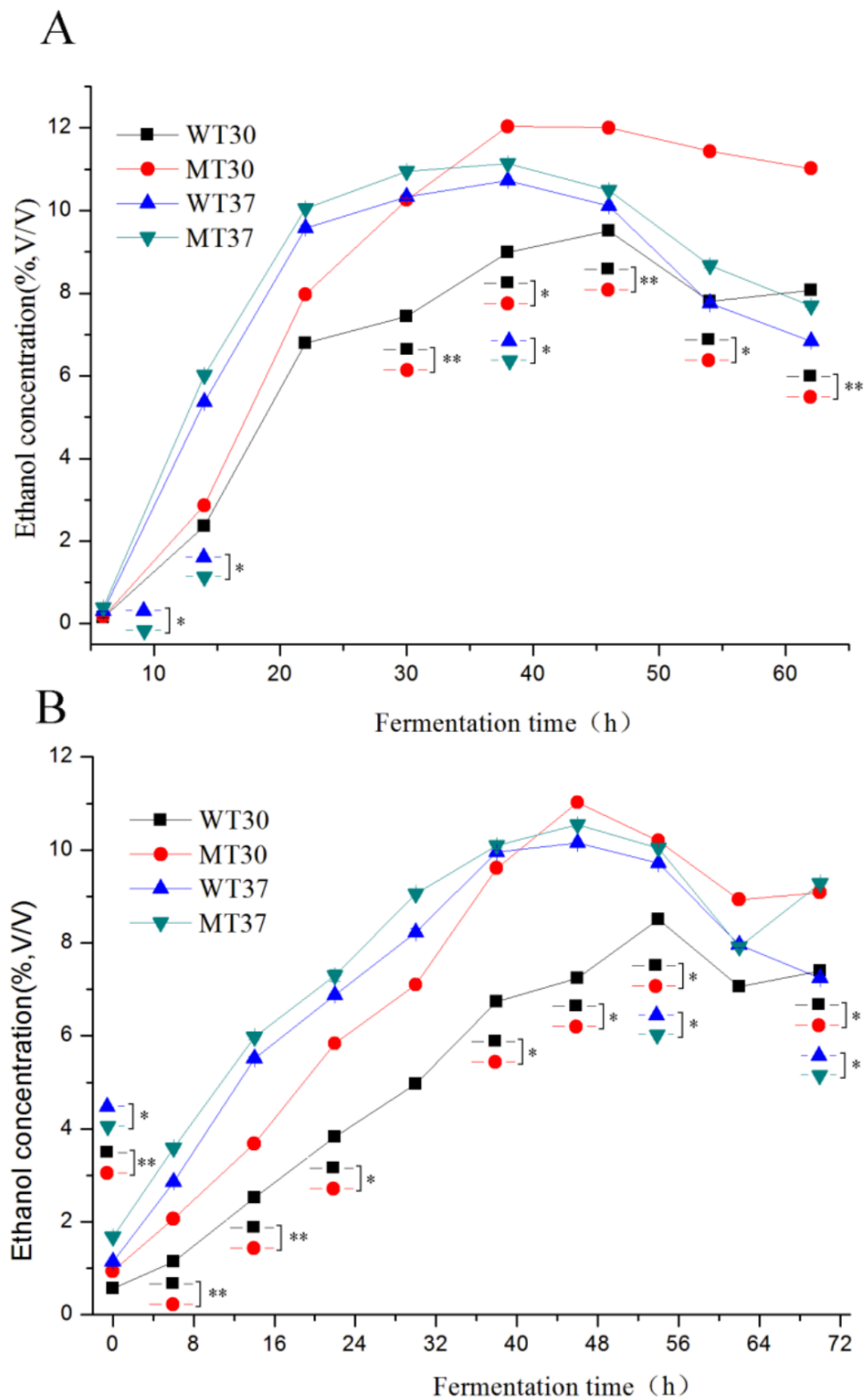


Figure 4

Ethanol production with sucrose (A) or molasses(B)

Ethanol content is determined by gas chromatography. The data shown represent the mean $\pm$ SD of three independent biological replicates.

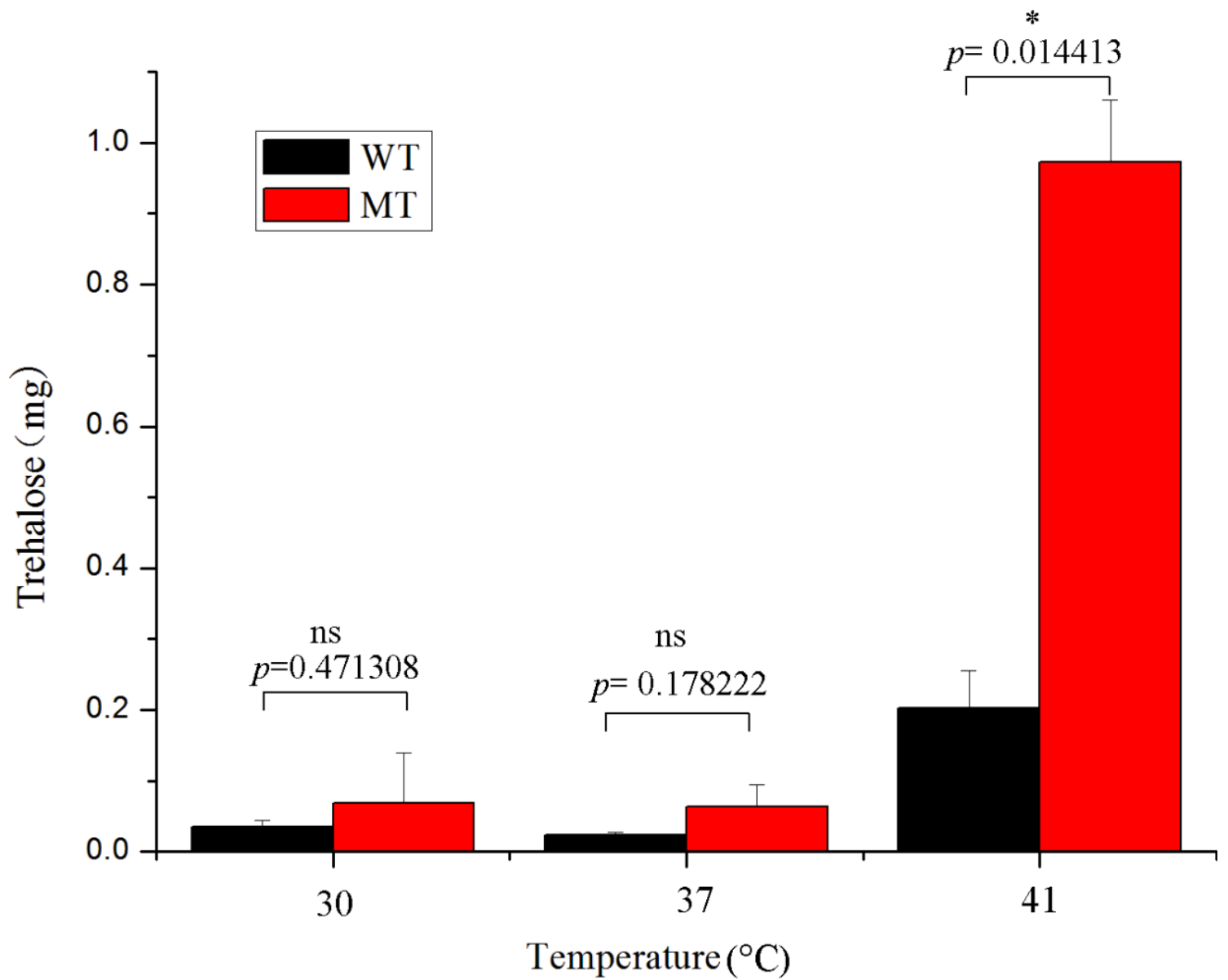


Figure 5

Trehalose content (per mg of DCW)

The data shown represent the mean $\pm$ SD of two or three independent biological replicates.

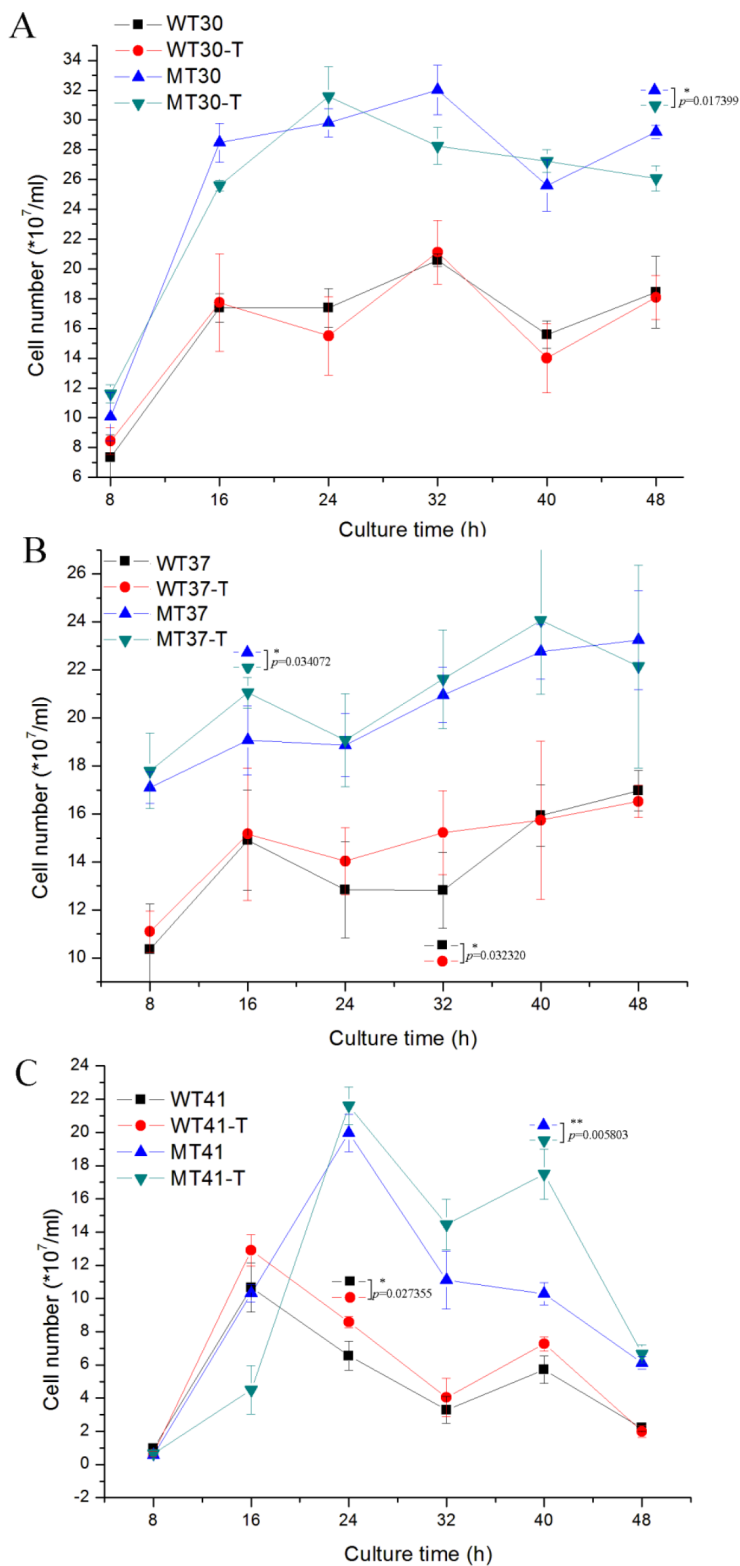


Figure 6

Growth curves of cultures with and without trehalose.

After two rounds of overnight culture, cells were harvested by centrifugation at 4°C. A total of 2×10^7 cells were inoculated into 10 mL of YPD medium in 50 mL centrifuge tubes, with or without trehalose supplementation (final concentration: 0.3 g/L). The cultures were incubated at 30 °C (A), 37 °C (B), and 41 °C

The SwissModel platform (<http://swissmodel.expasy.org/>) was used to perform homology modeling with the highest similarity sequence. Swiss-PdbViewer 4.1.0 was used to superimpose the Cdc15p structure of MT and WT. The α -helix encompassing residue 626 is highlighted in yellow. The region enriched with charged residues (residues 281-305)^[37] is shown in cyan(). Lower right panel: Rotational and zoom views of the mutated residue (Phe626, Leu626) in the α -helical. The side chains of Phe626 (WT) and Leu626 (MT) are colored green and red, respectively. For further details, see Fig.S5 and Structural model 1.

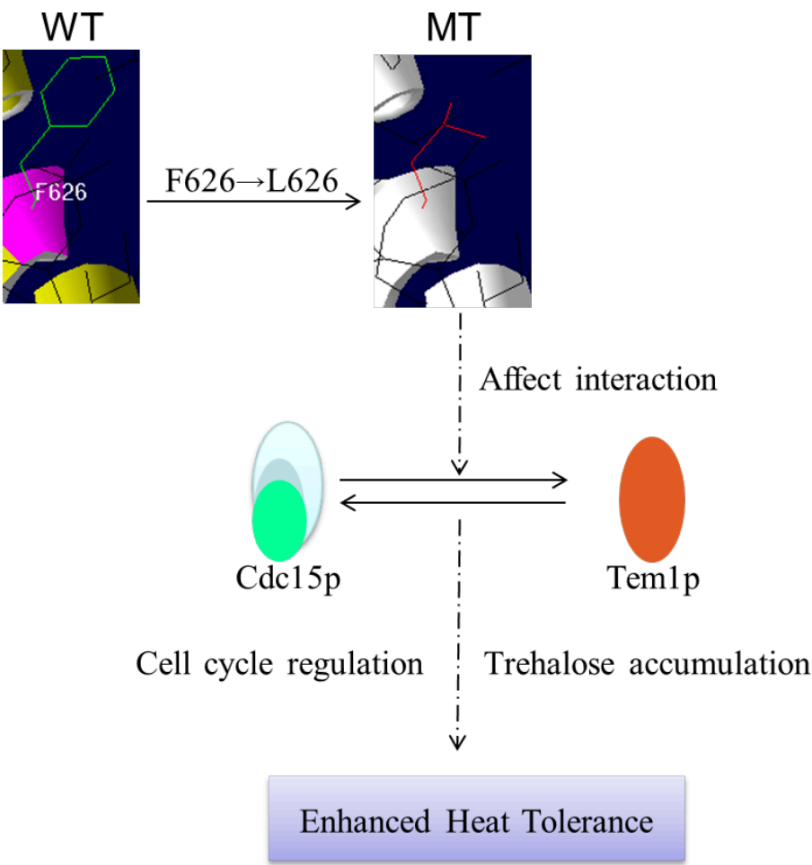


Fig.8 The proposed hypothetical model of how the mutation affects the thermotolerance

:The region enriched with charged residues (residues 281-305)^[37].

:The region able to interact individually with Tem1p(residues 281-362)

Figure 8

See image above for figure legend

Supplementary Files

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