Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Genome-Wide Editing Provides Insights into Role of Unsaturated Fatty Acids in Low Temperature Growth of the Psychrophilic Yeast Metschnikowia australis W7-5

Xin Wei
Ocean University of China
Miao Zhang
Ocean University of China
Zhe Chi
Ocean University of China
Guang-Lei Liu
Ocean University of China
Zhen-Ming Chi (chi@ouc.edu.cn)
Ocean University of China

Research Article

Keywords: Psychrophilic yeast, Low temperature, growth mechanism, Unsaturated fatty acid

Posted Date: June 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1710585/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Abstract

It has been confirmed that Fad12 (Δ^{12} fatty acid desaturase) and Fad15 (Δ^{15} fatty acid desaturase) were responsible for the synthesis of linoleic acid ($C_{18:2}$, $\Delta^{9,12}$) and linolenic acid ($C_{18:3}$, $\Delta^{9,12,15}$), respectively. In order to know their function in cold growth of the psychrophilic yeast *Metschnikowia australis* W7-5, the *FAD12* gene and *FAD15* gene were deleted, respectively. The intracellular linoleic acid percentage of the obtained *Δfad12* mutant was decreased from 27.1–1.5% while the percentage of $C_{18:1}$ fatty acid was increased from 28.3–55.7%. The growth rate of the *Δfad12* mutant was significantly reduced when it was cultured at 5 ℃ and 25 ℃ compared with that of the wild type strain W7-5 under the same conditions. But at 15°C, the mutant grew as well as its wild type strain W7-5. Although $C_{18:3}$ fatty acid of the *Δfad15* strain were not detected, there was no significant difference between the growth of *Δfad15* and that of the W7-5 strain at different temperatures. After the *FAD12* gene was supplemented, the growth at different temperatures and intracellular fatty acid compositions of the supplementing strain were restored compared to those of the strain W7-5. These results suggested that only linoleic acid synthesized by the psychrophilic yeast, not linolenic acid synthesized, played an important role in adaptation to low temperature (5°C) and high temperature (25°C) of the psychrophilic yeast, Meanwhile, it was found that cell wall in the mutant *Δfad12* grown at 5 and 25°C was also negatively affected after the mutant could not synthesize $C_{18:2}$ fatty acids. This caused the reduced cell growth rate of the mutant *Δfad12* grown at 5 and 25°C.

Introduction

It has been well known that psychrophiles grow faster at 15°C or below and are unable to grow above 25°C environment while psychrotrophs not only can grow at around 0°C, but also can grow well above 20°C (Alcaıno et al. 2015). Therefore, psychrophiles grows the best at 15°C. In fact, at this temperature, they do not need to be adapted to such an environment. Only do they grow at lower temperature such as 5 ℃ or at higher temperature such as 25 ℃, they do need to be adapted to these extreme environments. So, we think that we should be careful when we use the word “adaptation of psychrophiles”. It has been well documented that 80% of the biosphere in the earth has temperature below 5 ℃ and has not been explored. Therefore, psychrophiles and psychrotrophs are widely distributed in the nature environments (Deegenaars and Materson 1998). They can play key roles in the interactions between different organisms, the biodegradation of organic matter such as cleaning up of the oil spill, the cycling of essential nutrients and mineralizing of wastes in the cold environments. Their enzymes and metabolites have highly potential applications in food, chemical and pharmaceutical industries. So, it is very significant to study the cold life mechanisms of psychrophiles, especially those of psychrophilic yeasts because they represent eukaryotic cells in the nature. Although many psychrophilic fungi and yeasts have been isolated from different cold environments in recent years, little is known about their cold growth mechanisms at molecular levels. So far, the majority of studies of fungal cold growth mechanisms have focused on the cold stress response of mesophilic model organism, such as *Saccharomyces cerevisiae*. They respond to cold stress by inhibiting general protein synthesis, inducing cold shock proteins,

antifreeze and cold-active enzymes, producing compatible substances such as glycerol and trehalose, increasing in unsaturated membrane lipids (Russell, 2008), the production of RNA chaperones to suppress the formation of undesired secondary RNA structures (Maggi et al., 2013). However, in our previous study (Wei et al. 2021), it has been confirmed that trehalose, glycogen, and glycerol had no function in growth of the psychrophilic yeast at different temperatures. At the same time, some researchers also tried to resolve the cold growth mechanism of the obligate psychrophilic fungus *Mrakia psychrophile* through genomic, transcriptomic, and proteomic analysis (Su et al., 2016). But these data only can provide some initial insights into the cold-growth strategies of psychrophilic yeasts. In order to elucidate their true cold-growth strategies, the whole genome editing techniques must be used to delete and complement the relevant genes in the cells of psychrophilic yeasts based on our previous studies (Wei et al., 2021). It is generally regarded that keeping plasma membrane fluidity, such as synthesizing high level of polyunsaturated fatty acids, restructuring their membrane lipid composition in response to environmental changes to keep lipids in a lamellar crystalline phase are essential for psychrophilic yeasts to grow in cold environments. However, it is still completely unknown which unsaturated fatty acid can play an important role in cold growth at low temperatures and how the unsaturated fatty acid affects their cold growth.

Figure 1 shows that different desaturases and elongases are responsible for biosynthesis of different unsaturated fatty acids. In this study, in order to clarify role of each unsaturated fatty acid in cold growth of psychrophilic yeast *Metschnikowia australis* W7-5, different desaturases including the *FAD91* gene encoding Δ^9 desaturase1, *FAD92* gene encoding Δ^9 desaturase2, *FAD12* gene encoding Δ^{12} desaturase and *FAD15* gene responsible for Δ^{15} desaturase were deleted and complemented in *M. australis* W7-5 and fatty acid compositions and cell growth of different mutants and complementing strains were measured to show the true role of different unsaturated fatty acids in their cold growth. However, the *FAD91* gene and the *FAD92* gene encoding Δ^9 desaturase in all organisms is essential (Stukey et al. 1989 and 1990). This means that the monoenoic products palmitoleic ($C_{16:1}$) or oleic ($C_{18:1}$) acids are required for cell growth. It has been confirmed that Δ^9 desaturase catalyzes the insertion of a double bond between carbons 9 and 10 of the saturated fatty acyl substrates, palmitoyl($C_{16:0}$)- and stearoyl ($C_{18:0}$)-CoA, yielding the monoenoic products palmitoleic ($C_{16:1}$) or oleic ($C_{18:1}$) acids. Therefore, if the mutants in which the *FAD91* gene or the *FAD92* gene is deleted were tried to be isolated, the mutants must grow in the medium with oleic (or palmitoleic) acid. In this case, it is impossible investigate the role of palmitoleic ($C_{16:1}$) or oleic ($C_{18:1}$) acids in adaptation of the psychrophiles to cold environments.

Materials And Methods

Psychrophilic Yeast Strain, Plasmids and Media

The psychrophilic yeast used in this study was *M. australis* W7-5 isolated from the sea mud at Antarctica (Wei et al. 2021). The plasmids pGM simple-T fast and pLB-simple were used for amplification of the plasmids in *E. coli* DH5α. The knock-out plasmid Ma-NAT-loxp carrying nourseothricin resistance gene

(*NAT* gene), the knock-in plasmid Ma-NATX13-loxP carrying *NAT* gene, and the plasmid Ma-HPTX13-CRE carrying hygromycin B resistance gene (*HPT* gene) and Cre recombinase gene, specific for genome editing in *M. australis* W7-5 were constructed in our previous studies (Wei et al. 2021). The YPD medium for cultivation of the psychrophilic yeast contained 10.0 g/l yeast extract, 20.0 g/l peptone, and 20.0 g/l glucose and the solid YPD containing different concentrations of sorbitol or Congo red were used to examine growth of the unsaturated fatty acid mutants and their wild type strain W7-5. The genes and their functions used in this study are shown in Supplementary file 1.

Isolation and Sequencing of the Genomic DNAs and Molecular Identification of the M. australis W7-5

The genomic DNAs of *M. australis* W7-5 were isolated and purified based on the methods described by Chi et al (2012). The purified DNAs were detected by the agarose gel electrophoresis and quantified by a Qubit® 2.0 Fluorometer (Thermo Scientific). The libraries for single-molecule real-time (SMRT) sequencing were constructed with an insert size of 20 kb using a SMRT bell TM Template kit (version 1.0). For the Illumina HiSeq sequencing, sequencing libraries were generated using a NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. The whole genomic DNAs of *M. australis* W7-5 were sequenced using a PacBio Sequel platform and Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd, China. In order to ensure the accuracy of the subsequent analysis results, the low-quality reads were filtered (≤ 500 bp) to obtain clean data. The errors in the primary assembly were identified and corrected with a BLASR v5.1 (Chaisson and Tesler 2012). All the genes in the genome were predicted using an Augustus 3.2.1 and were functionally annotated using a BLAST2GO. The whole genome was online mined and analyzed using BLASTP. The whole-genome based phylogenetic tree of including *M. australis* W7-5 (GenBank: JAGSXI000000000) and other yeast strains was conducted through a composition vector (CV) approach on the CVTree3 website (http://tlife.fudan.edu.cn/cvtree/cvtree/).

Construction of the Disruption Vectors and Expression Vectors

The genomic DNA of *M. australis* strain W7-5 was prepared as described above. According to the sequenced genomic DNA (GenBank: JAGSXI000000000) of *M. australis* W7-5, the primers (Supplementary file 2) were designed to PCR amplify the gene *FAD91* encoding Δ^9 fatty acid desaturase 1, the *FAD92* gene encoding Δ^9 fatty acid desaturase 2, the gene *FAD12* encoding Δ^{12} fatty acid desaturase and the gene *FAD15* encoding Δ^{15} fatty acid desaturase using the genomic DNA of *M. australis* strain W7-5 as the template. Their accession numbers of these cloned genes are shown in Supplementary file 1. The 3'-arms and 5'-arms of the *FAD91* gene, the *FAD92* gene, the *FAD12* gene, the *FAD15* gene were PCR amplified using the primers shown in Supplementary file 2 and the genomic DNA of *M. australis* strain W7-5 as template. The cloned 3'-arms and 5'-arms were digested with the DNA restriction enzymes shown in Supplementary file 2 and the digests were ligated into plasmid Ma-NAT-loxP carrying the *NAT* gene digested with the same DNA restriction enzymes, forming Ma-NAT-loxP-ΔFAD91 (carrying 3'-arms and 5'-arms of the *FAD91* gene) (Supplementary file 3A), Ma-NAT-loxP-ΔFAD92 (carrying 3'-arms and 5'-arms of the *FAD92* gene) (Supplementary file 3B), Ma-NAT-loxP-ΔFAD12 (carrying 3'-arms

and 5'-arms of the *FAD12* gene) (Supplementary file 3C), Ma-NAT-loxP-FAD15 (carrying 3'-arms and 5'-arms of the *FAD15* gene) (Supplementary file 3D), The *FAD12* gene was PCR amplified using the primers (FAD12-F/FAD12-R) and the PCR products were digested with the corresponding enzymes in Supplementary file 2 and the digests were ligated into the knock-in plasmid MaNATX13 to yield Ma-NATX13-loxP-FAD12 (Supplementary file 3E).

Transformation of M. australis Strain W7-5 and Isolation of Various Deletants and Transformants

Preparation of the competent cells of *M. australis* strain W7-5 and transformation of *M. australis* strain W7-5 was carried out using the high efficiency transformation by electroporation as described by Wei et al (2021). The linear DNA fragments 5'-arm-PolyA-NAT-PGK-3'-arm were PCR amplified using the primers FAD91-5F/FAD91-5F, FAD92-5F/FAD92-3R, FAD12-5F/FAD12-3R, FAD15-5F/FAD15-3R (Supplementary file 2) and the plasmids Ma-NAT-loxP-ΔFAD91, Ma-NAT-loxP-ΔFAD92, Ma-NAT-loxP-ΔFAD12, Ma-NAT-loxP-ΔFAD15 constructed above as the templates. Similarly, the plasmids Ma-NATX13-loxP-FAD12 was digested with the enzyme *Sma*I to obtain the linear DNA fragments 18SrDNA-PGK-FAD12-polyA-NAT-PGK-26SrDNA. The linear DNA fragments 5'-arm-PolyA-NAT-PGK-3'-arm were transformed into the competent cells of *M. australis* strain W7-5 by using the electroporation method under the optimal conditions of voltage 2000 V, $OD_{600\ nm}$ of the yeast culture = 1.0, and the amount of DNA = 10.0 μg as described by Wei et al. (2021) and the mutants *Δfad12* in which the *FAD12* gene was totally removed and *Δfad15* in which the *FAD15* gene was completely abolished were obtained. However, the mutants in which the *FAD91* gene and the *FAD92* gene were removed could not be acquired because the absence of palmitoleic ($C_{16:1}$) or oleic ($C_{18:1}$) acids in the medium. After the mutant *Δfad12* was obtained, the linear DNA fragment PGK-HPT-polyA-CRE-PGK from the plasmid Ma-HPTX13-CRE was introduced into the disruptant cells to remove the *NAT* gene by the transiently expressed Cre recombinase as described by Wei et al (2021). Both the *NAT* gene and the *HPT* gene were lost after these disruptants were cultivated for 4 h and the mutant *Δfad12-cre* was obtained. At the same time, the linear DNA fragments 18SrDNA-PGK-FAD12-polyA-NAT-PGK-26SrDNA from the plasmid Ma-NATX13-loxP-FAD12 (Supplementary file 3E) transformed into the mutant *Δfad12-cre*, to acquire the complementing strain (FAD12-H). Finally, the linear DNA fragment PGKHPT-polyA-CRE-PGK from the plasmid Ma-HPTX13-CRE was again introduced into the FAD12-H to remove the *NAT* gene by the transiently expressed Cre recombinase and the *HPT* gene was lost automatically.

Determination of the Compositions of Fatty Acids in the Extracted Oil

All the disruptants, the complementing strain (FAD12-H) and their wild type strain were grown aerobically in the liquid YPD medium at 15°C for three days, The yeast cells in the culture were collected and washed by centrifugation at 5000 · g and 4°C for 10 min with sterile saline water for three times. The washed cells were dried at 80°C until the cell dried weight was constant. In order to analyze fatty acids compositions, the dried cells were added to 10.0 ml of 6.0 M hydrochloric acid solution, and the mixture was incubated in water bath at 80 ℃ for 4 h. Then, 7.0 ml of 60% methanol and 7.0 ml of chloroform were added to the mixture and the fatty acids were extracted by centrifugation at 5000 · g and 4°C for 10 min. Then, the extracted atty acids were dissolved in 2.0 ml of chloroform, 2.5 ml of 2% (v/v) sulfuric acid/methanol

solution was added to the mixture. Then, the mixture was incubated in water bath at 80 ℃ for 1 h. After cooling, 2.0 ml of saturated NaCl solution was added and mixed with shaking. Fatty acid methyl esters were extracted with 1.0–2.0 ml of n-hexane, and the upper layer with the fatty acid methyl ester was filtrated with 0.22 μm filter membrane to remove the impurity. Gas chromatography analysis of the fatty acid methyl esters obtained was carried out by using 5890-II (Agilent Company, USA). The chromatography column was a fused silica AC2.0 capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness); injector temperature, 250°C; carrier gas, N_2, 1.0 ml per min; temperature program, 50°C, held for 2.0 min from 150 to 200°C at 15°C per min, held for 2.0 min, then to 250°C at 2°C per min, held for 5.0 min. The fatty acid with $C_{19:0}$ was used as an internal standard (Li et al. 2010).

Analysis of Expression of Various Genes

The wild-type strain *M. australis* strain W7-5, different disruptants, and complementing strain obtained above were aerobically grown in 50.0 ml of the YPD medium at 15°C and 180 rpm for 2 days and total RNAs of them were extracted using an E.Z.N.A. Fungal RNA Kit. The yeast RNAs were reversely transcripted into cDNA using a PrimeScriptTM RT Reagent Kit (Perfect Real Time). The transcriptional levels of various genes in the wild-type strain *M. australis* strain W7-5, different disruptants, and complementing strain were determined using a Real time fluorescence quantitative PCR analyzer (QIAGEN, Germany) and the primers shown in Supplementary file 4.

Cell Growth in the Liquid YPD Medium at Different Temperatures

All the disruptants, complementing strain and their wild type strain were grown aerobically in the liquid YPD medium, effects of different temperatures (5°C, 15°C and 25°C) on their cell growth (OD_{600nm}) were examined. When the cells were grown for three days at 5°C, 15°C and 25°C, the yeast cells were harvested and washed by centrifugation at 5000 · g for 10 min with the sterile saline water. One part of the washed cells was treated at 80°C for 20 min. The washed yeast cells treated and untreated were suspended in 600.0 μl of the Annexin V-FITC binding solution and the cell suspension was thoroughly mixed with 5.0 μl of the Annexin V-FITC. Then, 10.0 μl of PI solution was added to the cell suspension and mixed well. The new cell suspension was incubated in the dark for 10–15 min. After that, the new cell suspension was centrifuged at 8000 × g for 5 min and the pellets obtained was resuspended in the Annexin V-FITC binding solution and the new cell suspension was observed under the fluorescence microscope (Olympus U-LH100HG, Japan) and images were recorded using the CellSense Standard software.

Cell Growth on the YPD Plates with Different Concentration of Congo Red and Sorbitol

At the same time, to test susceptibility to the cell wall disturbing compound (Congo red) and osmotic pressure stabilizer sorbitol, the yeast cells of *M. australis* strain W7-5, the disruptants and the complementing strain obtained above were cultivated on the YPD plates with Congo red (its concentrations were 200 μg/ml, 300 μg/ml, and 400 μg/ml) and sorbitol (its concentrations were 1.5 M, 2.0 M, and 2.5 M) at 5, 15 and 25°C for 4 days. After that, cell growth on the plates and cell morphology were observed and photographed.

Results

Sequencing of the Genomic DNA of the Psychrophilic Yeast M. australis Strain W7-5

The genomic DNA sequence of the *M. australis* strain W7-5 showed that the size of its genome was 14.52 Mbp, the whole genome contained 440 scaffolds and the GC content of the genome was 47.19% (Table 1). The whole genome sequence of the *M. australis* strain W7-5 was deposited at the NCBI and the GenBank accession number was JAGSXI000000000. Analysis using the software August 3.2.1 showed that the whole genome contained 4,813 encoding genes and the average length of each gene was 1,565 bp (Table 1). All the genes used in this study were shown in Supplementary file 1

Table 1
Assembly of the genomic DNA of *M. australis* W7-5 and its annotation

Statistic	Value per *M.australis*
	W7-5
Assembly statistics	
Assembly length (Mbp)	14.52
Scaffold length total (bp)	14,488,758
Number of scaffolds	440
Scaffold N50 (bp)	766,967
Contig length total (bp)	14,488,064
Number of contigs	443
Contig N50 (bp)	766,967
GC content (%)	47.19
Gene statistics	
Number of genes	4,813
Total Gene size (bp)	7,533,042
Average Gene Length (bp)	1,565
Functional annotations	
Genes with NR annotation	4,623
Genes with KEGG annotation	2,644
Genes with KOG annotation	3,303
Genes with Swissprot hit	4,036
Genes with GO annotation	2,902
Genes with Pfam annotation	3,978
Genes with TrEMBL annotation	4,630
Special database annotations	
Genes with CAZyme annotation	133
Genes with TCDB annotation	150
Genes with PHI nnotation	1,372

Deletion of the *FAD91* gene, *FAD92* gene, *FAD12* gene, *FAD15* gene

According to the sequenced genome *M. australis* W7-5, the *FAD91* gene, *FAD92* gene, *FAD12* gene, *FAD15* gene shown in Supplementary file 1, related to unsaturated fatty acid biosynthesis (Fig. 1), were deleted as described in Materials and methods. Unfortunately, the mutants in which the *FAD91* gene or the *FAD92* gene were abolished could not be obtained because such mutants could not grow in the medium in which was not supplemented with palmitoleic ($C_{16:1}$) or oleic ($C_{18:l}$) acids. These were reasonable because palmitoleic ($C_{16:1}$) or oleic ($C_{18:l}$) acids are required for growth and survival of all organisms (Brock et al. 2006). But the *FAD12* gene and *FAD15* gene could be easily removed and the corresponding *Δfad12* mutant and *Δfad15* mutant were obtained, respectively. Therefore, only the *Δfad12* mutant and the *Δfad15* mutant were used in subsequent investigations.

Cell Growth of the *Δfad12* Mutant and the *Δfad15* Mutant at Different Temperatures

Therefore, the *Δfad12* mutant, the *Δfad15* mutant and their wild type strain *M. australis* W7-5 were grown in the liquid YPD medium at 5°C, 15°C and 25°C and their cell growth (OD_{600nm}) were monitored during the cell growth. The results in Fig. 2 clearly showed that at optimal temperature (15°C), the two mutants grew the same as their wild type strain. However, at 5°C and 25°C, the *Δfad12* mutant grew much more slowly than its wild type strain W7-5. In addition, the *Δfad15* mutant still grew the same as its wild type strain W7-5 at 15°C (the optimal temperature for their growth), 5°C and 25°C. This strongly confirmed that only abolished gene *FAD12* gene could negatively affect cell growth at 5°C and 25°C. This meant that only linoleic acid ($C_{18:2}$) could affect cell growth (adaptation) of the psychrophilic yeast at 5°C and 25°C.

After the normal *FAD12* gene was expressed in the *Δfad12* mutant, the cell growth of the complementing strain FAD12-H obtained grew almost the same as its wild type strain *M. australis* W7-5 (Fig. 2). This meant complementation of the normal *FAD12* gene could restore cell growth of the mutant.

In order further to confirm this, the YPD medium was supplemented with linoleic acid and without linoleic acid, then, the *Δfad12* mutant and its wild type strain *M. australis* W7-5 were cultivated in the media at 5°C, 15°C and 25°C. The results in Fig. 3 indicated that cell growth of the *Δfad12* mutant in the medium with linoleic acid was better than that of the *Δfad12* mutant grown in the medium without linoleic acid. These strongly confirmed that only linoleic acid could indeed play an important role in adaptation of the psychrophilic yeast to low temperature (5°C) and high temperature (25°C).

The Cellular Fatty Acids Compositions of the Mutants, Their Wild Type Strain *M. australis* W7-5 and Complementing strain FAD12-H

The results in Table 2 highlighted that the percentage of linoleic acid ($C_{18:2}$) in the mutant *Δfad12* was greatly reduced while the percentage of $C_{18:1}$ fatty acid in the mutant *Δfad12* were obviously increased compared to those of $C_{18:1}$ and $C_{18:2}$ fatty acids in its wild type strain *M. australis* W7-5. This was

reasonable when biosynthesis of $C_{18:2}$ fatty acid was stopped, the biosynthesis of $C_{18:1}$ fatty acid was enhanced according to Fig. 1. In the mutant *Δfad15*, no $C_{18:3}$ fatty acid was detected and the percentages of any other fatty acids were almost the same as those of its wild type strain *M. australis* W7-5. At the same time, complementation of the normal *FAD12* gene in the *Δfad12* mutant made the complementing strain FAD12-H synthesize almost the same compositions of the fatty acids as its wild type strain *M. australis* W7-5 (Table 2). This meant that disable of the *FAD12* gene and the *FAD15* gene indeed stopped biosynthesis of $C_{18:2}$ fatty acid and $C_{18:3}$ fatty acid in the cells as stated in Fig. 1. The results in Table 2 also showed that in the YPD medium used in this study, there was also a small amount of $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids. But $C_{16:1}$ and $C_{18:2}$ fatty acids were not detected in the YPD medium (Table 2). All the results were consistent with those shown in Figs. 2, 3 and 4. So the YPD medium could not affect $C_{18:2}$ fatty acid biosynthesis in any cells of the psychrophilic yeast.

Table 2
The fatty acids compositions of *M. australis* W7-5, *Δfad12, Δfad15* and FAD12-H

Strain	**$C_{16:0}$(%)**	**$C_{16:1}$(%) (δ-9)**	**$C_{18:0}$(%)**	**$C_{18:1}$(%) (δ-9)**	**$C_{18:2}$(%) (δ-9,12)**	**$C_{18:3}$(%) (δ-9,12,15)**
W7-5	20.7 ± 1.1	3.9 ± 0.3	3.4 ± 0.2	28.3 ± 1.3	27.1 ± 0.8	9.1 ± 0.1
Δfad12	19.8 ± 1.3	5.5 ± 0.2	2.6 ± 0.2	55.7 ± 2.3	1.5** ± 0.1	5.48 ± 0.2
Δfad15	24.7 ± 1.2	3.6 ± 0.1	5.1 ± 0.3	31.7 ± 3.3	28.7 ± 2.2	ND
FAD12-H	20.9 ± 1.3	3.7 ± 0.2	3.3 ± 0.1	28.9 ± 2.1	27.0 ± 023	8.7 ± 0.3
Δfad12-Y	17.6 ± 0.7	6.3 ± 0.1	ND	58.4 ± 1.5	11.3 ± 0.3	1.5 ± 0.1
YPD	25.0 ± 1.5	ND	16.6 ± 0.2	14.6 ± 1.3	ND	ND

Data are given as mean ± SD, n = 3, * $P < 0.05$, ** $P < 0.01$.* means difference; **means significant difference.

The Relative Transcriptional Levels of the Relevant Genes in the Mutants, Their Wild Type Strain M. australis **W7-5 and Complementing Strain FAD12-H**

The results in Table 3 also revealed that the *FAD12* gene in the *Δfad12* mutant and the *FAD15* gene in the *Δfad*15 mutant were totally removed and deletion of the *FAD12* gene and the *FAD15* gene also made most of any other gene expression greatly be reduced. In contrast, it can be clearly observed that expression of the *FAD92* gene in *Δfad12* mutant was greatly enhanced. This result was constant with the fact that the amount of $C_{18:1}$ fatty acid in *Δfad12* mutant was promoted (Table 2). However, complementation of the *FAD12* gene in the *Δfad12* mutant made all the genes in FAD12-H related to fatty acid biosynthesis greatly be enhanced. The results were also consistent with those shown in Table 2.

Table 3

The relative transcriptional levels of the genes in *M. australis* W7-5, *Δfad12, Δfad15* and FAD12-H

Genes	*FAD91*	*FAD92*	*FAD12*	*FAD15*
W7-5 (%)	100.0	100.0	100.0	100.0
Δfad15 (%)	46.4 ± 12.5**	44.4 ± 3.5**	46.1 ± 1.9**	4.0 ± 0.2**
Δfad12 (%)	35.9 ± 3.0**	1920819.7± 247341.3**	0.0 ± 0.0**	42.8 ± 6.8**
FAD12-H (%)	11619.1± 1016.5**	9920.5± 1077.4**	2573.6± 282.7**	13282.3± 866.0**
Data are given as mean ± SD, n = 3, * $P < 0.05$, ** $P < 0.01$.* means difference; **means significant difference.				

The Sensitivity of the Mutants and the Wild Type Strain W7-1 to Congo Red and Sorbitol

It has been known that Congo red can interact with β-1,3-glucan and chitin and binding of Congo red with high level β-1,3-glucan and chitin in yeast cell walls can result in inhibition of yeast cell wall assemble, disturbance of cell wall stability, and stop of cell growth (Wei et al., 2021; Yoshimi et al., 2017). It has been well known that cell wall can protect the cell from osmotic and mechanical stress and is essential for not only for the maintenance of cell shape and integrity, but also for progression through the cell cycle (Garcia-Rubio et al., 2020; Levin, 2011). So, it is very important to keep cell wall integrity, rigidity and the cellular structure. It can be seen from the results in Fig. 4 that there was a weak cell growth of only the mutant *Δfad12* on the plate with 1.5 M, 2.0 M and 2.5 M sorbitol at 5, 15 and 25°C, confirming that the mutant *Δfad12* was sensitive to high osmotic pressure, especially to 2.0 and 2.5 M sorbitol at 25°C. This may mean that cell wall in the mutant *Δfad12* grown at 25°C was also negatively affected when the mutant could not synthesize $C_{18:1}$ fatty acid.

However, the results in Fig. 4 also showed that there was a weak cell growth of only the mutant *Δfad12* grown on the plate with 300 and 400 μg/ml of Congo red at 25°C, indicating that biosynthesis of β-1,3-glucan and chitin in yeast cell walls of the mutant *Δfad12* was enhanced when it was grown on the plate at 25°C.

However, cell morphology of the mutant *Δfad12* grown at different conditions and different temperatures was not affected compared to that of its wild type strain W7-5 (Fig. 5).

Only Cell Growth Rate of the Mutant Δfad12 Grown at 5°C and 25°C was Negatively Affected

It can be clearly observed from the results in Fig. 2 that the mutant *Δfad12* grew very slowly at 5°C and 25°C. In order to know what had happened to the cells of the mutant *Δfad12* grown at 5°C and 25°C, the

washed cells of the mutant *Δfad12* grown at 5°C, 15°C and 25°C were treated at 80°C for 20 min. Both the nuclei of the heated cells and unheated cells were strained with PI. It can be obviously observed from the results in Fig. 6 that only after the grown yeast cells of the mutant *Δfad12* were treated at 80°C for 20 min, they could be stained by PI, indicating that the treatment of the grown yeast cells of the mutant *Δfad12* caused membrane damage so that their nuclei could be stained by PI while the untreated cells kept cell integrity and alive. This meant that lack of $C_{18:2}$ fatty acid only could lead to the reduced growth rate of the yeast cells grown at 5°C and 25°C may by slowing down the cell division at 5°C and 25°C, but not affect cell growth of the mutant at 15°C.

Discussion

Table 1 showed the basic characteristics of the genome of the psychrophilic yeast *M. australis* strain W7-5 used in this study. It has been reported that the assembled draft of *M. australis* strain UFMG-CM-Y6158 isolated from a marine macroalgae, *Acrosiphonia arcta* (Chlorophyta), collected in Admiralty Bay of King George Island in Keller Peninsula, Antarctica consisted of 14.3 Mbp over 160 contigs (> 505 bp) with a G + C content of 47.2%. The longest contig was 1,116,518 bp long, and the N50 contig length was 542,232 bp in *M. australis* strain UFMG-CM-Y6158 (Batista et al. 2017). *M. australis* strain UFMG-CM-Y6158 has 4,442 protein-coding genes (Batista et al. 2017). Therefore, the genome of *M. australis* strain W7-5 used in this study was similar to that of *M. australis* strain UFMG-CM-Y6158.

Figure 2 showed that only linoleic acid could indeed play an important role in adaptation of the psychrophilic yeast to low temperature (5°C) and high temperature (25°C) because only the *Δfad12* mutant in which the percentage of $C_{18:2}$ fatty acid was greatly reduced (Table 2) grew very slowly at 5°C and 25°C. However, at the optimal temperature (15°C), the *Δfad12* mutant grew as well as its wild type strain W7-5 (Fig. 2). In *S. cerevisiae* a decrease in temperature induces the expression of many genes involved in transcription and translation, some of which display a cold-sensitivity phenotype (Aguilera et al. 2007). It has been reported that the genes encoding Δ^9 and Δ^{12} fatty acid desaturases (FAD) were also upregulated when the Antarctic yeast, *Glaciozyma antarctica* PI12 was grown at the low temperature of − 12°C (Athirah Yusof et al. 2021).

Similarly, linoleic acid ($C_{18:2}$) also increased in the *Aspergillus versicolor* and *Geomyces pannorum* strains, as well as in the *S. salinum*, *Geomyces vinaceus* and *G. pannorum* strain from Antarctica when grown at 8 °C (Maggi et al. 2013). It also has been found that linoleic (25–30%) acid was predominated in the fatty acid profiles of species of the psychrophilic yeasts *Mrakia* sp., *Leucosporidium* sp. and *Rhodotorula* sp. The polyunsaturated FA (PUFA) ($C_{18:2}$ and $C_{18:3}$) content in *Glaciozyma antarctica* PI12 increased by 1–2% to fuel the membrane fluidity particularly at the freezing temperature of − 12°C (Wang et al. 2017).

By contrast, at temperatures close to the maximum for growth, oleic (20–40%) and linoleic (30–50%) acids were the major components (Wang et al. 2017). It was found that Ole1, the only fatty acid desaturase known in *S. cerevisiae*, seemed to be important for cold growth (Aguilera et al. 2007). In

contrast, oleic acid quantities were higher both in the strains of *Cladosporium cladosporioides* and *Scolecobasidium salinum* and in the Antarctic strain of *Cadophora fastigiata* when these strains were grown at 8 °C than 25 °C. The *A. versicolor* strains and the Antarctic strains of *C. fastigiata* and *C. cladosporioides* displayed a significant amount of linolenic acid ($C_{18:3}$), which was higher at 8 °C than at 25 °C. The Antarctic strains of *M. alpina* and *M. antarctica* revealed high amounts of arachidonic acid ($C_{20:4}$), which increased in strains cultivated at 8 °C (Maggi et al. 2013). However, the present study found that linoleic acid synthesized by Fad12, not linolenic acid synthesized by Fad15, played an important role in the cold adaptation of *M. australis* W7-5 to low (5°C) and high (25°C) temperature (Fig. 2, Table 2). Furthermore, it has been reported that exposure to low temperature protects *S. cerevisiae* cells against freeze injury through the cold-induced accumulation of trehalose, glycerol and heat-shock proteins (Aguilera et al. 2007). The results also show that changes in membrane fluidity of *S. cerevisiae* cells are the primary signal triggering the cold shock response (Aguilera et al. 2013). This and those obtained in our previous studies (Wei et al. 2021) demonstrated that the cold growth mechanisms of *M. australis* W7-5 used in this study was completely different from those of *S. cerevisiae* and any other fungal strains from the Antarctic. This was the first time to show that only linoleic acid can play an important role in adaptation of psychrophilic yeasts to low (5° C) and high temperature (25°C) at molecular levels.

In order to know why its growth rate of the *Δfad12* mutant at 5°C and 25°C was greatly reduced (Fig. 2), the sensitivity of cell growth to sorbitol and Congo red was tested. Figure 5 indicated that there was a weak cell growth of only the mutant *Δfad12* grown on the plate with 1.5 M, 2.0 M and 2.5 M sorbitol at 5 and 25°C, and grown on the plate with 300 and 400 μg/mL of Congo red at 25°C. All these results suggested that when the *Δfad12* mutant could not synthesize $C_{18:2}$ fatty acids (Table 2), its cell wall synthesis, cell wall integrity, cell wall rigidity and the cellular structure was also negatively affected, resulting in its reduced growth rate at 5°C and 25°C (Fig. 2).

Figure 6 highlighted that lack of $C_{18:2}$ fatty acid only could lead to the reduced growth rate of the yeast cells grown at 5°C and 25°C by slowing down the cell division and could not affect cell growth of the mutant at 15°C. Indeed, reduction of growth rates was one of adaptation strategies which the psychrophilic yeasts had (Wang et al. 2017). It has been reported that inhibition of PUFA biosynthesis by knockout of the Δ^{12} fatty acid desaturase gene (*RKD12*) in mesophilic yeast *Rhodosporidium kratochvilovae* YM25235 influenced cold adaptation of the strain YM25235 by decreasing the polyunsaturated fatty acids content in cell membranes and reducing the growth rate and membrane fluidity of YM25235 at low temperature (Wang et al. 2017).

Conclusions

In summary, this study demonstrated that $C_{18:2}$ fatty acid could play an important role in adaptation of the psychrophilic yeast to low temperature (5°C) and high temperature (25°C). When the *Δfad12* mutant could not synthesize $C_{18:2}$ fatty acid (Table 2), its cell wall synthesis, cell wall integrity, cell wall rigidity and the cellular structure was also negatively affected, resulting in its reduced growth rate at 5°C and

25°C. But when it was grown at the optimal temperature (15°C), $C_{18:2}$ fatty acid could not play any role in cell growth.

Declarations

Acknowledgments This study was financially supported by the National Natural Science Foundation of China (Grant No. 31970058).

Funding This study was financially supported by the National Natural Science Foundation of China (Grant No. 31970058).

Availability of Data and Materials All data generated or analyzed during this study are included in this article and its supplementary files.

Author Contribution W. X and Z. M performed the experiments and analyzed the data; G. L. L and Z. C designed partial study and Z. M. C wrote the manuscript.

Compliance with Ethical Standards

Competing Interests The authors declare that they have no competing interests.

Ethics Approval and Consent to Participate Not applicable.

Consent for Publication Not applicable.

References

1. Aguilera J, Randez-Gil F, Prieto JA (2007) Cold response in *Saccharomyces cerevisiae*: new functions for old mechanisms. FEMS Microbiol Rev 31: 327–41
2. Alcaıno J, Cifuentes V, Baeza M (2015) Physiological adaptations of yeasts living in cold environments and their potential applications. World J Microbiol Biotechnol 31: 1467–1473
3. Athirah Yusof N, Fazlin Hashim NH, Bharudin I (2021) Cold Adaptation strategies and the potential of psychrophilic enzymes from the antarctic yeast, *Glaciozyma antarctica* PI12. J Fungi *7*: 528
4. Batista TM, Hilário HQ, Moreira RG, Furtado C, Godinho VM, Rosa LH, Franco, RG, Rosa CA (2017) Draft genome sequence of *Metschnikowia australis* strain UFMG-CMY6158, an extremophile marine yeast endemic to Antarctica. Genom Announ 5: e00328–e00317
5. Brock TJ, Browse J, Watts JL (2006) Genetic regulation of unsaturated fatty acid composition in *C. elegans*. PLoS Genet 2: e108
6. Chaisson M, Tesler JG (2012) Mapping single molecule sequencing reads using basic local alignment with successive refinement (BLASR): application and theory. BMC Bioinform 13: 238
7. Chi Z, Wang XX, Ma ZC, Buzdar MA, Chi ZM (2012) The unique role of siderophore in marine-derived A*ureobasidium pullulans* HN6.2. Biomet 25: 219–230

8. Deegenaars M, Materson K (1998) Heat shock response in psychrophilic and psychrotrophic yeast from Antarctica. Extremophil 2: 41–49
9. Garcia-Rubio R, de Oliveira HC, Rivera J, Trevijano-Contador N (2020) The Fungal cell wall: *Candida*, *Cryptococcus*, and *Aspergillus* Species. Front Microbiol 10: 2993
10. Levin DE (2011) Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genet 189: 1145–75
11. Li M, Liu GL, Chi Z, Chi ZM (2010) Single cell oil production from hydrolysate of cassava starch by marine-derived yeast *Rhodotorula mucilaginosa* TJY15a. Bioma Bioen 4: 101–7
12. Maggi O, Tosi S, Angelova M, Lagostina E, Fabbri A, Pecoraro L, Altobelli E, Maria Picco A, Savino E, Branda E, Turchetti B, Zotti MA, Vizzini AP, Buzzini P (2013) Adaptation of fungi, including yeasts, to cold Environments. Plant Biosyst 147: 247–258
13. Russell NJ (2008) Membrane components and cold sensing. fundamentals of cold-adapted enzymes. In: Margesin R, Schinner F, Marx JC, Gerday C, editors. Psychrophiles: from biodiversity to biotechnology. Berlin: Springer-Verlag. Pp: 177–190
14. Stukey JE, McDonough VM, Martin CE (1989) Isolation and. characterization of *OLEl*, a gene affecting fatty acid desaturation from *Saccharomyces cereuisiae*. J Biol Chem 264: 16537–16544
15. Stukey JE, McDonough VM, Martin CE (1990) The *OLEl* gene of *Saccharomyces cerevisiae* encodes the A9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. J Biol Chem 265: 20144–20149
16. Su Y, Jiang X, Wu W, Wang M, Imran Hamid M, Miang M, Liu X (2016) Genomic, transcriptomic, and proteomic analysis provide insights into the cold adaptation mechanism of the obligate psychrophilic fungus *Mrakia psychrophile*. G3 Gene Genome Genet 6: 3603
17. Wang J, Chen W, Nian H, Ji X, Lin L, Wei Y, Zhang Q (2017) Inhibition of polyunsaturated fatty acids synthesis decreases growth rate and membrane fluidity of *Rhodosporidium kratochvilovae* at low temperature. Lipids 52: 729–735
18. Wei X, Chi Z, Liu GL, Hu Z, Chi ZM (2021) Integrity in growth of the psychrophilic yeast *Metschnikowia australis* W7-5 at different temperatures. Microb Ecol 81: 52–66
19. Yoshimi A, Miyazawa K, Abe K (2017) Function and biosynthesis of cell wall α-1,3-glucan in fungi. J Fung 3: 63

Figures

Figure 1

Biosynthesis of different unsaturated fatty acid in eucaryotic cells. FAS: fatty acid synthetase

Figure 2

Cell growth of (OD_{600nm}) of the *Δfad12* mutant, the *Δfad15* mutant, the complementing strain FAD12-H and their wild type strain *M. australis* W7-5 at 5°C, 15 °C and 25 °C. Data are given as mean ± SD, n=3.

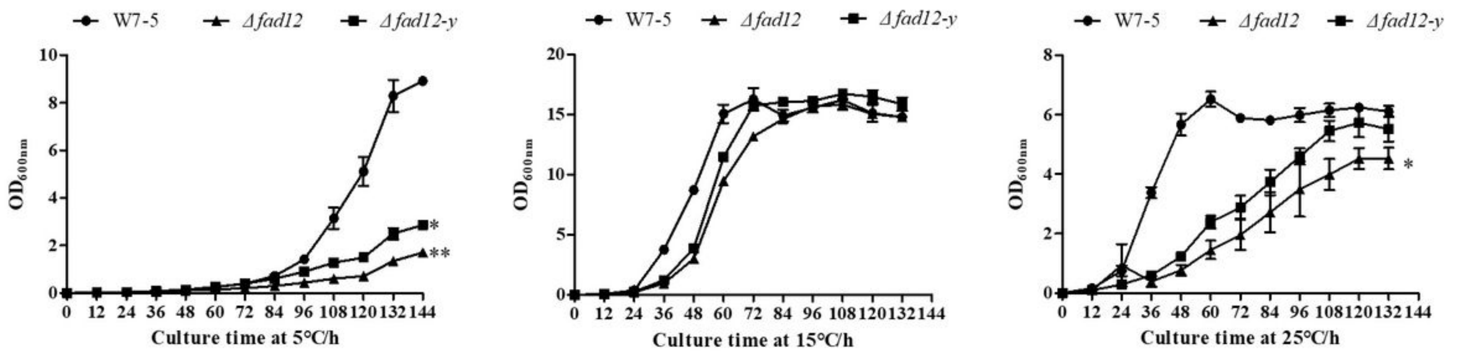

Figure 3

Effects of added linoleic acid on cell growth of the *Δfad12* mutant. *Δfad12*-y meant the growth of *Δfad12* grown in the medium supplemented with linoleic acid. Data are given as mean ± SD, n=3.

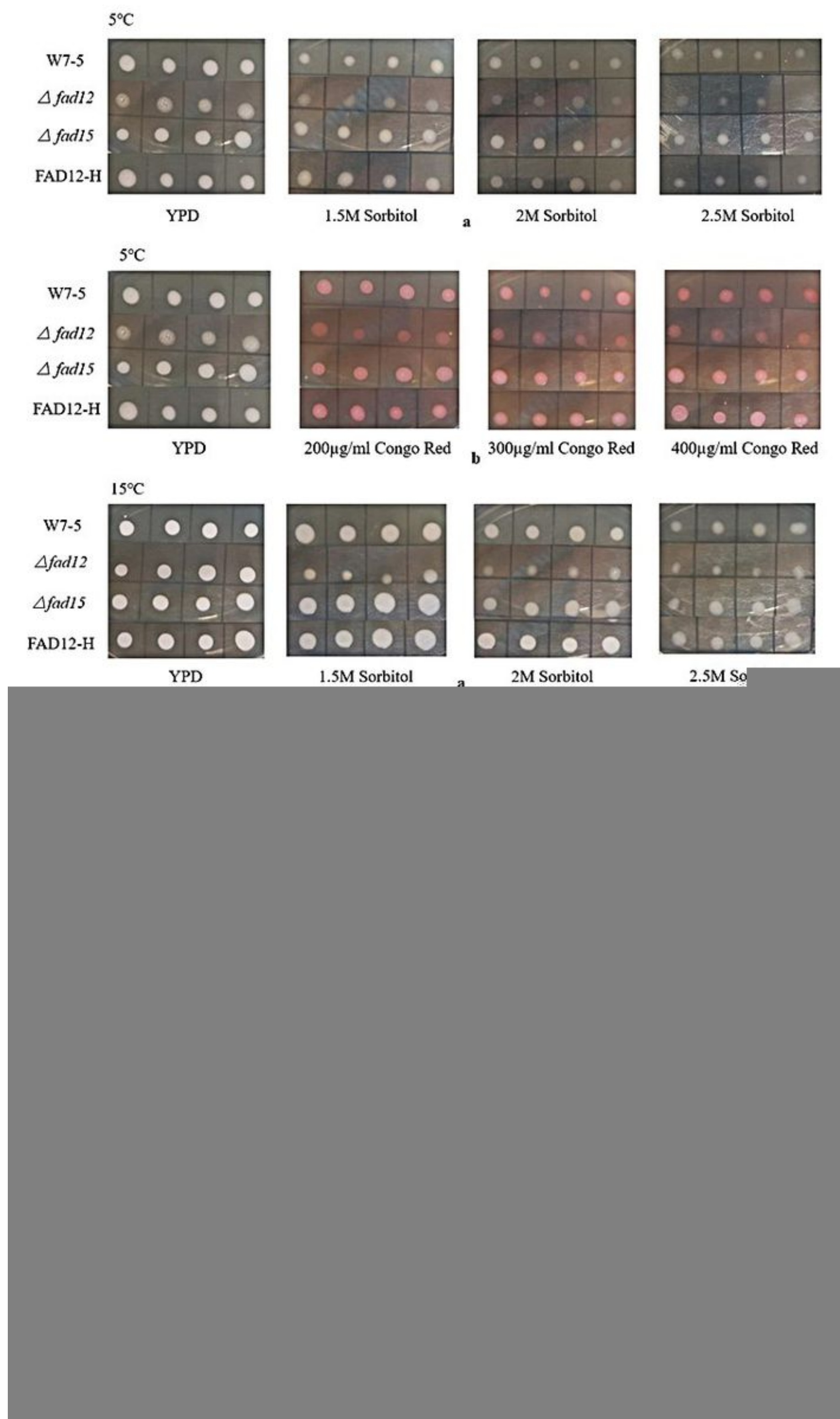

Figure 4

Effects of different concentration of sorbitol (A) and Congo red (B) and different temperatures on cell growth of different disruptants, complementing strain and their wild type strain.

Figure 5

Cell morphology of the mutant *Δfad12* and its wild type strain W7-5 grown at 5 °C, 15 °C and 25 °C

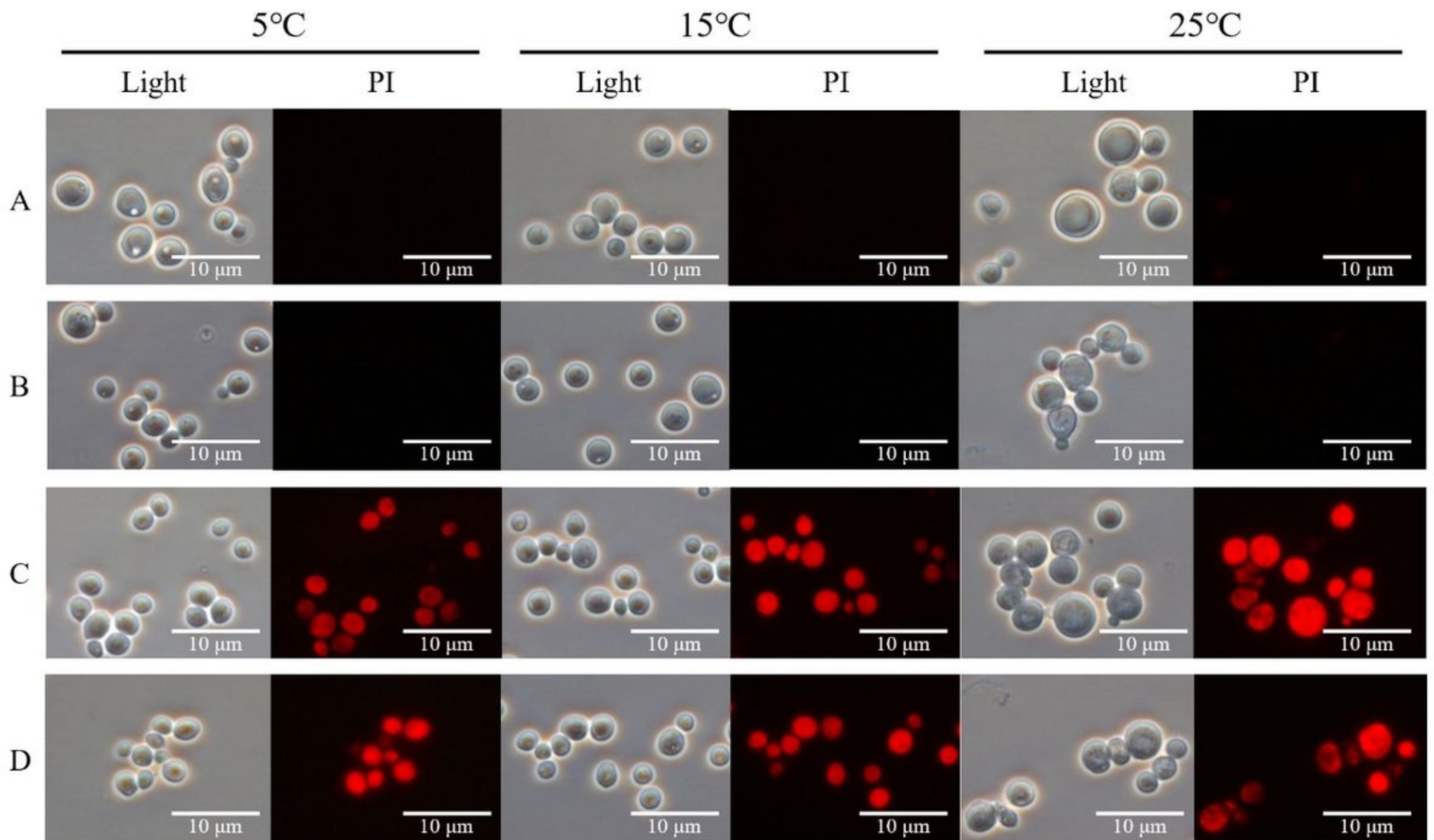

Figure 6

The PI staining of the heated yeast cells (C and D) of the mutant *Δfad12* grown at 5 °C, 15 °C and 25 °C and the PI staining of the unheated yeast cells (A and B) of the mutant *Δfad12* grown at 5°C, 15 °C and 25 °C. The yeast cells were taken under the normal light and under UV light, respectively.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- WeiSupplementaryfile2.doc
- WeiSupplementaryfile1.doc
- WeiSupplementaryfile3.doc
- WeiSupplementaryfile4.doc