fermentation

MDPI

Article

Discovery and Functional Evaluation of Heat Tolerance Genes in the Nonconventional Yeast *Yarrowia lipolytica*

Mengchan Liu and Hairong Cheng *

State Key Laboratory of Microbial Metabolism, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China; lmc19940607@sjtu.edu.cn
* Correspondence: chrqrq@sjtu.edu.cn

Abstract: *Yarrowia lipolytica*, a GRAS (generally recognized as safe) nonconventional yeast, has been used widely in industrial fermentation to produce chemicals, fuels, and functional sugars such as erythritol and mannitol. Although *Y. lipolytica* is a promising organism for bioconversion and has substantial potential in industrial production, its utilization is restricted by the high cost of cooling during the fermentation process; the optimum growth or fermentation temperature of *Y. lipolytica* is 28–30 °C, which is lower than that of some fermenting species. Therefore, it is necessary to breed a thermoresistant *Y. lipolytica* for use in a fermentation system. Here, we report a new thermoduric *Y. lipolytica* strain (a thermoresistant clone, HRC) that can grow at 35 °C—higher than the starting strain *Y. lipolytica* CGMCC7326 (maximum growth temperature at 33 °C)—by laboratory adaptive evolution. Based on the transcriptome analysis of the mutant strain HRC and the parental strain *Y. lipolytica* CGMCC7326 at different temperatures, 22 genes with increased expression at high temperatures were identified and 10 of them were overexpressed in *Y. lipolytica* CGMCC7326. HRC1, HRC2, and HRC3 (with YALI0B21582g, YALI0C13750g, and YALI0B10626g overexpressed, respectively) were assessed for growth at higher temperatures. This revealed that these three genes were related to thermotolerance. This study provides insights into the metabolic landscape of *Y. lipolytica* under heat stress, enabling future metabolic engineering endeavors to improve both thermoresistance and sugar alcohol production in the yeast *Y. lipolytica*.

Keywords: *Yarrowia lipolytica*; thermoresistance; transcriptome analysis; laboratory evolution

Citation: Liu, M.; Cheng, H. Discovery and Functional Evaluation of Heat Tolerance Genes in the Nonconventional Yeast *Yarrowia lipolytica*. *Fermentation* **2023**, *9*, 509. https://doi.org/10.3390/fermentation9060509

Academic Editors: Bartłomiej Zieniuk and Dorota Nowak

Received: 7 May 2023
Revised: 21 May 2023
Accepted: 22 May 2023
Published: 25 May 2023

1. Introduction

With global climate change and the energy crisis, microbial fermentation using renewable substrates and waste carbon is emerging as one of the best alternatives to bypass conventional chemical syntheses, enabling sustainable bio-based chemical production [1,2]. Within this arena, yeast hosts have proven to be promising microbial factories for specialty chemical production due to their capacity for fermentation [3–5]. Among these desirable microbial hosts, several nonconventional yeasts [5] have been selected, with *Yarrowia lipolytica* garnering the strongest interest and showing utility in biotechnology applications [6].

Y. lipolytica, an oleaginous yeast, has been studied and widely reviewed for its production of fatty acid-based fuels and chemicals such as lipids and biofuels [7–11], succinic acid [12], α-ketoglutaric acid [13], and itaconic acid [14]. Recently, erythritol—a naturally abundant sweetener—has drawn increasing attention because it prevents an increase in blood glucose, improves glycemic control, and exerts antioxidative properties [15]. It has been regarded as a safe and healthy sugar substitute for humans and is used as a sweetener in calorie-reduced foods, candies, and bakery products. Notably, *Y. lipolytica* is widely used for industrial erythritol production [16,17].

Although *Y. lipolytica* is a promising host for bioconversion and has substantial potential for industrial production, its utilization is restricted by some shortcomings. The most significant one is that the optimum growth temperature of *Y. lipolytica* is 28–30 °C; this

is lower than that of some other species, such as the thermotolerant yeast *Kluyveromyces marxianus*, which can grow well at temperatures over 45 °C [18]. A large amount of heat is produced in fermentation during production, exposing yeast strains to elevated temperatures and significantly reducing cell viability and sugar alcohol production. Cooling systems generally maintain the fermentation temperature at 30 °C, which leads to high energy costs [19]. To mitigate this, it is necessary to breed thermoresistant *Y. lipolytica* for the fermentation system.

Efforts have been made to improve the thermotolerance of yeasts such as *Saccharomyces cerevisiae* [20–22]. An in-depth understanding of the mechanism of yeast thermotolerance lays the foundation for breeding robust strains. Since the discovery of the heat shock phenomenon in prokaryotes and eukaryotes, the heat shock response has garnered much attention [23]. Some molecules have been shown to contribute to heat resistance in yeast, including Hsps, antioxidant enzymes, trehalose, ATPase, and ubiquitin ligase. Hsps are important for most organisms to resist high temperatures since they assist in the refolding of damaged proteins, clear irreversibly aggregated proteins, and improve the thermal stability of soluble proteins in stressed cells [24]. Furthermore, antioxidant enzymes remove the damage caused by ROS (including superoxide and hydrogen peroxide), which are generated in heat stress [24,25]. Trehalose is a storage carbohydrate for yeast and can stabilize biological membranes, proteins, and nucleic acids under stress conditions [26,27]. Notably, when *S. cerevisiae* is exposed to high temperatures, trehalose biosynthesis is upregulated [28–30]. It was reported that ATPase is crucial for thermoresistance in yeast. Reduced expression of Pma1, the major H^+-ATPase in yeast, led to a significantly reduced thermoresistance [24,31]. Further, ubiquitin ligase plays a role in protecting cells against proteotoxic stress caused by high temperature [32]. Rsp5, an E3 ubiquitin-protein ligase, has been shown to clear cytosolic misfolding proteins under heat stress in yeast [33,34].

Based on the mechanism of yeast thermoresistance, some heat-resistant devices have been introduced into *Y. lipolytica* to improve its thermoresistance [35,36]; however, there are a limited number of heat-resistant devices that could be used. Thus, it is crucial to develop new heat-resistant devices that can be applied to improve the thermoduric capacity of *Y. lipolytica*. Here, we report a new thermoduric *Y. lipolytica* strain (a heat-resistant clone, HRC) that can grow at 35 °C—higher than the original strain *Y. lipolytica* CGMCC7326 (maximum temperature of 33 °C) after adaptive evolution in the laboratory. More importantly, the HRC strain has a comparable capacity to produce erythritol and mannitol. To reveal the genetic determinants of the possible tolerance improvement, an RNA-seq analysis was carried out. We analyzed the abundance of genes that were differentially regulated according to the comparative transcriptomics analysis for the thermotolerant strain HRC and the original strain *Y. lipolytica* CGMCC7326 [37] by cluster analysis. We identified 22 genes with increased expression at high temperature by RT-qPCR and overexpressed 10 of them in *Y. lipolytica* CGMCC7326. HRC1, HRC2, and HR3 (with YALI0B21582g, YALI0C13750g, and YALI0B10626g overexpressed, respectively) were assessed for growth at higher temperatures. This revealed that these three genes were related to thermotolerance. The proteins encoded by YALI0B21582g and YALI0C13750g are transcription factors, and YALI0B10626g is an unknown gene. Our data provide insights into the metabolic landscape of *Y. lipolytica* under heat stress, enabling future metabolic engineering endeavors to improve both thermoresistance and sugar alcohol production in *Y. lipolytica*.

2. Materials and Methods

2.1. Yeast Strains, Medium, and Culture Conditions

The *Escherichia coli* and *Y. lipolytica* strains used in this research are listed in Table S1. The *E. coli* strains were grown at 37 °C in LB medium supplemented with 100 μg/mL ampicillin (Amp). The *Y. lipolytica* strains were grown at 30 °C in YPD (YPD: yeast extract 10 g/L, tryptone 5 g/L, glucose 20 g/L) or YNB medium (10 g/L yeast nitrogen base without amino acids, 5 g/L ammonia sulfate) supplemented with 200 μg/mL mycophenolic

acid. Fermentation medium for *Y. lipolytica*: glucose 270 g/L, yeast extract 8 g/L, peptone 2 g/L, magnesium sulfate 0.1 g/L, zinc chloride 0.01 g/L.

2.2. Experimental Evolution

Laboratory adaptive evolution was performed as previously described with minor modifications [38]. *Y. lipolytica* CGMCC7326 cells from a single colony were streaked on solid YPD medium plates and incubated at 30 °C for 24 h. Next, single colonies were selected and inoculated into 2 mL liquid YPD to incubate at 30 °C for 24 h. Cells were then collected and gradient diluted to plated on solid YPD medium and placed at 35 °C for 72 h. After that, large single colonies were selected and inoculated into 2 mL liquid YPD at 30 °C for 24 h. The procedure was repeated 10 times, and large colonies were picked for the aerobic fermentation and thermoresistance tests.

2.3. Cell Growth Profiling

Strains were inoculated in 30 mL of YPD medium for 24 h under 220 rpm agitation at 30 °C. Cells were then transferred to an initial OD_{600nm} of 0.4 into a new flask containing 30 mL of fresh medium under 220 rpm agitation at the indicated temperatures. The OD_{600nm} values were recorded every 6 or 8 h until the strains reached stationary phase. Each test was performed in biological triplicates. Then, the average OD_{600nm} of triplicate samples was calculated, and the cell growth profiles were depicted in GraphPad Prism 6.01.

2.4. Estimation of Tolerance to Temperature Stress

The strains were first inoculated on a YPD plate and cultured at 30 °C for 15 h. Then, plated cells were transferred into 30 mL of YPD medium and cultured at 30 °C for 24 h to ensure that they reached the stationary phase. The cells were then diluted using sterilized water to an OD_{600nm} of 10. A cell suspension of 2.5 µL from the heated sample was serially diluted tenfold, spotted onto a YPD plate, and cultured at 35 °C for 24 h.

2.5. Transcriptome Analysis

The cells of *Y. lipolytica* CGMCC7326 were collected at an OD_{600nm} of 12 after culturing at 30 °C and 33 °C. The cells of the thermoduric strain HRC were collected at an OD_{600nm} of 12 after culturing at 30 °C, 33 °C, and 35 °C (Figure 1a). Cell pellets were resuspended in deionized water. After centrifugation at 6000× *g* for 10 min, the cell pellets were flash frozen in liquid nitrogen and stored at −80 °C. The samples were prepared in triplicate.

RNA extraction and sequencing were carried out at BGI Gene Technology Co., Ltd. (Wuhan, China). The mRNA: PolyA-tail messenger RNAs (mRNAs) were enriched by magnetic beads with oligo(dT). Ribosomal RNA was hybridized with DNA probes, and RNase H was used to selectively digest the DNA/RNA hybridization chain. Then, the DNA probe was digested by DNase I. The obtained mRNA was segmented and reverse transcribed with random N6 primers to synthesize the complementary DNA. Synthesized double-stranded DNA was flattened and phosphorylated at the 5′ end. The 3′ end formed a sticky end with a protruding "A", which was connected to a bubbly joint with a protruding "T" at the 3′ end. The ligation products were amplified by polymerase chain reaction (PCR) with specific primers. The PCR products were heat denatured into single-stranded DNA and then cyclized with a bridge primer to obtain a single-stranded circular DNA library. To analyze the original sequencing data, we first filtered out raw reads with low quality, contaminated joints, and high unknown base content. The clean reads were then blasted against the reference genome GCA_000002525 (NCBI database). After that, new transcripts were predicted, and SNP/InDel and differential splicing genes were detected. The new transcripts with protein-coding potential were added to the reference gene sequence, and the gene transcription level was calculated. Relevant data have been uploaded to the NCBI database (PRJNA948389).

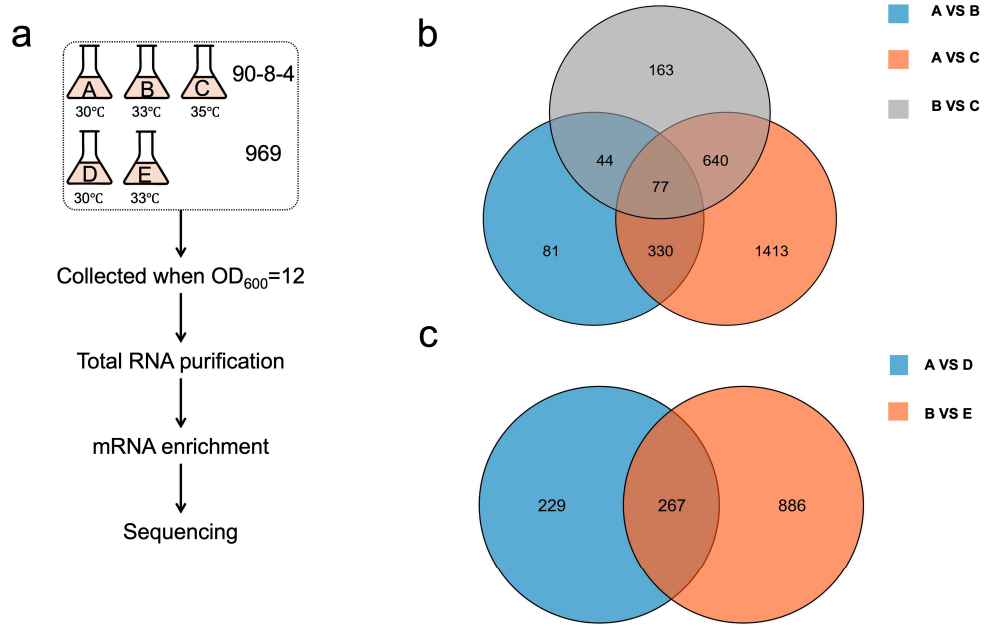

Figure 1. Transcriptomic analysis of HRC and *Y. lipolytica* CGMCC7326 at different temperatures. (**a**). Flow chart of sample preparation and sequencing. (**b**). Venn diagram comparing the differences in gene expression between Group A and Group B, Group A and Group C, and Group B and Group C. (**c**). Venn diagram comparing the differences in gene expression between Group B and Group E and Group A and Group D.

2.6. RNA Isolation and Transcript-Level Quantification

Cultures were grown in shaken flasks containing rich medium. Cells were collected at an OD_{600nm} of 2.0 and stored at −80 °C in TRIzol solution from Sangon Biotech (Shanghai, China). Total RNA was extracted using liquid nitrogen and the TRIzol kit. cDNA was obtained using HiScript® III-RT SuperMix for qPCR with gDNA wiper (Vazyme Biotech Co., Ltd., Nanjing, China). These cDNA samples were used as templates for quantitative real-time PCR analysis (qRT-PCR) with the specific primer sets listed in Table S1. The qRT-PCRs were performed using ChamQ™ Universal SYBR®qPCR Master Mix (Vazyme Biotech Co., Ltd.) and an ABI7500 Real-Time PCR system. The qPCRs proceeded as follows: initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 62 °C for 30 s, and elongation at 72 °C for 20 s. Specific amplification was confirmed by analysis of melting curves from 65 °C to 95 °C. Gene expression was normalized to that of the 18S rRNA gene (ΔCT method). The differences in gene expression between the transformed and control strains of *Y. lipolytica* CGMCC7326 were calculated by the $2^{-\Delta\Delta CT}$ method. All samples were analyzed in triplicate.

2.7. Overexpression of Selected Genes to Verify Their Thermoresistance in Y. lipolytica CGMCC7326

The ten selected genes (Table S2) were PCR amplified from the genomic DNA of strain CGMCC7326 using the primers listed in Table S1. Forward (F) and reverse (R) primers (named according to gene nomenclature) were designed to introduce *Kpn*I sites into the resulting amplicons. Then, genes were expressed using *Kpn*I-treated pSWV-guab expression cassettes (5′-26S *rDNA-hp4d-gene of interest-TT-guab-TT-26S rDNA*-3′) (Sequences 1–11 in the Supplementary Information). *Y. lipolytica* CGMCC7326 was streaked on a YPD plate and incubated at 30 °C for 24 h. Then, yeast cells were collected and resuspended in 200 µL of transformation buffer (400 g/L PEG 4000, 150 mM lithium acetate (pH 6.0), 150 mM dithiothreitol, and 0.2 mg/mL single-stranded salmon sperm DNA). Samples of 3–5 µg of a pSWV-guab expression cassette were added to the transformation system. The system was then vortexed thoroughly and incubated at 39 °C for 60 min with shaking at regular intervals to prevent cells from sinking to the bottom of the tube. After incubation,

cells were spread directly on a YNB medium plate (with 200 μg/mL mycophenolic acid added) for 5 days, and transformants of 10 strains were streaked on solid YNB medium (with 200 μg/mL mycophenolic acid added) and cultivated at 35 °C for 30 h. The growth of these transformants was observed to judge the thermoresistance of the transformants. Transformants grown on this medium were verified by PCR using the primers listed in Table S1.

3. Results

3.1. Thermotolerance Improvement after Adaptive Evolution

First, we tested the temperature sensitivity of *Y. lipolytica* CGMCC7326. It grew well at 30 °C, slowly at 33 °C, and was almost inviable at 35 °C (Figure S1). To obtain thermoresistant strains by laboratory adaptive evolution, the wild-type strain was streaked on solid YPD medium plates and incubated at 30 °C for 24 h. Next, single colonies were selected and inoculated into 2 mL liquid YPD at 30 °C for 24 h; cells were then collected, gradient diluted, and plated onto solid YPD medium and placed at 35 °C for 72 h. After that, large single colonies were selected and inoculated into 2 mL liquid YPD at 30 °C for 24 h. The procedure was repeated 10 times, a HRC was selected, and its thermotolerance was verified by measuring the growth curve at 35 °C (Figure S1). According to the growth curve, *Y. lipolytica* CGMCC7326 and HRC both grew well at 30 °C, with the upper level of OD_{600nm} nearly reaching 25 in 20 h (with the specific growth rate of 0.19 h^{-1} vs. 0.17 h^{-1} for CGMCC7326 and HRC). When the temperature increased to 33 °C, *Y. lipolytica* CGMCC7326 grew slower than HRC, with the maximum OD_{600nm} reaching approximately 22 in 30 h, whereas the HRC still grew well, with the peak value of OD_{600nm} reaching 25 in 20 h (with the specific growth rate of 0.14 h^{-1} vs. 0.20 h^{-1} for CGMCC7326 and HRC). When the temperature was 35 °C, *Y. lipolytica* CGMCC7326 was almost inviable, and HRC grew slightly slower than at 30 °C and 33 °C, with the upper level of OD_{600nm} reaching approximately 23 after cultivation for 25 h (with the specific growth rate of 0.03 h^{-1} vs. 0.17 h^{-1} for CGMCC7326 and HRC). A spot assay at 35 °C (Figure S2) also showed that *Y. lipolytica* CGMCC7326 grew more poorly than HRC, which demonstrated that the thermoresistance of HRC was remarkably improved after laboratory evolution.

3.2. Differential Expression Analysis through Comparative Transcriptome Gene Quantification

To thoroughly understand the HRC mechanism of thermotolerance, we designed an elaborate RNA-seq analysis to compare the differences in gene expression for HRC and *Y. lipolytica* CGMCC7326 at different temperatures. The HRC strain was cultured at 30 °C (Group A), 33 °C (Group B), and 35 °C (Group C) separately, while the *Y. lipolytica* CGMCC7326 strain was cultured at 30 °C (Group D) and 33 °C (Group E). *Y. lipolytica* CGMCC7326 was not included in the 35 °C group because it was almost inviable at 35 °C (Figure S1). After reaching the midpoint of log phase (OD_{600nm} = 12), the total mRNA was enriched and sequenced (Figure 1a).

Transcriptome analysis of the strains was performed with RNA-seq technology using the Illumina Hi-Seq platform. First, we analyzed the change in gene transcription in HRC strains with altered expression at higher temperatures. Comparative transcriptomic analyses were applied between Groups A, B, and C. In total, 532 and 2460 genes were significantly different when comparing the 33 °C and 35 °C groups to the 30 °C group, respectively. Additionally, 924 genes were differentially expressed between the 33 °C group and the 35 °C group. Across these 3 comparisons, 77 genes had no differential expression. Gene expression was also compared between *Y. lipolytica* CGMCC7326 and HRC strains at different temperatures. At 30 °C, only 496 genes were expressed differently; however, 1153 genes were expressed differently in HRC at 33 °C (Figure 1b,c), suggesting that HRC gene expression was remodeled for thermoresistance. Furthermore, comparison of gene expression in Group C and Group D revealed that there were 2864 differentially expressed genes (DEGs), including 1339 upregulated genes and 1252 downregulated genes. These results provided useful information about the genes that were engaged in the heat

stress response in *Y. lipolytica* CGMCC7326. GO enrichment analysis of Group C and Group D indicated that most DEGs were enriched in organic acid metabolic process, carboxylic acid metabolic process, oxoacid metabolic process, and small molecule metabolic process (Figure S3a). KEGG analysis showed that DEGs were significantly enriched in the mitogen-activated protein kinase (MAPK) signaling pathway, biosynthesis of antibiotics, biosynthesis of amino acids, pyruvate metabolism, arginine and proline metabolism, and carbohydrate metabolism (Figure S3b).

Based on our definition of DEGs as having a $|\log2 \text{ ratio}| \geq 1$ and p value < 0.05, 22 significant genes (Table S3) were selected, including 4 membrane proteins and 18 soluble proteins. The transcriptomic results were further validated by RT-qPCR. The mRNA levels of these genes were almost all upregulated in the HRC after thermal stimuli, except YALI0C19404g, YALI0B10626g, and YALI0F11121g (Figure 2a,b). Almost all the selected genes were induced when comparing HRC strains with *Y. lipolytica* CGMCC7326 at 33 °C (Figure 2c). Furthermore, the mRNA levels of 13 genes (Table S4) were significantly upregulated (more than twofold) at 30 °C in HRC strains compared to *Y. lipolytica* CGMCC7326, suggesting that some thermoresistance-related genes are nearly continuously activated in the HRC strains, even without thermal stimuli. These genes might be helpful to stabilize the intracellular environment and increase cell growth during upcoming stress.

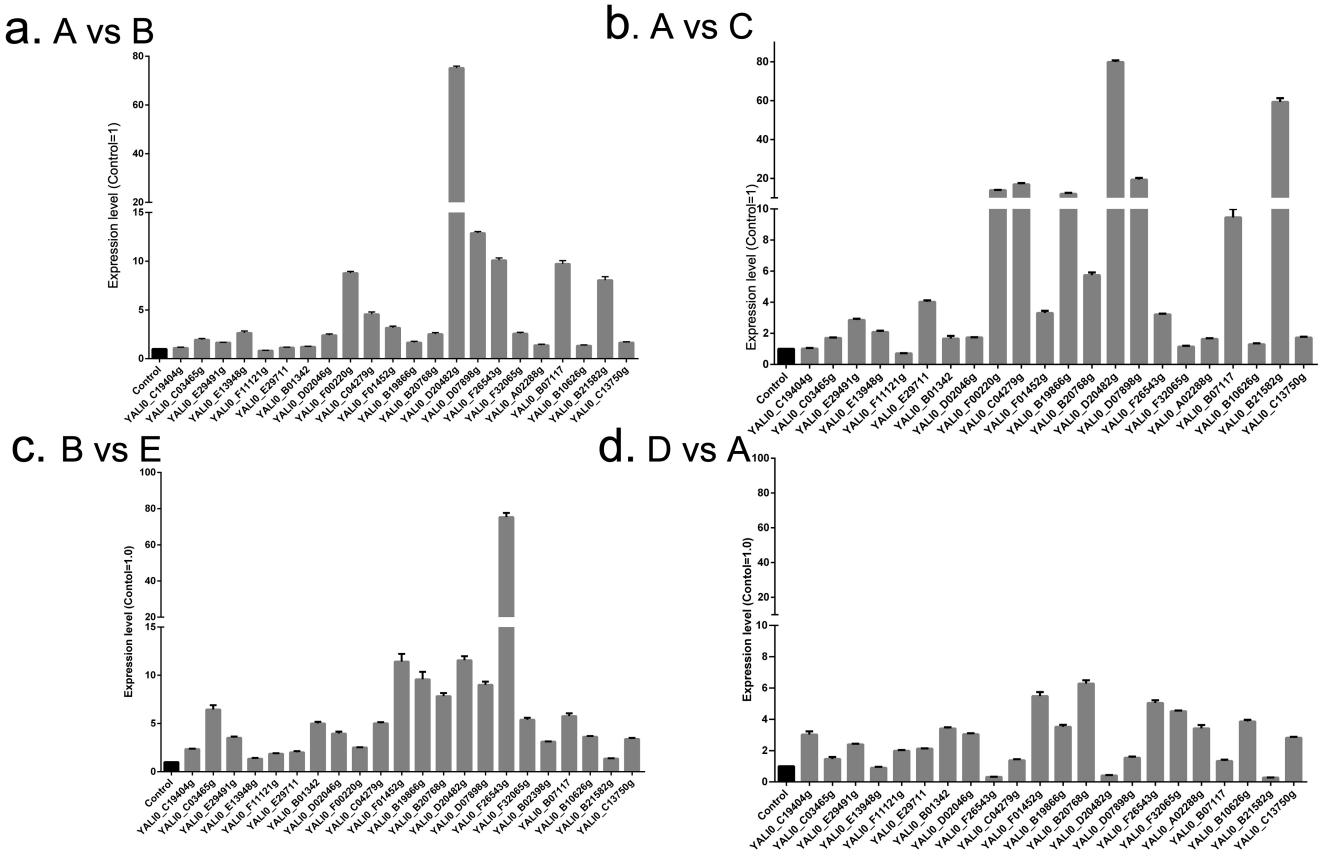

Figure 2. RT-qPCR analysis for differential gene expression of HRC and *Y. lipolytica* CGMCC7326 in different groups. (**a**). HRC at 30 °C 33 °C. (**b**). HRC at 30 °C 35 °C. (**c**). HRC vs. *Y. lipolytica* CGMCC7326 at 33 °C. (**d**). *Y. lipolytica* CGMCC7326 vs. HRC at 30 °C. In each sample, 18S was used as an internal control. Total RNA was isolated from cells cultured at 48 h in biological triplicate.

3.3. Improving the Thermotolerance of Wild-Type Y. lipolytica CGMCC7326

Among the twenty-two genes (Table S3) that were validated by RT-qPCR to verify relative expression levels (Figure 2), we found that nine genes were highly expressed in Groups B and C when compared to the wild-type Groups D and E (Table S2); these data may demonstrate that the nine genes were induced by temperature stimuli. YALI0C13750g was

another gene that attracted our attention, since it is the homolog of the msn4 transcriptional activator in *S. cerevisiae*, which is proven to be related to the heat response [39].

These ten genes were selected to further validate the thermotolerant effects. The ten genes were overexpressed in wild-type *Y. lipolytica* CGMCC7326 to determine if the cells could exhibit thermotolerance (Figure 3a). Among the 10 genes (Table S2), overexpression of YALI0B21582g, YALI0C13750g, and YALI0B10626g improved the thermotolerance of *Y. lipolytica* CGMCC7326 (Figure 3a). YALI0B21582g and YALI0C13750g belong to the MAPK signaling pathway, which plays a part in responding to environmental fluctuations, especially osmotic and heat stresses [39]. The overexpression of the three genes was verified by PCR (Figure 3b). YALI0B21582g—also called Mhy1—contains C2H2 zinc finger motifs and functions as a transcription factor in response to environmental stress [40]. YALI0C13750g encodes a protein homolog of the *S. cerevisiae* msn4 transcriptional activator. Msn4 is known to promote the formation of pseudohyphae in yeast [40]. Consistent with previous reports, YALI0C13750g can dramatically induce the formation of mycelia, especially in the HRC2 strain (Figure S4), and it is a novel gene involved in the yeast-to-hyphae transition of the dimorphic yeast *Y. lipolytica.* The third gene, YALI0B10626g, has an unknown functionality.

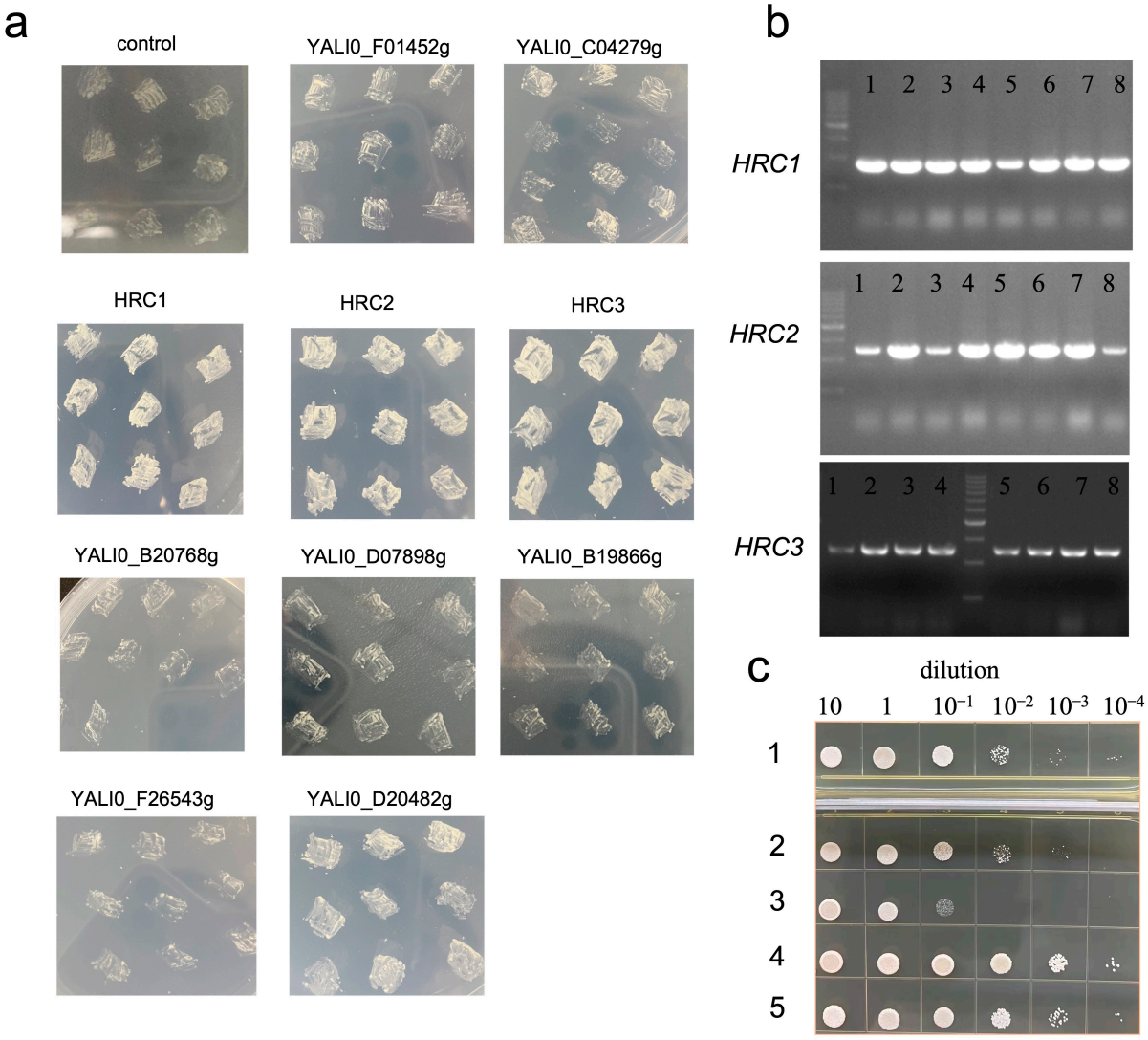

Figure 3. Thermotolerance of 10 overexpressed strains. (**a**). The overexpressed transformants picked were spread on YNB plates at 35 °C. (**b**). PCR verification of eight transformants of HRC1, HRC2 and HRC3. (**c**). Spot assay at 35 °C. (1) HRC1, (2) HRC2, (3) *Y. lipolytica* CGMCC7326, (4) HRC, (5) HRC3.

The thermoresistance properties of YALI0B21582g, YALI0C13750g, and YALI0B10626g were further confirmed with the spot assay. After gradient diluted, the cells were dropped on the solid YPD medium and cultured at 35 °C for 72 h, HRC1, HRC2, HRC3 and HRC grew well, while the wild-type strain *Y. lipolytica* CGMCC7326 grew poorly and could not survive when diluted to 10^{-2} (Figure 3c). A growth curve at 34 °C showed that *Y. lipolytica* CGMCC7326 grew slowly with a cell density of the upper level of OD_{600nm} reaching nearly 2, but HRC1, HRC2, and HRC3 grew well with a cell density of the upper level of OD_{600nm} reaching nearly 20 in 18 h, which demonstrated that the overexpression of these three genes conferred *Y. lipolytica* CGMCC7326 thermotolerance. However, HRC grew faster than HRC1, HRC2, and HRC3, with the maximum OD_{600nm} reaching nearly 25 in 18 h, which implied that there may be other heat-resistant devices that have not been discovered yet (Figure 4).

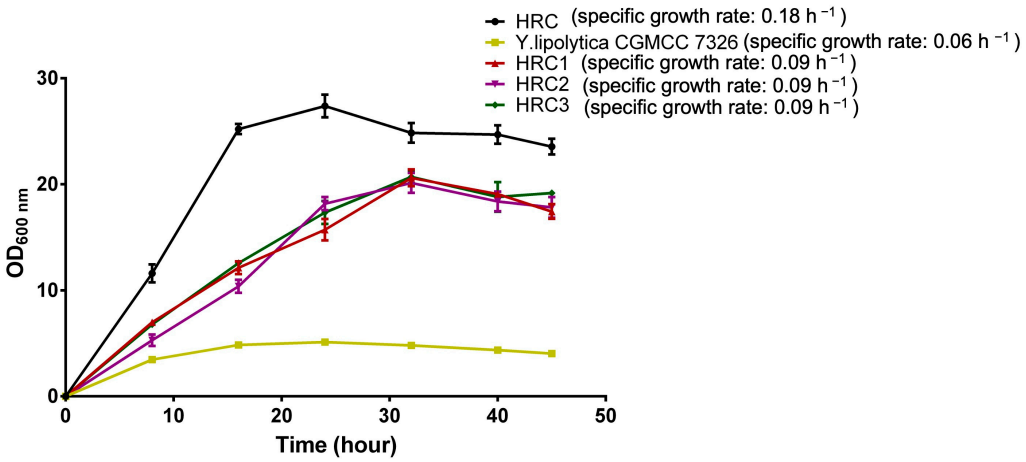

Figure 4. Growth curve of *Y. lipolytica* CGMCC7326 and HRC, HRC1, HRC2, and HRC3 at 34 °C.

3.4. Polyols Synthesis Analysis of Y. lipolytica CGMCC7326 and HRC

To evaluate the synthesis ability of erythritol and mannitol by the 2 strains, fermentation was carried out in a 250 mL flask containing 270 g/L glucose at 30 °C and 33 °C. The fermentation times of *Y. lipolytica* CGMCC 7326 and HRC were nearly consistent at 30 °C (Figure 5a). At 30 °C, the concentration of erythritol produced by *Y. lipolytica* CGMCC 7326 was 178.4 4.2 g/L, and the mannitol concentration was 10.4 2.1 g/L at 84 h. HRC produced 32.8 g/L mannitol, which is threefold that of *Y. lipolytica* CGMCC 7326; however, the erythritol titer of HRC was 152.7 5.2 g/L (Figure 5c). When the temperature was elevated to 33 °C, we found that the mannitol titer was increased in both *Y. lipolytica* CGMCC 7326 and HRC to 14.4 2.2 g/L at 96 h and 43.7 4.2 g/L at 92 h, respectively. Thus, we suspected that the strains reacted to thermal stimuli by increasing mannitol production. For erythritol, the titers of *Y. lipolytica* CGMCC 7326 and HRC decreased to 170.2 3.5 g/L and 141.2 3.7 g/L, respectively, indicating that erythritol synthesis is sensitive to high temperature.

However, the fermentation ability of the three strains HRC1, HRC2, and HRC3 was not as good as that of *Y. lipolytica* CGMCC 7326 and HRC, with less sugar alcohols and delayed fermentation time. Interestingly, most HRC2 cells switched to mycelia formation during fermentation (Figure S3).

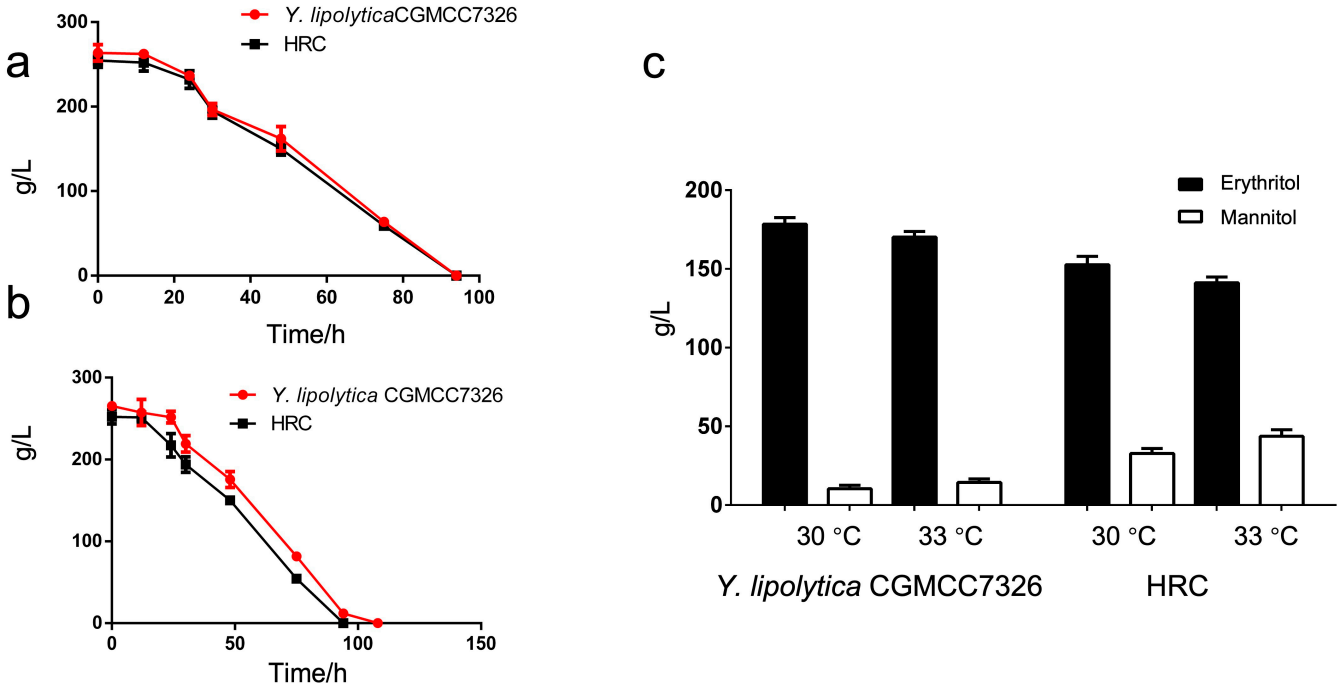

Figure 5. Glucose utilization and erythritol and mannitol production of *Y. lipolytica* CGMCC7326 and HRC. (**a**) Glucose utilization of *Y. lipolytica* CGMCC7326 and HRC at 30 °C. (**b**) Glucose utilization of *Y. lipolytica* CGMCC7326 and HRC at 33 °C. (**c**) Erythritol and mannitol production of *Y. lipolytica* CGMCC7326 and HRC at 30 °C and 33 °C.

4. Discussion

Recent advances in improving yeasts' thermoresistance have mainly focused on random and rational strategies of conferring thermotolerance; these are based on yeasts' own heat-resistance mechanisms and the mechanisms of heat resistance in thermophiles. Random strategies have focused on classical strain improvement methods such as natural breeding, mutagenesis, adaptation, and modern biotechnology breeding, including genome shuffling [24]. Evolutionary engineering approaches are very useful for developing thermotolerance in yeast. Qiu et al. selected a *Y. lipolytica* strain with improved thermoduric capabilities and erythritol production by using laboratory evolution [38]. Rational strategies emphasize the use of thermophiles. Heat-resistant proteins and heat-inducible genes from thermophiles can be introduced into yeasts to improve their thermotolerance. Hsps and ubiquitin are important for the cell to respond to high temperatures. Hsp- and ubiquitin-encoding genes from *Thermoanaerobacter tengcongensis* were introduced into *S. cerevisiae* to improve thermoduric capacity and overexpressed with a strong constitutive *FBA1* promoter by Liu et al. [41]. The engineered strain *T.te-Gros2* showed the best thermotolerance when cultured at 42 °C for 72 h. Notably, Liang et al. [35] introduced potential thermotolerance genes that encode Hsps, stress response proteins, ATPases, ubiquitin ligases, antioxidant enzymes, and nucleic acid protectors into *Y. lipolytica*. The results showed that expression of Hsp10, San1, and Ctt1 enhanced the growth of *Y. lipolytica* at 35 °C. Additionally, Wang et al. [36] and Hosein Shahsavarani et al. [33] overexpressed *Rsp5* in *Y. lipolytica* and *S. cerevisiae*, respectively, which contributed to the increase in the upper limit of yeast thermotolerance. This is because *Rsp5* is an essential gene encoding E3 ubiquitin-protein ligase, which improves heat resistance by targeting cytosolic misfolded proteins following heat stress in yeast [42].

In our study, we obtained the heat-tolerant strain HRC by laboratory adaptive evolution. On the basis of transcriptome analysis and RT-qPCR data, we found that the transcription levels of YALI0B21582g, YALI0C13750g, and YALI0B10626g were substantially upregulated in HRC compared to the wild-type strain when the temperature was

increased (Table S2). In particular, the overexpression of these genes confers a thermotolerant phenotype to the wild-type strain (Figures 3 and 4).

Using bioinformatic analyses, we found that YALI0B21582g and YALI0C13750g belong to MAPK signaling pathways. In particular, MAPK and its related transcription factors and interacting proteins are the primary modulators of the heat shock response. In 1993, a kinase was found to be essential for *S. cerevisiae* to survive in high osmolarity environments [43]. This led to the discovery of the HOG pathway, a MAPK pathway that is crucial to the responses to a wide range of stress conditions in fungi [44]. MAPK signaling pathways are essential sensor mechanisms that transduce environmental inputs to biochemical events, ensuring adaptation to new physiological situations. These pathways are conserved in eukaryotic organisms and control proliferation and stress responses [44,45]. Hog1, the central kinase of the HOG signaling pathway, is activated by heat stress and plays a crucial role in resisting heat stress [39]. Activated Hog1 helps promote recovery from cellular damage caused by heat stress [46].

Most genes induced by heat stress are controlled by msn2 and msn4, C2H2-type zinc-finger proteins downstream of Hog1 [47]. Msn2 and msn4 regulate general stress responses and therefore can regulate most heat-resistance genes [48,49]. In *S. cerevisiae*, they regulate the expression of the *Nth1* gene that encodes trehalose, thus regulating trehalose hydrolysis under different stress conditions. Additionally, they maintain trehalose concentrations under stress by regulating trehalose synthesis and hydrolase expression [50]. Msn2 can also regulate lipid metabolism, altering membrane fluidity when faced with high temperatures [51]. In particular, YALI0B21582g and YALI0C13750g are transcription factors containing the (C2H2) zinc finger motif [52]. The two genes are functional homologs of msn2 and msn4 in *S. cerevisiae*. Interestingly, overexpression of YALI0C13750g induces the formation of mycelia, and it is a novel gene involved in the yeast-to-hyphae transition of dimorphic yeast (Figure S4). Previous studies have shown that Msn2p and Msn4p can be constitutively synthesized during growth, and they are activated by translocation from the cytosol into the nucleus in response to stress conditions such as heat shock, carbon source starvation, osmotic stress, and the presence of ethanol or sorbate [53,54]. Therefore, we speculated that overexpression of YALI0B21582g and YALI0C13750g promotes their translocation to the nucleus, which helps strains survive heat stress.

However, the function of YALI0B10626g is unknown, and the characteristics of the gene should be studied in future research. Given that *Y. lipolytica* CGMCC7326 was also used in industry to produce functional sugar alcohols, engineering of the heat tolerant strain HRC to improve sugar alcohol production has substantial industrial implications.

To adapt the changing environment, yeasts also take other actions through a thermal response system that includes conservative signaling pathways, transcriptional regulatory systems, physiological and biochemical processes, and phenotypic changes [55]. The heat resistance mechanisms employed by yeast mainly consist of gene expression responses, Hsps, trehalose, ATPase, ubiquitin, and antioxidant enzymes [24]. These mechanisms might also be conserved in *Y. lipolytica*, and other heat-resistant devices may need to be further discovered.

5. Conclusions

In summary, we obtained the thermoduric strain HRC through laboratory adaptive evolution. The strain grows well at 35 °C and can produce more mannitol than the wild-type strain, *Y. lipolytica* CGMCC 7326, which grows well at 28–30 °C. Using transcriptome analyses, we discovered that heat stress alters the transcriptional profile of many genes related to the MAPK signaling pathway, as well as cell wall and cytomembrane remodeling. Among these genes, 22 were validated by RT-qPCR, showing that these genes were upregulated with elevated temperature and may be associated with thermoduric conditions. Of the twenty-two genes, overexpression of three genes—YALI0B21582g, YALI0C13750g, and YALI0B10626g—led to the acquisition of thermoresistance. Among them, YALI0B21582g and YALI0C13750g belong to MAPK signaling pathways. Our study highlights the im-

portance of engineering thermoresistant phenotypes to improve sugar alcohol production in *Y. lipolytica*, which may reduce the cooling cost in large-scale fermentation in industry. Our data provided insights into the metabolic landscape of *Y. lipolytica* under heat stress, enabling future metabolic engineering endeavors to improve both thermoresistance and alcohol production in *Y. lipolytica*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9060509/s1, Figure S1: Growth curve of HRC and Y. lipolytica CGMCC7326 at different temperatures; Figure S2: Spot assay of *Y. lipolytica* CGMCC7326 and HRC; Figure S3: Differentially expressed genes (DEGs) analysis of D group (HRC at 35 °C) vs. C Group (CGMCC7326 at 30 °C); Figure S4: Morphological characteristics of HRC2 and *Y. lipolytica* CGMCC7326. Table S1: Strains, gene cassettes, and primers used in this study; Table S2: Ten selected genes which are overexpressed in *Y. lipolytica* CGMCC7326; Table S3: relative expression level of 22 genes; Table S4: 13 genes were significant upregulated in HRC strains at 30 °C compared with the *Y. lipolytica* CGMCC7326.

Author Contributions: Methodology, formal analysis, investigation and writing, M.L.; Conceptualization, supervision and funding acquisition, H.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Key Research and Development Program of China with grant number [2018YFA0900700] and the APC was also funded by this grant.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Acknowledgments: We would like to thank Joseph Elliot at the University of Kansas for her assistance with English language and grammatical editing of the manuscript.

Conflicts of Interest: The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Shields-Menard, S.A.; Amirsadeghi, M.; French, W.T.; Boopathy, R. A review on microbial lipids as a potential biofuel. *Bioresour. Technol.* **2018**, *259*, 451–460. [CrossRef] [PubMed]
2. Ekas, H.; Deaner, M.; Alper, H.S. Recent advancements in fungal-derived fuel and chemical production and commercialization. *Curr. Opin. Biotechnol.* **2019**, *57*, 1–9. [CrossRef] [PubMed]
3. Liu, L.Q.; Redden, H.; Alper, H.S. Frontiers of yeast metabolic engineering: Diversifying beyond ethanol and Saccharomyces. *Curr. Opin. Biotech.* **2013**, *24*, 1023–1030. [CrossRef]
4. Lian, J.; Mishra, S.; Zhao, H. Recent advances in metabolic engineering of Saccharomyces cerevisiae: New tools and their applications. *Metab. Eng.* **2018**, *50*, 85–108. [CrossRef] [PubMed]
5. Wagner, J.M.; Alper, H.S. Synthetic biology and molecular genetics in non-conventional yeasts: Current tools and future advances. *Fungal Genet. Biol.* **2016**, *89*, 126–136. [CrossRef]
6. Abdel-Mawgoud, A.M.; Markham, K.A.; Palmer, C.M.; Liu, N.; Stephanopoulos, G.; Alper, H.S. Metabolic engineering in the host Yarrowia lipolytica. *Metab. Eng.* **2018**, *50*, 192–208. [CrossRef] [PubMed]
7. Sheng, J.; Feng, X. Metabolic engineering of yeast to produce fatty acid-derived biofuels: Bottlenecks and solutions. *Front. Microbiol.* **2015**, *6*, 554. [CrossRef]
8. Ledesma-Amaro, R.; Nicaud, J.M. Yarrowia lipolytica as a biotechnological chassis to produce usual and unusual fatty acids. *Prog. Lipid Res.* **2016**, *61*, 40–50. [CrossRef]
9. Adrio, J.L. Oleaginous yeasts: Promising platforms for the production of oleochemicals and biofuels. *Biotechnol. Bioeng.* **2017**, *114*, 1915–1920. [CrossRef]
10. Yan, J.; Han, B.; Gui, X.; Wang, G.; Xu, L.; Yan, Y.; Madzak, C.; Pan, D.; Wang, Y.; Zha, G.; et al. Engineering Yarrowia lipolytica to Simultaneously Produce Lipase and Single Cell Protein from Agro-industrial Wastes for Feed. *Sci. Rep.* **2018**, *8*, 758. [CrossRef]
11. Lazar, Z.; Liu, N.; Stephanopoulos, G. Holistic Approaches in Lipid Production by Yarrowia lipolytica. *Trends Biotechnol.* **2018**, *36*, 1157–1170. [CrossRef]

12. Yuzbashev, T.V.; Yuzbasheva, E.Y.; Sobolevskaya, T.I.; Laptev, I.A.; Vybornaya, T.V.; Larina, A.S.; Matsui, K.; Fukui, K.; Sineoky, S.P. Production of succinic acid at low pH by a recombinant strain of the aerobic yeast Yarrowia lipolytica. *Biotechnol. Bioeng.* **2010**, *107*, 673–682. [CrossRef] [PubMed]
13. Kamzolova, S.V.; Morgunov, I.G. alpha-Ketoglutaric acid production from rapeseed oil by Yarrowia lipolytica yeast. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 5517–5525. [CrossRef] [PubMed]
14. Blazeck, J.; Hill, A.; Jamoussi, M.; Pan, A.; Miller, J.; Alper, H.S. Metabolic engineering of Yarrowia lipolytica for itaconic acid production. *Metab. Eng.* **2015**, *32*, 66–73. [CrossRef] [PubMed]
15. Chukwuma, C.I.; Mopuri, R.; Nagiah, S.; Chuturgoon, A.A.; Islam, M.S. Erythritol reduces small intestinal glucose absorption, increases muscle glucose uptake, improves glucose metabolic enzymes activities and increases expression of Glut-4 and IRS-1 in type 2 diabetic rats. *Eur. J. Nutr.* **2018**, *57*, 2431–2444. [CrossRef]
16. Lange, F.T.; Scheurer, M.; Brauch, H.J. Artificial sweeteners—A recently recognized class of emerging environmental contaminants: A review. *Anal. Bioanal. Chem.* **2012**, *403*, 2503–2518. [CrossRef]
17. Fickers, P.; Cheng, H.; Sze Ki Lin, C. Sugar Alcohols and Organic Acids Synthesis in Yarrowia lipolytica: Where Are We? *Microorganisms* **2020**, *8*, 574. [CrossRef]
18. Kosaka, T.; Tsuzuno, T.; Nishida, S.; Pattanakittivorakul, S.; Murata, M.; Miyakawa, I.; Lertwattanasakul, N.; Limtong, S.; Yamada, M. Distinct Metabolic Flow in Response to Temperature in Thermotolerant *Kluyveromyces marxianus*. *Appl. Environ. Microbiol.* **2022**, *88*, e0200621. [CrossRef]
19. Schenk, C.; Schulz, V. Energy-optimal control of temperature for wine fermentation based on a novel model including the yeast dying phase. *IFAC Pap.* **2015**, *48*, 452–457. [CrossRef]
20. Garcia-Rios, E.; Lairon-Peris, M.; Muniz-Calvo, S.; Heras, J.M.; Ortiz-Julien, A.; Poirot, P.; Rozes, N.; Querol, A.; Guillamon, J.M. Thermo-adaptive evolution to generate improved Saccharomyces cerevisiae strains for cocoa pulp fermentations. *Int. J. Food Microbiol.* **2021**, *342*, 109077. [CrossRef]
21. Caspeta, L.; Chen, Y.; Ghiaci, P.; Feizi, A.; Buskov, S.; Hallstrom, B.M.; Petranovic, D.; Nielsen, J. Biofuels. Altered sterol composition renders yeast thermotolerant. *Science* **2014**, *346*, 75–78. [CrossRef] [PubMed]
22. Foster, B.; Tyrawa, C.; Ozsahin, E.; Lubberts, M.; Krogerus, K.; Preiss, R.; van der Merwe, G. Kveik Brewing Yeasts Demonstrate Wide Flexibility in Beer Fermentation Temperature Tolerance and Exhibit Enhanced Trehalose Accumulation. *Front. Microbiol.* **2022**, *13*, 747546. [CrossRef] [PubMed]
23. Richter, K.; Haslbeck, M.; Buchner, J. The heat shock response: Life on the verge of death. *Mol. Cell.* **2010**, *40*, 253–266. [CrossRef] [PubMed]
24. Gao, L.; Liu, Y.; Sun, H.; Li, C.; Zhao, Z.; Liu, G. Advances in mechanisms and modifications for rendering yeast thermotolerance. *J. Biosci. Bioeng.* **2016**, *121*, 599–606. [CrossRef]
25. Morano, K.A.; Grant, C.M.; Moye-Rowley, W.S. The response to heat shock and oxidative stress in Saccharomyces cerevisiae. *Genetics* **2012**, *190*, 1157–1195. [CrossRef] [PubMed]
26. Mahmud, S.A.; Hirasawa, T.; Shimizu, H. Differential importance of trehalose accumulation in Saccharomyces cerevisiae in response to various environmental stresses. *J. Biosci. Bioeng.* **2010**, *109*, 262–266. [CrossRef]
27. Mahmud, S.A.; Nagahisa, K.; Hirasawa, T.; Yoshikawa, K.; Ashitani, K.; Shimizu, H. Effect of trehalose accumulation on response to saline stress in Saccharomyces cerevisiae. *Yeast* **2009**, *26*, 17–30. [CrossRef]
28. Mahmud, S.A.; Hirasawa, T.; Furusawa, C.; Yoshikawa, K.; Shimizu, H. Understanding the mechanism of heat stress tolerance caused by high trehalose accumulation in Saccharomyces cerevisiae using DNA microarray. *J. Biosci. Bioeng.* **2012**, *113*, 526–528. [CrossRef]
29. Li, H.; Wang, H.-L.; Du, J.; Du, G.; Zhan, J.-C.; Huang, W.-D. Trehalose protects wine yeast against oxidation under thermal stress. *World J. Microbiol. Biotechnol.* **2010**, *26*, 969–976. [CrossRef]
30. Moon, M.H.; Ryu, J.; Choeng, Y.-H.; Hong, S.-K.; Kang, H.A.; Chang, Y.K. Enhancement of stress tolerance and ethanol production in Saccharomyces cerevisiae by heterologous expression of a trehalose biosynthetic gene from Streptomyces albus. *Biotechnol. Bioprocess. Eng.* **2012**, *17*, 986–996. [CrossRef]
31. Mason, A.B.; Allen, K.E.; Slayman, C.W. C-terminal truncations of the Saccharomyces cerevisiae PMA1 H+-ATPase have major impacts on protein conformation, trafficking, quality control, and function. *Eukaryot. Cell.* **2014**, *13*, 43–52. [CrossRef] [PubMed]
32. Theodoraki, M.A.; Nillegoda, N.B.; Saini, J.; Caplan, A.J. A Network of Ubiquitin Ligases Is Important for the Dynamics of Misfolded Protein Aggregates in Yeast. *J. Biol. Chem.* **2012**, *287*, 23911–23922. [CrossRef] [PubMed]
33. Shahsavarani, H.; Sugiyama, M.; Kaneko, Y.; Chuenchit, B.; Harashima, S. Superior thermotolerance of Saccharomyces cerevisiae for efficient bioethanol fermentation can be achieved by overexpression of RSP5 ubiquitin ligase. *Biotechnol. Adv.* **2012**, *30*, 1289–1300. [CrossRef] [PubMed]
34. Sommer, T.; Weber, A.; Jarosch, E. Rsp5/Nedd4 clears cells of heat-damaged proteins. *Nat. Cell. Biol.* **2014**, *16*, 1130–1132. [CrossRef]
35. Liang, P.; Li, J.; Wang, Q.; Dai, Z. Enhancing the thermotolerance and erythritol production of Yarrowia lipolytica by introducing heat-resistant devices. *Front. Bioeng. Biotechnol.* **2023**, *11*, 1108653. [CrossRef]
36. Wang, N.; Chi, P.; Zou, Y.; Xu, Y.; Xu, S.; Bilal, M.; Fickers, P.; Cheng, H. Correction to: Metabolic engineering of Yarrowia lipolytica for thermoresistance and enhanced erythritol productivity. *Biotechnol. Biofuels* **2020**, *13*, 189. [CrossRef]
37. Cheng, H.; Wang, S.; Bilal, M.; Ge, X.; Zhang, C.; Fickers, P.; Cheng, H. Identification, characterization of two NADPH-dependent erythrose reductases in the yeast Yarrowia lipolytica and improvement of erythritol productivity using metabolic engineering. *Microb. Cell. Fact.* **2018**, *17*, 133. [CrossRef]

38. Qiu, X.; Gu, Y.; Du, G.; Zhang, J.; Xu, P.; Li, J. Conferring thermotolerant phenotype to wild-type Yarrowia lipolytica improves cell growth and erythritol production. *Biotechnol. Bioeng.* **2021**, *118*, 3117–3127. [CrossRef]
39. Dunayevich, P.; Baltanas, R.; Clemente, J.A.; Couto, A.; Sapochnik, D.; Vasen, G.; Colman-Lerner, A. Heat-stress triggers MAPK crosstalk to turn on the hyperosmotic response pathway. *Sci. Rep.* **2018**, *8*, 15168. [CrossRef]
40. Hurtado, C.A.R.; Rachubinski, R.A. MHY1 encodes a C2H2-type zinc finger protein that promotes dimorphic transition in the yeast Yarrowia lipolytica. *J. Bacteriol.* **1999**, *181*, 3051–3057. [CrossRef]
41. Liu, Y.; Zhang, G.; Sun, H.; Sun, X.; Jiang, N.; Rasool, A.; Lin, Z.; Li, C. Enhanced pathway efficiency of Saccharomyces cerevisiae by introducing thermo-tolerant devices. *Bioresour. Technol.* **2014**, *170*, 38–44. [CrossRef] [PubMed]
42. Fang, N.N.; Chan, G.T.; Zhu, M.; Comyn, S.A.; Persaud, A.; Deshaies, R.J.; Rotin, D.; Gsponer, J.; Mayor, T. Rsp5/Nedd4 is the main ubiquitin ligase that targets cytosolic misfolded proteins following heat stress. *Nat. Cell. Biol.* **2014**, *16*, 1227–1237. [CrossRef] [PubMed]
43. Brewster, J.L.; de Valoir, T.; Dwyer, N.D.; Winter, E.; Gustin, M.C. An osmosensing signal transduction pathway in yeast. *Science* **1993**, *259*, 1760–1763. [CrossRef] [PubMed]
44. Roman, E.; Correia, I.; Prieto, D.; Alonso, R.; Pla, J. The HOG MAPK pathway in Candida albicans: More than an osmosensing pathway. *Int. Microbiol.* **2020**, *23*, 23–29. [CrossRef] [PubMed]
45. Widmann, C.; Gibson, S.; Jarpe, M.B.; Johnson, G.L. Mitogen-activated protein kinase: Conservation of a three-kinase module from yeast to human. *Physiol. Rev.* **1999**, *79*, 143–180. [CrossRef]
46. Winkler, A.; Arkind, C.; Mattison, C.P.; Burkholder, A.; Knoche, K.; Ota, I. Heat stress activates the yeast high-osmolarity glycerol mitogen-activated protein kinase pathway, and protein tyrosine phosphatases are essential under heat stress. *Eukaryot. Cell.* **2002**, *1*, 163–173. [CrossRef]
47. Pincus, D. Size doesn't matter in the heat shock response. *Curr. Genet.* **2017**, *63*, 175–178. [CrossRef]
48. Johnson, B.C.; Zibordi, G.; Brown, S.W.; Feinholz, M.E.; Sorokin, M.G.; Slutsker, I.; Woodward, J.T.; Yoon, H.W. Characterization and absolute calibration of an AERONET-OC radiometer. *Appl. Opt.* **2021**, *60*, 3380–3392. [CrossRef]
49. Stewart-Ornstein, J.; Nelson, C.; DeRisi, J.; Weissman, J.S.; El-Samad, H. Msn2 coordinates a stoichiometric gene expression program. *Curr. Biol.* **2013**, *23*, 2336–2345. [CrossRef]
50. Zahringer, H.; Thevelein, J.M.; Nwaka, S. Induction of neutral trehalase Nth1 by heat and osmotic stress is controlled by STRE elements and Msn2/Msn4 transcription factors: Variations of PKA effect during stress and growth. *Mol. Microbiol.* **2000**, *35*, 397–406. [CrossRef]
51. Li, P.S.; Fu, X.F.; Zhang, L.; Zhang, Z.Y.; Li, J.H.; Li, S.Z. The transcription factors Hsf1 and Msn2 of thermotolerant Kluyveromyces marxianus promote cell growth and ethanol fermentation of Saccharomyces cerevisiae at high temperatures. *Biotechnol. Biofuels* **2017**, *10*, 289. [CrossRef]
52. Sadeh, A.; Baran, D.; Volokh, M.; Aharoni, A. Conserved motifs in the Msn2-activating domain are important for Msn2-mediated yeast stress response. *J. Cell. Sci.* **2012**, *125*, 3333–3342. [CrossRef] [PubMed]
53. DeRisi, J.L.; Iyer, V.R.; Brown, P.O. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science* **1997**, *278*, 680–686. [CrossRef] [PubMed]
54. Gorner, W.; Durchschlag, E.; Martinez-Pastor, M.T.; Estruch, F.; Ammerer, G.; Hamilton, B.; Ruis, H.; Schuller, C. Nuclear localization of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Gene Dev.* **1998**, *12*, 586–597. [CrossRef] [PubMed]
55. Xiao, W.; Zhang, J.; Huang, J.; Xin, C.; Li, M.J.; Song, Z. Response and regulatory mechanisms of heat resistance in pathogenic fungi. *Appl. Microbiol. Biotechnol.* **2022**, *106*, 5415–5431. [CrossRef] [PubMed]