

Article

Upregulation of Key Metabolic and Stress-Response Genes Enhanced Growth and High-Temperature Ethanol Fermentation of Thermotolerant *Zymomonas mobilis* 200M and PYK

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Simple Summary

Bioethanol is a renewable fuel that helps reduce our dependence on fossil fuels and lowers carbon emissions. To increase the fuel yield and reduce production costs, the bacteria used in the fermentation process must remain active even under stressful industrial conditions. In this study, two heat-tolerant strains of the bacterium *Zymomonas mobilis* were tested for their growth and ethanol production under heat stress. We found that these new strains could grow and produce six times more ethanol at 40 °C (104 °F) compared to the original parental strain. They also showed a better ability to survive other industrial challenges, such as high acidity and alcohol levels. By studying their internal activity, we identified specific protective systems that these bacteria activate to withstand high temperatures. These findings are valuable to society because these newly developed bacteria can make biofuel production more efficient and sustainable. This leads to the development of cheaper green energy and a cleaner environment.

Abstract

Among various ethanologenic microorganisms, thermotolerant *Zymomonas mobilis* has emerged as a promising candidate for industrial ethanol production at elevated temperatures. However, the comparative fermentation efficiency and the underlying molecular mechanisms driving thermotolerance in newly developed strains remain largely unexplored, hindering their industrial application. In this study, the recently developed thermotolerant strains *Z. mobilis* 200M and *Z. mobilis* PYK exhibited critical high temperatures for growth approximately 2.0 and 2.5 °C higher than the wild-type, respectively. While 40 °C represents severe heat stress that completely inhibits the growth of the wild-type, the thermotolerant strains remained viable, exhibiting significantly shorter cell lengths under these conditions. This study provides the first evidence of their superior multi-stress tolerance toward heat, ethanol, acetic acid, formic acid, and H₂O₂. Furthermore, the

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thermotolerant strains exhibited significantly higher ethanol fermentation efficiencies than the wild-type. At 40 °C, *Z. mobilis* 200M produced approximately 5.8-fold and 3.0-fold more ethanol than the wild-type after 24 and 48 h, respectively, while *Z. mobilis* PYK yielded 6.4-fold and 3.1-fold increases. Novel transcriptional insights via RT-qPCR revealed the simultaneous overexpression of genes involved in ethanol production, protein quality control, and signal transduction, particularly during the exponential phase under heat stress. Collectively, these findings bridge the gap between strain development and molecular understanding, suggesting that the coordinated upregulation of these genetic pathways enhances the adaptive capacity and fermentation efficiency of these thermotolerant strains during sustained growth at 40 °C.

Keywords: high-temperature ethanol fermentation; stress-responsive genes; thermotolerant microorganisms; *Zymomonas mobilis*

1. Introduction

In the current transition toward a circular bioeconomy, bioethanol remains the most prevalent renewable liquid fuel globally. While traditionally utilized in vehicular transportation, its application has expanded into high-value sectors, including the synthesis of sustainable aviation fuels (SAF), pharmaceuticals, and bio-based fine chemicals [1–5]. As of 2024, global production capacity has reached approximately 31.1 billion gallons. Market leadership is maintained by the USA and Brazil (16.1 and 8.8 billion gallons, respectively), with India showing the most significant year-on-year growth at approximately 1.6 billion gallons [6]. With bioethanol now comprising a significant share of the global liquid fuel mix, its industrial expansion is increasingly driven by international mandates for low-carbon-intensity fuels [2,7,8].

Bioethanol can be produced from diverse sugar-based (sugarcane, molasses), starch-based (corn, cassava), and lignocellulosic-based feedstocks (agricultural residues and wood) [7–10]. However, commercial production is currently hindered by high production costs relative to those of conventional fuels. To overcome these challenges, high-ethanol fermentation technologies with a cost-effective platform are necessary. The bioethanol fermentation process typically relies on yeast, especially *Saccharomyces cerevisiae*. This conventional ethanologenic yeast yields relatively high ethanol yield and significantly high ethanol productivity. It can withstand relatively high sugar concentrations up to 250 g/L [11–13]. However, it faces a fundamental limitation in industrial operations, particularly under diverse environmental stressors, such as heat and ethanol stress, and the presence of growth inhibitors, for example, acetic acid [14]. Consequently, there is a need to explore alternative ethanologenic microbes capable of maintaining high ethanol production under these challenging industrial conditions.

Among the various ethanol producers, the Gram-negative, facultatively anaerobic bacterium *Zymomonas mobilis* has emerged as a promising candidate. This ethanologenic bacterium possesses numerous desirable characteristics that meet the requirements of industrial bioethanol production. These include rapid fermentation rates, high tolerance to both ethanol and sugar concentrations, wide pH ranges, and less byproduct formation [15–17]. One of the most prominent advantages of this ethanologenic bacterium lies in its unique metabolic pathway. Unlike *S. cerevisiae*, which relies on the Embden–Meyerhof–Parnas (EMP) pathway for glycolysis, *Z. mobilis* utilizes the more efficient Entner–Doudoroff (ED) pathway. This robust metabolic pathway is highly streamlined, yielding 50% less adenosine triphosphate (ATP) per mole of glucose for cell growth, thereby directing carbon flux toward ethanol production and increasing the theoretical ethanol yield to

approximately 98%. Additionally, it exhibits superior ethanol productivity relative to conventional yeast, *S. cerevisiae*, owing to its higher specific cell surface area and faster sugar uptake rate. Most importantly, this facultatively anaerobic bacterium exhibits a lower oxygen requirement for biomass maintenance compared to *S. cerevisiae*. While ethanol production in *Z. mobilis* is an anaerobic process, industrial yeast often requires controlled oxygen levels (microaeration) to synthesize essential components, such as ergosterol, to maintain cell membrane integrity. In contrast, *Z. mobilis* lacks these requirements, thereby facilitating large-scale anaerobic fermentation and reducing operational costs associated with aeration control and agitation [16].

Despite these physiological advantages, the industrial application of *Z. mobilis* remains more limited than that of *S. cerevisiae* due to several inherent drawbacks. A primary challenge is its narrow substrate spectrum. The wild type strain of *Z. mobilis* is restricted to fermenting glucose, fructose, and sucrose and lacks the natural ability to utilize pentose sugars (such as xylose and arabinose) that are prevalent in lignocellulosic biomass [15,16]. Furthermore, although it exhibits high ethanol tolerance, its growth is significantly more sensitive to the complex mixture of acetic acid, phenolic compounds, and furfural found in non-purified industrial lignocellulosic hydrolysates than that of robust industrial yeast strains [16]. These limitations necessitate extensive metabolic engineering and strain improvement to enhance its versatility and resilience for large-scale, low-cost cultivation [18].

Environmental stresses encountered during industrial bioethanol fermentation include temperature fluctuations, high ethanol and sugar concentrations, nutrient starvation, and elevated levels of inhibitory compounds, particularly those present in lignocellulosic hydrolysates, such as acetic acid, formic acid, and furfural. These stressful conditions significantly diminish microbial cell growth and viability, sugar utilization, and overall ethanol fermentation efficiency [19–22]. To address these challenges, researchers are developing robust *Z. mobilis* strains that exhibit multistress tolerance, including heat resistance. Several significant advancements in *Z. mobilis* strains have been reported. For instance, *Z. mobilis* ZM401 (acetate-tolerant) [23], *Z. mobilis* ZMF3-3 (furfural and acetic acid tolerant) [24], *Z. mobilis* AD41 (multi-stress tolerant) [21], *Z. mobilis* R301 (multi-stress tolerant) [25], and *Z. mobilis* FR2 (superior xylose assimilation) [26]. Building on these advances, work in our laboratory has focused on developing thermotolerant *Z. mobilis* strains. In a previous study, we successfully developed two potential thermotolerant *Z. mobilis* strains, i.e., *Z. mobilis* 200M (a thermoadaptive strain) and *Z. mobilis* PYK (a single-mutation strain in the pyruvate kinase gene). These two thermotolerant strains exhibited superior growth at 40 °C compared to the parental strain, *Z. mobilis* TISTR 548, which showed significantly inhibited growth under such thermal stress [27]. However, several critical gaps prevent their industrial application. First, although these thermotolerant strains were developed from *Z. mobilis* TISTR 548, their comparative ethanol fermentation efficiency has not been validated against the wild-type under elevated temperature. This comparison is essential to confirm that the gain in thermotolerance did not come at the expense of metabolic productivity. Second, the molecular mechanisms and regulatory pathways responsible for this enhanced thermotolerance remain unknown. Understanding these genetic drivers is vital for the targeted engineering of next-generation robust biocatalysts.

This research, therefore, aims to elucidate the growth patterns of these thermotolerant strains under various stress conditions, including heat, ethanol, acetic acid, formic acid, and H₂O₂. Further investigation of the dynamic gene-expression patterns underlying the stress response using reverse transcription quantitative PCR (RT-qPCR), particularly under thermal stress at 40 °C, is also proposed. This will help elucidate the adaptive mechanisms that enable robust performance in industrial fermentation environments.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

The wild-type *Z. mobilis* (TISTR548) was obtained from the Thailand Institute of Scientific and Technological Research (TISTR) (Pathum Thani, Thailand), while the thermotolerant strains [*Z. mobilis* 200M (thermo-adapted strain), and *Z. mobilis* PYK (single mutation of the *pyk* gene)] were developed and characterized by our group as previously described by Kosaka et al. [27]. All bacterial strains were cultured on yeast extract-peptone-glucose (YPG) agar and maintained at 4 °C, with subcultures performed monthly. For long-term storage, the cultures were maintained in 50% (*v/v*) glycerol at −80 °C.

2.2. Seed Culture Preparation

To prepare the seed culture, a single colony of *Z. mobilis* (wild-type or thermotolerant strain) was first cultured in a 30 mL test tube containing 5 mL of sterilized YPG medium. This initial culture was incubated in the controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea) at 30 °C with shaking at 100 rpm for 12 h. Once the medium reached an optical density at 550 nm (OD₅₅₀) of 0.8–1.0 (mid-exponential phase), the cells were subcultured by transferring them into a 250 mL Erlenmeyer flask containing 50 mL of fresh YPG medium to achieve an initial OD₅₅₀ of 0.05. After a final 8 h incubation under identical conditions (30 °C, 100 rpm), the resulting active cells were collected and used as the starter culture for all subsequent experiments.

2.3. Critical High Temperature by Two-Step Cultivation Assay

The maximum growth temperature, defined here as the critical high temperature (CHT), was determined for each *Z. mobilis* strain using a two-step cultivation assay [21,27]. This method was specifically employed to differentiate between transient cell survival and sustained physiological adaptation to elevated temperatures. The selection of temperatures between 38 °C and 41 °C was based on prior characterization of the wild-type strain TISTR 548, which identifies 38 °C as its upper thermal limit for growth. Consequently, testing at 40 °C and 41 °C was rationalized as a definitive challenge to verify the expanded thermal niche of the newly developed thermotolerant strains.

To initiate the assay, starter cultures (prepared as described in Section 2.2) were transferred into 30 mL test tubes containing 10 mL of fresh YPG medium to an initial optical density (OD₅₅₀) of 0.05. These cultures were statically incubated in a high-precision controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea) at stress-inducing temperatures (38–41 °C). Following 12 h of primary incubation, which allowed for initial thermal adaptation, these cultures were used to re-inoculate fresh YPG medium to an OD₅₅₀ of 0.05. These secondary cultures were then incubated at the same constant temperature for an additional 48 h. In this two-step scheme, the first cultivation step provides sufficient time for the strains to adjust their metabolic processes to heat, while the second step serves as the definitive test of their adaptive capacity. Sustained growth during this second phase confirms the strains' ability to proliferate under constant thermal stress, rather than merely surviving. Cell growth was monitored by measuring turbidity at OD₅₅₀ every 12 h. To ensure high-resolution accuracy and reproducibility, all experiments were conducted in triplicate, with manual thermometer verification used to confirm that temperature fluctuations remained within ±0.1 °C of the setpoint.

2.4. Effect of Heat Stress on Cell Morphology of *Zymomonas mobilis*

To characterize the effect of heat stress on cell morphology, starter cultures of *Z. mobilis* wild-type and thermotolerant strains were transferred to a 250 mL Erlenmeyer flask containing 100 mL YPG medium to an initial OD₅₅₀ of 0.05. All cultures were statically

incubated in the controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea) at 30 °C and 40 °C. Based on preliminary growth profile analysis and previous work [21,28], the mid-exponential phase was defined as 8 h, and the stationary phase as 24 h of incubation at both temperatures. The growth stage was confirmed by OD₅₅₀ measurements prior to harvesting to ensure consistent physiological states across all samples. Cells were harvested by centrifugation at 10,000 rpm for 5 min at both the mid-exponential and stationary growth phases. The cell pellets were first resuspended in 0.05 M sodium phosphate buffer (pH 7.0) and fixed using 2% glutaraldehyde for 30 min. The samples were then sequentially dehydrated using ethanol gradients of 10%, 20%, 50%, 75%, 95%, and 100% v/v, with 30 min of exposure at each concentration. Finally, after air-drying, the samples were sputter-coated with 1–2 nm of gold and examined using a TESCAN MIRA Field Emission Scanning Electron Microscope (FESEM, Brno, Czech Republic), as previously described [29].

2.5. Ethanol Production Ability of *Zymomonas mobilis*

Batch fermentations were conducted in 250 mL Erlenmeyer flasks to evaluate the comparative ethanol yield and production performance of *Z. mobilis* wild-type and thermotolerant strains. Active starter cultures (10% v/v) were inoculated into 100 mL of YPG medium supplemented with 160 g/L glucose. This glucose concentration was chosen for its industrial relevance in achieving high ethanol titers and to maintain consistency with previous performance benchmarks [28]. Furthermore, this concentration served as a baseline to assess the metabolic robustness of the thermotolerant mutants relative to the wild type under thermal stress prior to process optimization. The flasks were incubated statically at 30 °C and 40 °C, temperatures selected based on the known thermal limits of the parental strain [27,28], using a controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea). Samples were collected at 24 and 48 h to quantify ethanol concentrations. While this sampling frequency focused specifically on ethanol production rather than on detailed kinetic modeling or metabolite profiling, it enabled a targeted assessment of fermentation robustness. For analysis, samples were centrifuged (10,000 rpm, 4 °C, 5 min), and the ethanol concentration in the supernatant was analyzed via gas chromatography (GC-14B, Shimadzu, Kyoto, Japan). The volumetric ethanol productivity and ethanol yield were analyzed using the methods described by Nuanpeng et al. [11]. Experiments were performed in two independent runs, each containing two replicates.

2.6. Effect of Stress Conditions on Growth of *Zymomonas mobilis*

The stress tolerance of *Z. mobilis* wild-type and thermotolerant strains was evaluated using a two-step cultivation technique [21,27], designed to assess sustained growth at threshold stress levels. The experimental variables included temperature, ethanol, acetic acid, formic acid, and H₂O₂. Cultivations were performed in 250 mL Erlenmeyer flasks containing 100 mL of YPG medium, with static incubation in a controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea). To distinguish sustained growth from transient survival, growth was monitored by measuring OD at 550 nm every 12 h. Specifically, thermal stress was assessed at 40 °C, a temperature representing the thermal limit of the parental strain [27], while 30 °C served as the control. For chemical stress challenges, the YPG medium was supplemented with 7% (v/v) ethanol, 50 mM acetic acid, 0.6 g/L formic acid, or 0.1 mM H₂O₂. These chemical concentrations were selected based on the preliminary study and previous works [21,27]. All chemical stress experiments were conducted at both 30 °C and 40 °C to evaluate the synergistic effects of heat and chemical inhibition.

2.7. Gene Expression Analysis of *Zymomonas mobilis* Under Heat Stress Conditions

Total RNA was extracted from both the *Z. mobilis* wild-type and thermotolerant strains to investigate gene expression under elevated-temperature stress. Starter cultures of bacterial cells were transferred to a 250 mL Erlenmeyer flask containing 100 mL of YPG medium, with an initial OD₅₅₀ of 0.05. Cultures were statically incubated in a controlled incubator shaker (JSSI-300CL, JSR, Gongju, South Korea) at 30 °C and 40 °C and harvested at the exponential (8 h) and stationary (24 h) growth phases. For RNA isolation preparation, cell pellets were collected by centrifugation (10,000 rpm for 5 min at 4 °C) and washed twice with sterile distilled water. Total RNA was then isolated from the resulting cell pellets using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). To eliminate genomic DNA contamination, 1 µg of total RNA was treated with 1 Unit of RNase-free DNase I (Invitrogen, Thermo Fisher Scientific, USA) according to the manufacturer's protocol. The reaction mixture was incubated for 30 min at 37 °C, followed by heat inactivation at 65 °C for 10 min in the presence of EDTA. RNA concentration and purity were subsequently assessed with a BioDrop µLite (Denville Scientific Inc., Holliston, MA, USA).

Complementary DNA (cDNA) was synthesized from high-quality total RNA using the Maxime RT PreMix Kit (iNtRON Biotechnology, Seongnam, South Korea), following the manufacturer's instructions. Briefly, 1 µg of total RNA was added to a Maxime RT PreMix tube, and nuclease-free water was added to bring the final reaction volume to 20 µL. The reverse transcription reaction was incubated at 45 °C for 60 min, followed by enzyme inactivation at 95 °C for 5 min. The synthesized cDNA was then diluted with nuclease-free water to a working concentration of 10 ng/µL for use as a template in quantitative PCR (qPCR).

qPCR was performed in a total reaction volume of 20 µL. Each reaction consisted of 10 µL of RealMOD™ Green W² 2× qPCR Master Mix (iNtRON Biotechnology, Seongnam, South Korea), 1 µL of cDNA template, and a final concentration of 0.5 µM for both gene-specific forward and reverse primers (detailed in Supplementary Materials Table S1). The final volume was adjusted to 20 µL using nuclease-free water. qPCR amplification was performed on an Applied Biosystems™ QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's recommended protocol. The cycling conditions included an initial activation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 40 s, and extension at 72 °C for 60 s. A final extension step was carried out at 72 °C for 5 min. All qPCR reactions were performed in duplicates. The 16S ribosomal RNA (16S rRNA) was selected as the internal reference (endogenous control) for data normalization due to its stable expression under the tested conditions. Relative gene expression levels were calculated using the comparative Ct method ($2^{-\Delta\Delta C_t}$), where the target gene expression was normalized to the 16S rRNA levels and expressed relative to the control group.

2.8. Analytical Methods

Cell growth of *Z. mobilis* was quantified by measuring the cells' turbidity with a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) at a wavelength of 550 nm. Sugar concentration was measured using phenol-sulfuric acid method. The final ethanol concentration (P, g/L) was determined using Gas Chromatography (GC-14B, Shimadzu, Kyoto, Japan), which utilized a polyethylene glycol (PEG-20 M) packed column and a flame ionization detector (FID). The temperatures for the injector, detector, and column oven were 180 °C, 250 °C, and 150 °C, respectively. N₂ serves as the carrier gas, and 2-propanol (10% v/v) was included as the internal standard for accurate quantification. The N₂ pressure was controlled at 200 kPa. Based on these measurements, the volumetric ethanol productivity (Q_p, g/L.h) and ethanol yield (Y_p, g/g) were calculated according to the methods described by Nuanpeng et al. [11]. All experimental data are expressed as the mean ±

standard deviation (SD). To determine statistical significance, mean differences between treatment groups were analyzed using Duncan's multiple-range test (DMRT) at the $p < 0.05$ level, performed in SPSS for Windows (Version 28.0, IBM Corporation, Armonk, NY, USA).

3. Results and Discussion

3.1. Critical High Temperature for Growth of *Zymomonas mobilis*

The maximum growth temperature, defined in this study as the critical high temperature (CHT), represents the maximum thermal threshold at which microbial growth can be sustained; beyond this point, cells undergo irreversible physiological damage [30]. While CHT values are species-specific, ranging, e.g., from 38 °C in *Acetobacter pasteurianus* to 45 °C in *Escherichia coli* [27,30], establishing these precise limits for adapted strains is essential to quantify the adaptive thermal capacity of improved strains. In this study, we employed a two-step cultivation assay [21,27] to determine the CHT of *Z. mobilis* 200M and PYK, using the wild-type TISTR 548 as a reference. In this methodology, the presence of growth (measured by OD₅₅₀) during the second cultivation step serves as a robust indicator of successful adaptation, confirming the population's ability to maintain metabolic activity and proliferation under sustained thermal pressure.

The parental strain, *Z. mobilis* TISTR 548, possesses a well-documented optimal growth temperature of 30 °C [27,31]. Consistent with its known physiological profile, the wild-type strain exhibited significant vulnerability at temperatures exceeding 38 °C. While initial growth was observed at 39 °C, the strain failed to proliferate upon transfer to a second culture at the same temperature. A marked decrease in the specific growth rate of the wild type occurred above 38 °C, particularly during the second cultivation step, confirming its CHT as 38 °C. In contrast, the thermotolerant strains *Z. mobilis* 200M and PYK demonstrated robust, sustained growth up to 40 °C and 40.5 °C, respectively, failing only when challenged at 41 °C (Figure 1). These mutants also maintained higher specific growth rates than the wild type across both cultivation steps under identical thermal conditions (Supplementary Materials Table S2). These results conclusively show that, while the optimum temperature remains centered near 30 °C, the thermotolerant strains exhibit a significantly broader growth range and a higher CHT than the wild type. Specifically, *Z. mobilis* 200M showed an increase of 2.0 °C, while *Z. mobilis* PYK showed an even greater increase of 2.5 °C in their respective CHTs compared to the wild-type. These findings confirm that the thermotolerant *Z. mobilis* strains have successfully adapted to high-temperature environments. This thermal adaptation is likely mediated by coordinated mechanisms, including membrane stabilization, suppression of ROS via altered fatty acid profiles, and improved protein quality control [32,33]. From a commercial perspective, raising the maximum growth temperature in these strains enables fermentation at higher temperatures, thereby significantly reducing cooling energy requirements and enhancing the cost-effectiveness of producing bioproducts such as bioethanol, sorbitol, and succinate.

Notably, while the two-step cultivation method and OD monitoring effectively defined the CHT for these *Z. mobilis* strains, we acknowledge that OD measurements do not fully capture the nuances of cell viability and population dynamics. Future studies incorporating colony-forming unit (CFU) assays and higher-resolution kinetic monitoring would provide deeper insights into the precise physiological adaptation and survival mechanisms of these strains during the transition to higher temperatures.

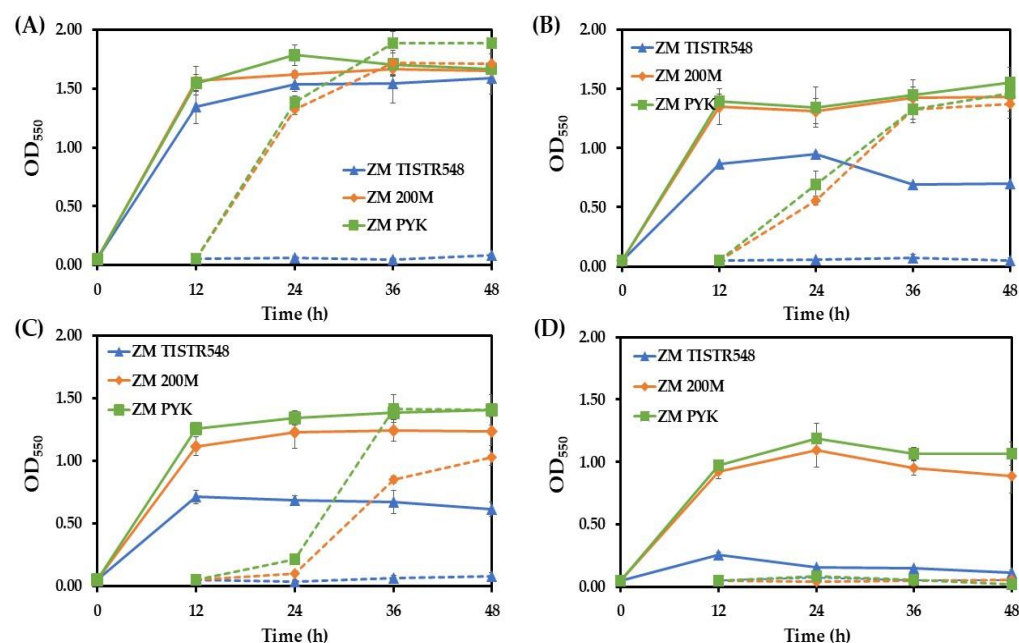


Figure 1. Growth of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains under different temperatures by a two-step cultivation technique. (A), 39 °C; (B), 40 °C; (C), 40.5 °C; (D), 41 °C.

3.2. Changes in Cell Morphology of *Zymomonas mobilis* Under Heat Stress

In response to heat stress, *Z. mobilis* exhibits complicated changes at both the physiological and molecular levels [22]. Among these changes, a key physiological response is morphological alteration, especially in cell shape and size. As demonstrated in this study, cells incubated at 30 °C were regular, rod-shaped, with a smooth cell surface during the mid-exponential growth phase, and cells were slightly longer at the stationary phase. While the qualitative morphological traits (rod-shaped and smooth surface) of all strains were nearly identical at 30 °C (Figure 2), significant quantitative differences in cell length emerged under heat stress.

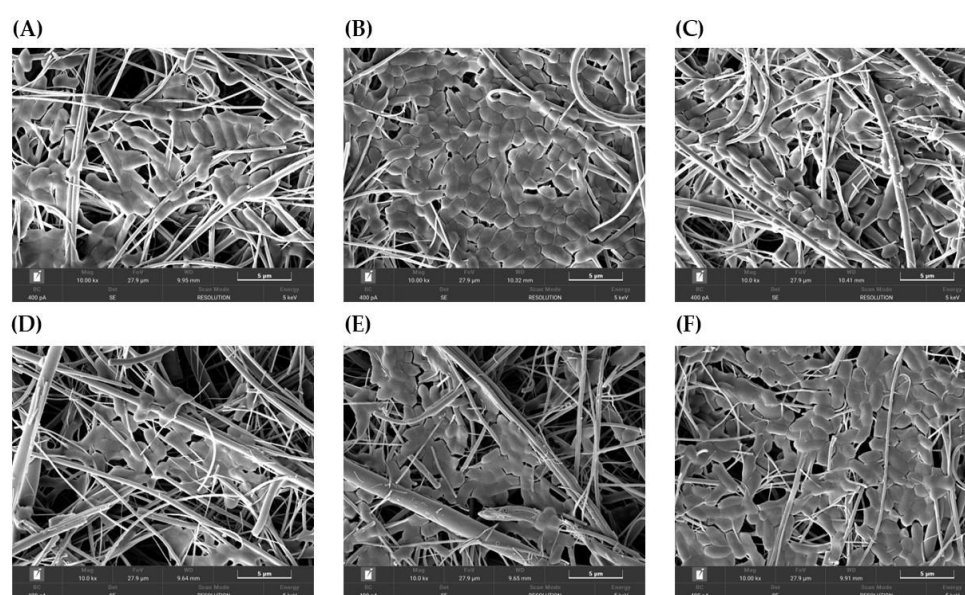


Figure 2. Cell morphology of *Zymomonas mobilis* TISTR548 (A,D), 200M (B,E), and PYK (C,F) after cultivation in YPG medium at 30 °C until growth reached mid-exponential (A–C) and stationary phases (D–F).

At 40 °C, cells become elongated (filamentous-like), specifically in the wild-type strain at both the mid-exponential and stationary growth phases (Figure 3). This phenomenon is consistent with that reported by Kosaka et al. [27], when the strain was subjected to a temperature close to its CHT. As summarized in Table 1, although the strains share a common rod-shaped morphology, the degree of filamentation varies significantly. *Z. mobilis* 200M and PYK exhibited cell sizes of 2.14–2.17 µm at the mid-exponential growth at 30 °C, which are relatively shorter than that of the wild-type (2.31 µm). In the stationary growth phase, all strains exhibited cells that were longer than those observed in the mid-exponential phase. The thermotolerant *Z. mobilis* 200M exhibited the shortest cell size (2.28 µm), while the wild-type strain TISTR548 had the longest (2.58 µm). The relatively longer cell length of bacterial cells in the stationary growth phase may be due to nutrient depletion in the cultivation medium. As reported by Asefi et al. [22], nutrient scarcity is one of the stressors that affects microbial cell morphology, including cell size and shape.

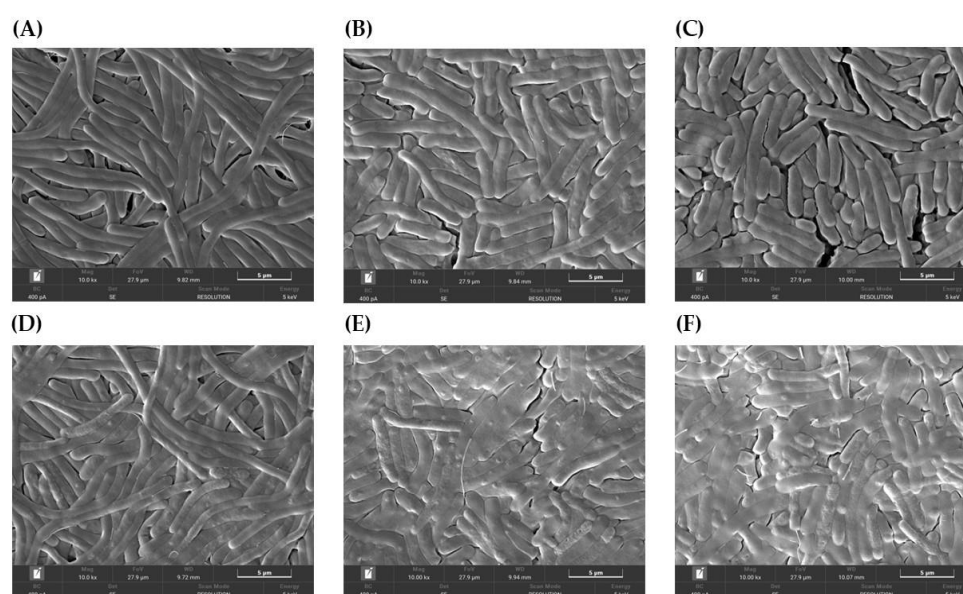


Figure 3. Cell morphology of *Zymomonas mobilis* TISTR548 (A,D), 200M (B,E), and PYK (C,F) after cultivation in YPG medium at 40 °C until growth reached mid-exponential (A–C) and stationary phases (D–F).

A significant increase in cell size was detected when cells were subjected to heat stress at 40 °C. The wild-type strain exhibited the longest cell size, exceeding 10 µm, at both the mid-exponential and the stationary growth phases. In contrast, the thermotolerant strains *Z. mobilis* 200M and PYK maintained significantly shorter cell sizes than the wild-type at both growth phases. Although the cell length at the stationary growth phase was longer than that at the mid-exponential growth phase, it was still shorter than that of the wild-type strain. This suggests that while the basic rod-like appearance remains, the ability to maintain regulated cell division under thermal stress is a distinguishing feature of the thermotolerant strains.

Changes in bacterial cell size and shape may be associated with the effects of heat stress on physiological response in this microorganism. As previously reported, heat stress negatively affects bacterial DNA replication and cell division by suppressing the expression of *minD* and *minC*, genes involved in these processes [32]. Consequently, the preservation of shorter cell lengths in *Z. mobilis* 200M and PYK at 40 °C serves as a vital physiological marker of their improved tolerance and robust cell division machinery compared to the wild-type. Heat stress may also expose bacterial cells to oxidative stress by increasing endogenous ROS, which not only have detrimental effects on membrane lipids,

proteins, and DNA but also presumably influence cell division. As demonstrated by Charoensuk et al. [32], overexpression of genes encoding ROS-scavenging enzymes results in smaller bacterial cell sizes under severe stress conditions, including thermal stress.

From a bioprocessing perspective, the maintenance of a stable, short rod-shaped morphology in thermotolerant strains 200M and PYK is a significant advantage. Filamentation, as observed in the wild-type at 40 °C, can increase broth viscosity and interfere with efficient nutrient mass transfer. Furthermore, because filamentous growth is often a precursor to cell death in *Z. mobilis* [27], the ability to maintain a regulated cell size is a key physiological indicator of the metabolic vigor required to sustain high ethanol yields during non-isothermal fermentation.

Table 1. Cell size of *Zymomonas mobilis* TISTR548, 200M, and PYK under heat stress at different growth phases.

Strain	Cell Size (μm)	
	Mid-Exponential	Stationary
30 °C		
<i>Z. mobilis</i> TISTR548	2.31 ± 0.31	2.58 ± 0.21
<i>Z. mobilis</i> 200M	2.14 ± 0.24	2.28 ± 0.20
<i>Z. mobilis</i> PYK	2.17 ± 0.14	2.44 ± 0.27
40 °C		
<i>Z. mobilis</i> TISTR548	>10	>10
<i>Z. mobilis</i> 200M	5.02 ± 0.40	7.08 ± 0.73
<i>Z. mobilis</i> PYK	4.23 ± 0.78	6.15 ± 0.49

3.3. High Ethanol Fermentation Efficiency of Thermotolerant Strains Under Heat Stress

Heat stress not only affects the growth and cell morphology of *Z. mobilis* but also reduces its ethanol fermentation efficiency. Although all strains produced relatively high ethanol concentrations, productivities, and yields at the optimal growth temperature (30 °C), the newly developed thermotolerant strains outperformed the wild type. As shown in Table 2, at 24 h post-fermentation (30 °C), *Z. mobilis* 200M and PYK produced ethanol concentrations of 56.41 g/L and 57.20 g/L, respectively, approximately 1.1-fold higher than the wild type. Correspondingly, ethanol productivities and yields were also 1.1-fold greater. This trend persisted at 48 h, with the thermotolerant strains consistently maintaining superior fermentation parameters.

At 40 °C, the wild-type strain exhibited a drastic decline in fermentation performance compared to the newly thermotolerant strains. Its ethanol concentrations at 24 and 48 h were only 8.68 and 16.37 g/L, representing 5.8- and 2.9-fold reductions compared to its performance at 30 °C. Conversely, the thermotolerant strains were less affected by thermal stress. Their maximum ethanol titers (49.51–55.63 g/L) were 5.8- to 6.4-fold higher than the wild type at 24 h, and 3.0- to 3.1-fold higher at 48 h. Similarly, productivities were 3.0- to 6.4-fold higher, and yields were 2.4- to 5.1-fold greater than those of the wild type under identical conditions (Table 2). These findings highlight the potential of these resilient strains to enhance bioethanol production in industrial settings subject to high-temperature fluctuations.

The marked decline in ethanol fermentation efficiency of the wild-type strain at 40 °C may be attributed to the profound physical and biological damage caused by heat stress on the microbial cells. As previously reported, heat stress can denature several key biological components, including DNA, RNA, and proteins. Moreover, this stress compromises cellular homeostasis by disrupting membrane structure, decreasing membrane integrity, and promoting membrane leakage. As a result, essential nutrients, as well as coenzymes and cofactors required for enzyme activity in the carbon catabolism and

ethanol production pathway, are lost, thereby disrupting metabolic activity and reducing ethanol fermentation capability [27]. Therefore, understanding these cellular responses is vital for developing resilient strains that maintain fermentation performance under heat stress.

Notably, ethanol yields for all strains decreased at 48 h post-fermentation at both 30 °C and 40 °C. This decline may be due to the reassimilation of ethanol into acetaldehyde by alcohol dehydrogenase (ADH2) [31] or the diversion of remaining sugars toward secondary metabolites such as sorbitol [22]. Further investigation is required to validate these hypotheses.

Table 2. Comparative analysis of ethanol production by *Zymomonas mobilis* TISTR548, 200M, and PYK under heat stress using 160 g/L glucose as a carbon source.

Strain	30 °C			40 °C		
	P	Qp	Yp/s	P	Qp	Yp/s
24 h						
<i>Z. mobilis</i> TISTR548	50.69 ± 0.27 ^c	2.11 ± 0.01 ^b	0.43 ± 0.00 ^b	8.68 ± 0.56 ^e	0.36 ± 0.02 ^d	0.09 ± 0.01 ^e
<i>Z. mobilis</i> 200M	56.41 ± 0.55 ^a	2.35 ± 0.02 ^a	0.46 ± 0.01 ^a	50.69 ± 0.28 ^{bc}	2.11 ± 0.01 ^b	0.44 ± 0.01 ^a
<i>Z. mobilis</i> PYK	57.20 ± 0.56 ^a	2.39 ± 0.02 ^a	0.48 ± 0.01 ^a	55.63 ± 0.56 ^a	2.32 ± 0.02 ^a	0.46 ± 0.01 ^a
48 h						
<i>Z. mobilis</i> TISTR548	47.93 ± 0.28 ^e	1.00 ± 0.01 ^d	0.36 ± 0.01 ^d	16.37 ± 0.28 ^d	0.34 ± 0.01 ^d	0.15 ± 0.00 ^d
<i>Z. mobilis</i> 200M	49.31 ± 0.56 ^d	1.03 ± 0.01 ^d	0.37 ± 0.01 ^d	49.51 ± 0.28 ^c	1.03 ± 0.01 ^c	0.36 ± 0.01 ^c
<i>Z. mobilis</i> PYK	53.26 ± 0.57 ^b	1.11 ± 0.01 ^c	0.40 ± 0.00 ^c	51.29 ± 1.11 ^b	1.06 ± 0.02 ^c	0.39 ± 0.00 ^b

P, ethanol concentration (g/L); Qp, ethanol productivity (g/L.h); Yp/s, ethanol yield (g/g). Means ± SD with different letters in each column are significantly different at $p \leq 0.05$ based on DMRT analysis.

3.4. Growth Characterization of *Zymomonas mobilis* Under Different Stress Conditions

During fermentation, microbial cells face various environmental stressors that typically compromise cell growth and viability as well as ethanol production efficiency. One of the most significant challenges is elevated temperature. This stressor often arises from both the reaction's exothermic nature and the mechanical heat generated by the equipment [21,27]. To assess how elevated temperature impacts microbial cell viability, the growth of *Z. mobilis* wild-type and newly developed thermotolerant strains was compared using a two-step cultivation method. As illustrated in Figure 4 and Supplementary Materials Table S3, the growth patterns and the specific growth rate of bacterial cells varied significantly with the applied temperature.

At the optimal growth temperature (30 °C), both wild-type and thermotolerant strains exhibited robust, comparable growth across both cultivation stages. However, the growth performance diverged sharply at 40 °C. While the wild-type strain (*Z. mobilis* TISTR 548) showed initial growth comparable to that of the thermotolerant mutants in the first cultivation step, it failed to proliferate upon transfer to the second culture, indicating severely compromised cell viability. Notably, the wild-type specific growth rate was approximately 1.8-fold lower than that of the thermotolerant strains (Supplementary Materials Table S3). In stark contrast, the thermotolerant *Z. mobilis* 200M and PYK maintained vigorous growth throughout both cultivation steps at 40 °C. This sustained viability confirms a greater CHT for the growth of these thermotolerant strains than the wild type. This superior resilience, achieved either via adaptive laboratory evolution (*Z. mobilis* 200M) or genetic engineering (*Z. mobilis* PYK), is essential for maintaining industrial performance under thermal stress.

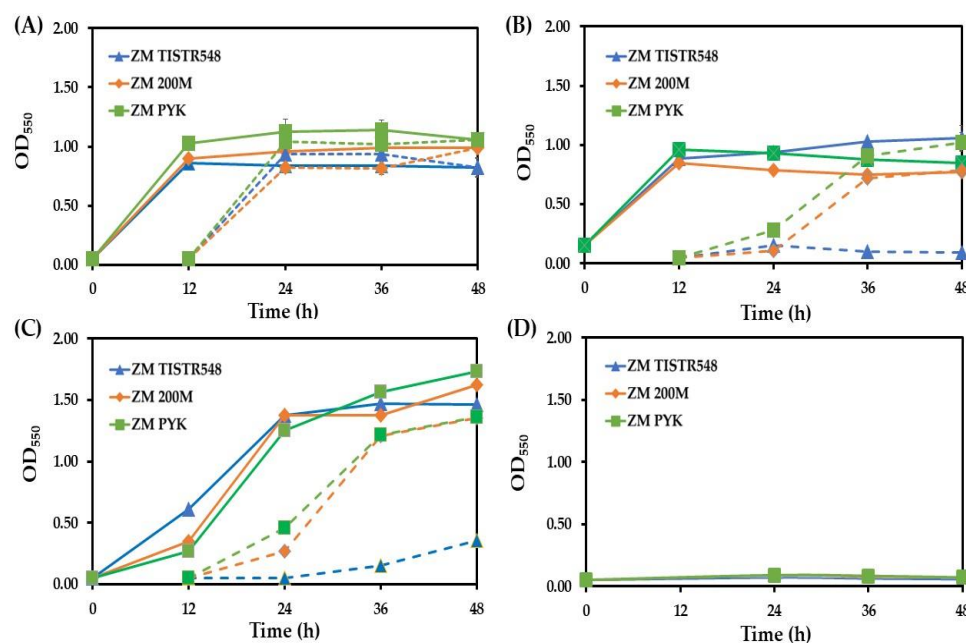


Figure 4. Effect of elevated temperature (A,B) and ethanol (C,D) stress at 30 °C (A,C) and 40 °C (B,D) on growth profiles of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains.

Ethanol represents another critical stressor during fermentation, exerting multiple deleterious effects on microbial cells. While low ethanol concentrations typically inhibit growth rates and reduce cell volume, higher concentrations have been shown to induce oxidative stress via ROS accumulation, ultimately leading to cell death [33]. Furthermore, high ethanol levels increase membrane fluidity, impair glucose utilization, and diminish energy production, all of which significantly hinder microbial metabolism and fermentation capability [22].

To evaluate physiological responses to ethanol stress, the *Z. mobilis* wild-type and newly developed thermotolerant strains were cultured in medium supplemented with 7% (v/v) ethanol. This concentration was selected as a benchmark based on previous studies identifying it as a critical inhibitory threshold for the wild-type strain [25,31]. Cultivations were performed at both 30 °C (control) and 40 °C (thermal stress) for 48 h using a two-step cultivation technique. At 30 °C, all strains exhibited similar growth profiles in the first step, but the thermotolerant strains 200M and PYK demonstrated significantly higher growth rates in the second step. Their specific growth rates were approximately 1.7- to 1.9-fold greater than that of the wild type. However, at 40 °C, a condition representing the combined stressors of heat and ethanol, a significant decline in growth performance was observed across all strains. In the first cultivation step, the thermotolerant strains maintained a slightly higher specific growth rate (1.7- to 1.8-fold) compared to the wild type; however, none of the strains sustained growth upon transfer to the second culture (Figure 4 and Supplementary Materials Table S3). This complete growth inhibition at 40 °C in 7% (v/v) ethanol, in contrast to the survival observed when these stressors are applied individually, provides experimental evidence of a potent synergistic effect between thermal and ethanol stress.

This synergism is consistent with established models of microbial inhibition, as ethanol and heat stress share common deleterious mechanisms [22,32,33]. These combined stressors likely disrupt the plasma membrane, leading to the leakage of essential cofactors and metabolites. Furthermore, the simultaneous effects of ethanol and heat accelerate ROS

accumulation and oxidative damage to proteins and nucleic acids, ultimately exceeding the cell's repair capacity and resulting in the total growth inhibition observed at 40 °C.

Lignocellulosic biomasses, such as corn cob, wheat straw, rice straw, sugarcane bagasse, sawdust, and cassava pulp, are considered promising feedstocks for second-generation bioethanol production [34,35]. However, pretreatment of these feedstocks frequently generates inhibitory compounds, which can be categorized into five major groups: furan aldehydes, aromatic compounds, alcohols, inorganic compounds, and organic acids [36,37]. Although the composition of these inhibitors varies with the biomass source and pretreatment method, our previous analysis of sugarcane bagasse hydrolysate identified weak organic acids as the predominant inhibitory species [38]. Among these inhibitors, acetic and formic acids are significantly prevalent. These acids have been demonstrated to significantly impair microbial growth, cell viability, metabolic activity, and ethanol fermentation efficiency [22,39]. As demonstrated by Liu et al. [40], acetic acid inhibits microbial growth through several mechanisms, including the accumulation of cellular ROS, reduction in cellular pH, disruption of membrane function, and diversion of ATP from growth and cellular maintenance processes.

To simulate the stressors inherent in a bagasse-derived system, this study evaluated the impact of weak acids on the growth of wild-type and thermotolerant *Z. mobilis* strains. Concentrations of 50 mM acetic acid and 0.6 g/L formic acid were selected based on typical levels encountered in our bagasse pretreatment process [38]. Cells were cultivated at 30 °C (control) and 40 °C (thermal stress) in YPG medium supplemented with either acid. As shown in Figure 5 and Supplementary Materials Table S3, all strains exhibited similar growth profiles and specific growth rates during the initial cultivation step in acetic acid at 30 °C. However, the thermotolerant strains demonstrated 1.2- to 1.3-fold higher specific growth rates during the subsequent cultivation stage. At 40 °C, the thermotolerant *Z. mobilis* strains significantly outperformed the wild type in both cultivation steps. Notably, the specific growth rates of the thermotolerant strains were 1.8- to 2.2-fold higher than those of the wild type, with the greatest difference observed during the second cultivation step.

A similar growth profile was observed when cells were treated with 0.6 g/L formic acid. At 30 °C, the wild-type and thermotolerant strains exhibited comparable growth in the first culture; however, the thermotolerant strains demonstrated superior growth in the subsequent cultivation step, with specific growth rates approximately 1.4- to 1.5-fold higher than those of the wild type. Under combined thermal and formic acid stress (40 °C), the thermotolerant strains showed a pronounced growth advantage, particularly during the second cultivation, achieving specific growth rates 1.7- to 1.8-fold higher than the wild type (Figure 5 and Supplementary Table S3). These results clearly indicate that thermotolerant strains resist not only high temperatures and ethanol stress but also key lignocellulosic inhibitors, including acetic and formic acids. This multi-stress tolerance makes them promising candidates for second-generation ethanol production under high-temperature conditions. Notably, our findings align with previous reports on *Z. mobilis* ZM481 and its sodium acetate-tolerant mutants (ZMA-142 and ZMA-167) [40], as well as the thermal-adapted mutant ZM AD41 [21].

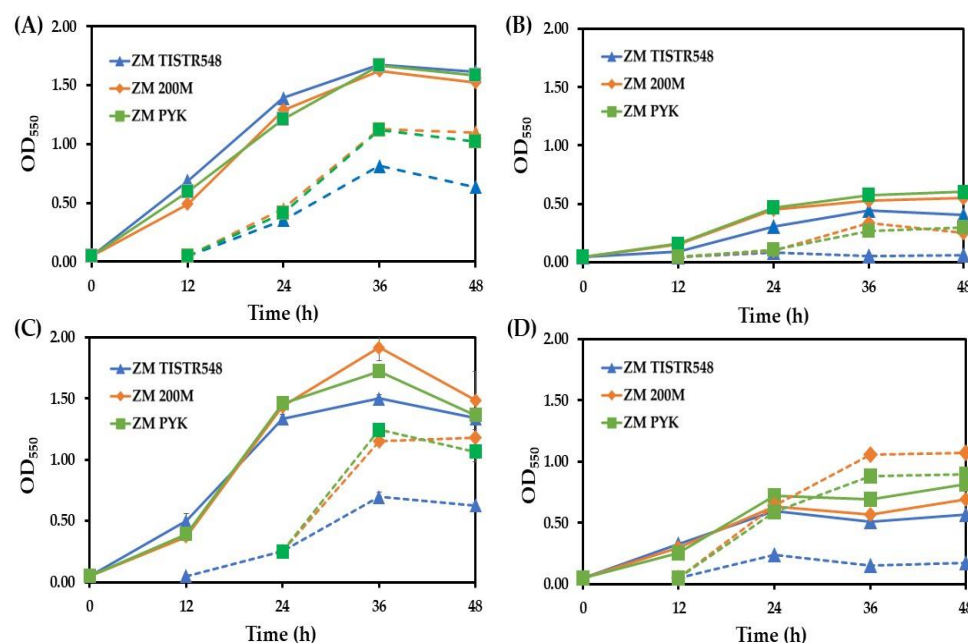


Figure 5. Effect of acetic acid (A,B) and formic acid (C,D) stress at 30 °C (A,C) and 40 °C (B,D) on growth profiles of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains.

During fermentation, H₂O₂ and other ROS are generated endogenously as metabolic byproducts. The accumulation of these species is significantly exacerbated by environmental stressors, such as high temperatures and elevated ethanol concentrations [32,33]. This internal oxidative stress impairs microbial growth by damaging membrane lipids and denaturing cellular macromolecules, such as proteins and DNA [41]. Consequently, evaluating the sensitivity of strains to exogenous H₂O₂ serves as a critical indicator of their capacity to withstand oxidative damage under severe fermentation conditions.

In this study, the H₂O₂ sensitivity of the *Z. mobilis* wild-type and thermotolerant strains was evaluated in YPG medium containing 0.1 mM H₂O₂ at 30 °C and 40 °C. At 30 °C, thermotolerant strains 200M and PYK exhibited superior growth profiles compared to the wild type across both cultivation steps (Figure 6A). Although the specific growth rates did not differ significantly (Supplementary Table S3), the wild-type strain experienced a growth delay of approximately 12 h under H₂O₂ stress. This suggests that the thermotolerant strains are more resilient to oxidative stress at optimal growth temperatures. However, the deleterious effects of H₂O₂ were far more pronounced at elevated temperatures. As shown in Figure 6B, growth for all strains was delayed by 24 h during the initial cultivation at 40 °C, and no growth was detected in the second culture. These findings indicate that both the wild-type and thermotolerant strains remain sensitive to H₂O₂-induced oxidative stress, particularly when compounded by thermal stress. This complete growth inhibition likely results from a potent synergistic interaction, in which elevated temperatures may impair the stability of antioxidant enzymes, such as catalase and superoxide dismutase, thereby diminishing the cell's capacity to neutralize exogenous H₂O₂ [32,41].

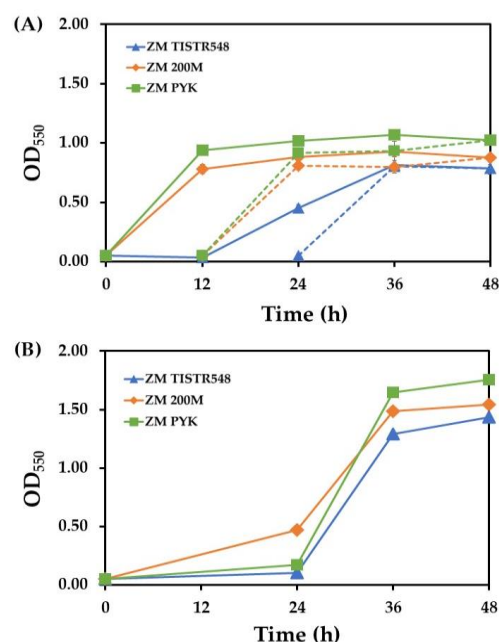


Figure 6. Effect of H₂O₂ stress at 30 °C (A) and 40 °C (B) on growth profiles of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains.

The molecular mechanisms in response to acid and oxidative stress are complex and involve multiple genes related to membrane stabilization, transport, regulation, ROS-scavenging enzymes, and heat shock proteins (HSPs). For example, Liu et al. [40] demonstrated that the regulator hfq and the putative terminator sequences of ZMO0117, which are involved in hydroxylamine reductase synthesis, contribute to acetate tolerance in *Z. mobilis* by modulating stress-response pathways. Anggarini et al. [42] reported that over-expression of ROS-related genes (*sod*, *cat*, *ahpC1*, *ahpC2*, and *ZZ6_0186*) and HSP genes (*dnaK*, *hsp20*, *clpP*, *clpB*, and *clpS*) promoted the growth of *Z. mobilis* under challenging conditions. The former gene products can enhance ROS detoxification, while the latter can help protect cellular proteins from stress-induced damage. Therefore, the ability of thermotolerant *Z. mobilis* 200M and PYK to tolerate acetic acid, formic acid, and oxidative stress at elevated temperatures may be attributed to the expression of specific stress response genes. Notably, newly developed thermotolerant strains of *Z. mobilis* show strong potential for high-temperature ethanol fermentation, particularly under acetic acid, formic acid, and H₂O₂ stress.

3.5. RT-qPCR Analysis of Gene Expression Under Heat Stress

Based on transposon mutagenesis in *Z. mobilis* TISTR548, the wild-type strain used in this study, nine groups of genes are essential for survival at CHT. These include genes involved in general metabolism, membrane stabilization, transporters, DNA repair and modification, tRNA and rRNA modification, protein quality control, translational control, cell division, and transcriptional regulation [32]. The molecular mechanisms underlying heat-stress responses in the thermotolerant strains *Z. mobilis* 200M and PYK remain uncharacterized. Therefore, a comparative analysis of gene expression was performed using RT-qPCR. In this study, a set of genes was selected that are associated with the ethanol production pathway, carbon and energy metabolism, transporters, protein quality control, general stress response, translation control, and other genes involved in signal transduction and protein–protein interactions, previously identified as upregulated under heat stress by transcriptomic analysis.

As summarized in Figure 7, expression levels of nearly all genes tested at 30 °C in both exponential- and stationary-phase cells were relatively low compared with those at 40 °C. Of the three strains, thermotolerant *Z. mobilis* 200M exhibited the lowest gene expression at this control temperature. Three genes, including *frk* (encoding fructokinase), *adhB* (encoding alcohol dehydrogenase II), and *pdc* (encoding pyruvate decarboxylase), were upregulated at 30 °C in exponential-growth cells, particularly in newly thermotolerant *Z. mobilis* PYK as compared to the wild-type strain. However, in the stationary phase, their expression levels returned to normal levels comparable to those of the wild type. Since these genes are crucial for ethanol production, the high ethanol production efficiency of this newly developed thermotolerant strain, as summarized in Table 2, may be linked to their upregulation.

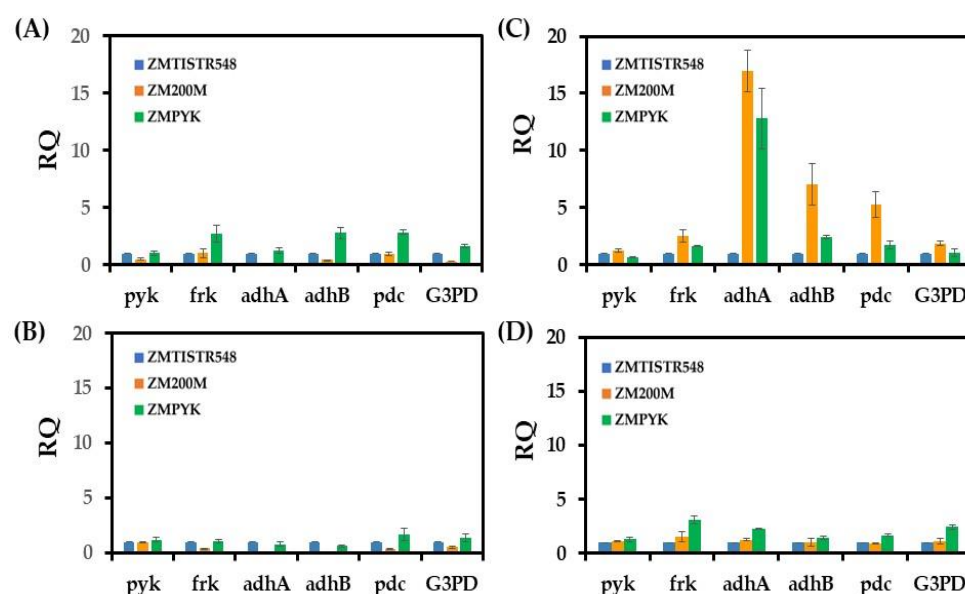


Figure 7. Relative expression level of genes involved in the ethanol production pathway of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

In the thermotolerant strains, a significant upregulation of nearly all targeted genes, except for *pyk* and *g3pd*, was observed during the exponential growth phase at 40 °C. Among the strains tested, *Z. mobilis* 200M exhibited the highest transcriptional response under this thermal stress. The expression levels of all genes declined as cells entered the stationary phase. Notably, *adhA* (encoding alcohol dehydrogenase I) showed the highest expression, followed by *adhB* and *pdc*. These findings align with Charoenpunthuwong et al. [28], who identified *adhA*, *adhB*, and *pdc* as key stress-responsive genes induced by elevated temperature and ethanol stress. Similar transcriptional upregulations of *adh* genes under thermal stress have been reported in other ethanologenic microbes, including the conventional yeast *S. cerevisiae* and the non-conventional yeast *Pichia kudriavzevii* [43,44]. Our results suggest that *adhA*, *adhB*, and *pdc* function as heat-inducible genes in *Z. mobilis*, and their upregulation at 40 °C likely contributes to the enhanced ethanol fermentation efficiency observed in the thermotolerant strains relative to the wild type.

Figure 8 shows the expression of genes involved in carbon and energy metabolism in *Z. mobilis* wild-type and thermotolerant strains. At 30 °C, relatively low expression levels were observed for almost all genes in the exponential growth of bacterial cells. However, when cells entered the stationary growth phase, a significant increase in the expression levels of ZZ6_RS00060, which encodes an aldo-keto reductase, and ZZ6_RS00835, which encodes a CocE/NonD family hydrolase, was observed, particularly in the

thermotolerant *Z. mobilis* PYK. As previously reported, the gene encoding aldo-keto reductase is induced by phenolic aldehyde inhibitors and plays a role in converting toxic aldehydes into less toxic alcohols in *Z. mobilis* cells [45]. In addition, the CocE/NonD hydrolase, a member of the aminopeptidase family, has also been shown to be upregulated under stress conditions in *Sediminibacterium* sp. [46]. Although the biological functions of these proteins under stress conditions in *Z. mobilis* remain uncharacterized, further investigation is warranted. On the other hand, as reported by Asefi et al. [22], nutrient shortage is among the stressors that can induce the expression of stress-responsive genes, particularly those involved in carbon and energy metabolism. Based on these findings, it is speculated that the upregulation of these genes may be associated with nutrient depletion, although direct evidence in *Z. mobilis* is currently lacking.

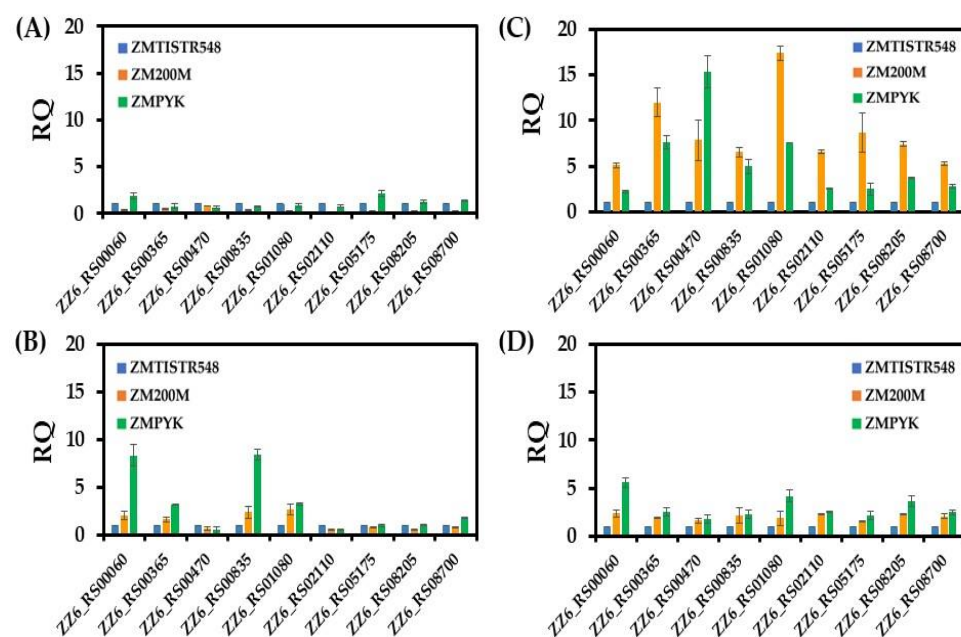


Figure 8. Relative expression level of genes involved in carbon and energy metabolism of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

Interestingly, upon thermal stress at 40 °C, all targeted genes were significantly upregulated, particularly during the exponential growth phase. Specifically, genes encoding NAD(P)-dependent oxidoreductase (ZZ6_RS01080) and uroporphyrinogen-III C-methyltransferase (ZZ6_RS00365) showed the highest expression in the thermotolerant *Z. mobilis* 200M, while that encoding 2-hydroxyacid dehydrogenase (ZZ6_RS00470) was uniquely upregulated in the thermotolerant *Z. mobilis* PYK. Although transcriptional levels generally declined as cells entered the stationary phase, genes ZZ6_RS00060, ZZ6_RS01080, and ZZ6_RS08205 (encoding an inositol monophosphatase) maintained relatively high expression levels.

While the biological functions of these specific gene products remain uncharacterized in *Z. mobilis*, we speculate that their heat-induced upregulation serves several critical survival mechanisms. First, they may assist in maintaining the intracellular NAD⁺/NADH ratio by providing alternative pathways for cofactor recycling when primary fermentation enzymes are impaired by high temperatures. Second, these enzymes may be involved in carbon flux diversion and the management of keto-acid intermediates, thereby preventing the toxic accumulation of metabolic byproducts under thermal stress. Furthermore, these gene products may contribute to the synthesis of protective membrane lipids and hopanoids, which are essential for stabilizing the cell envelope against heat-induced

fluidity. Finally, they likely play a role in neutralizing ROS and thermal degradation products that accumulate under severe stress conditions [22,32,47].

The transport of nutrients and cofactors across membranes, and the maintenance of the proton motive force (PMF), are vital for sustaining cellular energy and homeostasis under environmental stress. As shown in Figure 9, genes encoding a putative TonB-dependent transport system (ZZ6_RS07320, ZZ6_RS07325, ZZ6_RS08160, and ZZ6_RS08185) were highly upregulated during the exponential growth phase at 30 °C, an effect particularly pronounced in the thermotolerant *Z. mobilis* PYK. In response to thermal stress at 40 °C, these TonB-related genes, alongside others encoding putative proteins for general solute, peptide, and nitrogen transport (ZZ6_RS01595, ZZ6_RS04240, ZZ6_RS04635, and ZZ6_RS08470), exhibited significant upregulation in both thermotolerant strains relative to the wild type. Interestingly, the expression of nearly all these transport-related genes returned to baseline levels during the stationary phase, mirroring the expression profile of the wild-type strain. These findings suggest that enhanced nutrient and cofactor acquisition is a phase-specific requirement for maintaining growth under thermal pressure.

Under heat stress, the efficient trafficking of nutrients, cofactors, and metabolic intermediates (such as oligopeptides) becomes essential to compensate for increased metabolic turnover and potential membrane leakiness. The TonB-dependent system, typically associated with the uptake of iron and complex carbohydrates, likely plays a specialized role in scavenging essential micronutrients and maintaining the integrity of the cell envelope when the PMF is challenged by high temperatures. This observation aligns with Charoensuk et al. [32] and Li et al. [48], who reported the upregulation of Fe²⁺/Mn²⁺ transporters and TonB-related proteins in *Z. mobilis* TISTR548 and ZM4, respectively, under critical thermal stress. Our results further support the hypothesis that reinforcing the transportome is a conserved strategy for thermotolerance in *Z. mobilis*.

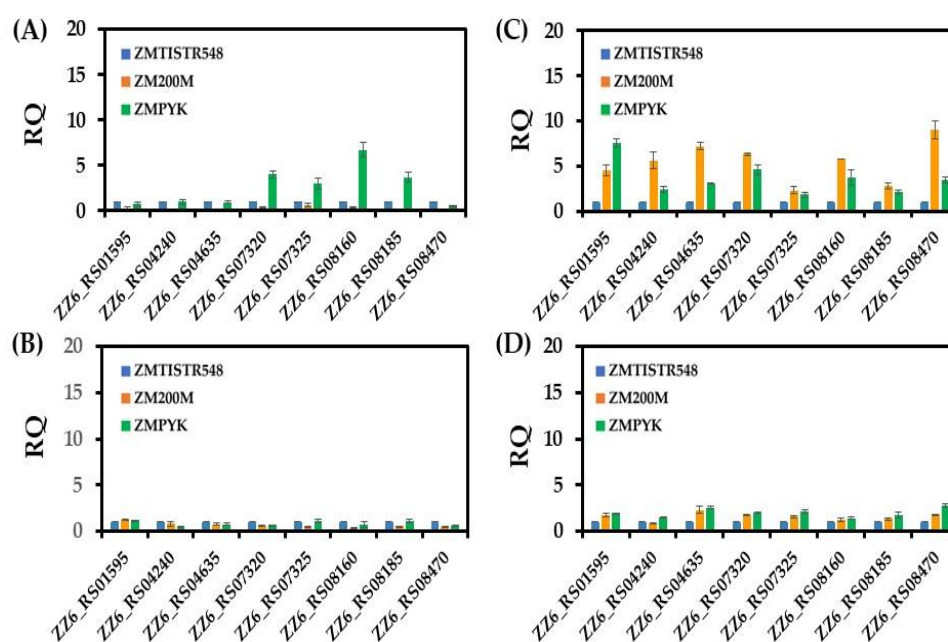


Figure 9. Relative expression level of genes involved in membrane transport of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

The upregulation of genes involved in protein quality control and proteostasis is a conserved response to environmental stress in *Z. mobilis* and other microorganisms. Previous studies have identified specific components of this machinery, such as Zinc-

dependent peptidases and the ATP-dependent Clp protease subunit (ClpA), as essential for the survival and growth of *Z. mobilis* at critical thermal limits [32,42]. Expanding upon these observations, our study demonstrates that a suite of genes encoding the ATP-dependent Clp protease (*clpA*), M1 family metallopeptidase (ZZ6_RS07045), zinc metallopeptidase (ZZ6_RS08640), and a putative protein-disulfide reductase (*dsb*) (ZZ6_RS08800) were significantly upregulated under thermal stress at 40 °C. This induction was particularly pronounced during the exponential growth phase of the thermotolerant strains. Notably, while expression declined in the stationary phase, these levels remained slightly higher in the thermotolerant mutants than in the wild type. In contrast, no significant differential expression was observed between the strains during growth at the optimal temperature of 30 °C (Figure 10).

These gene products constitute a robust proteolytic and refolding network designed to mitigate the effects of thermal proteotoxicity. As reported by Aleshin et al. [49], these enzymes act by identifying and degrading denatured or misfolded proteins that aggregate within the cytoplasm during heat stress. Furthermore, the presence of protein-disulfide reductase (Dsb) suggests an enhanced capacity to manage oxidative protein damage and ensure correct disulfide bond formation, which is frequently disrupted at high temperatures. Consequently, the superior growth and fermentation performance of our thermotolerant *Z. mobilis* strains at 40 °C appear to be driven by the coordinated upregulation of these proteostatic genes, which prevents the toxic accumulation of protein aggregates.

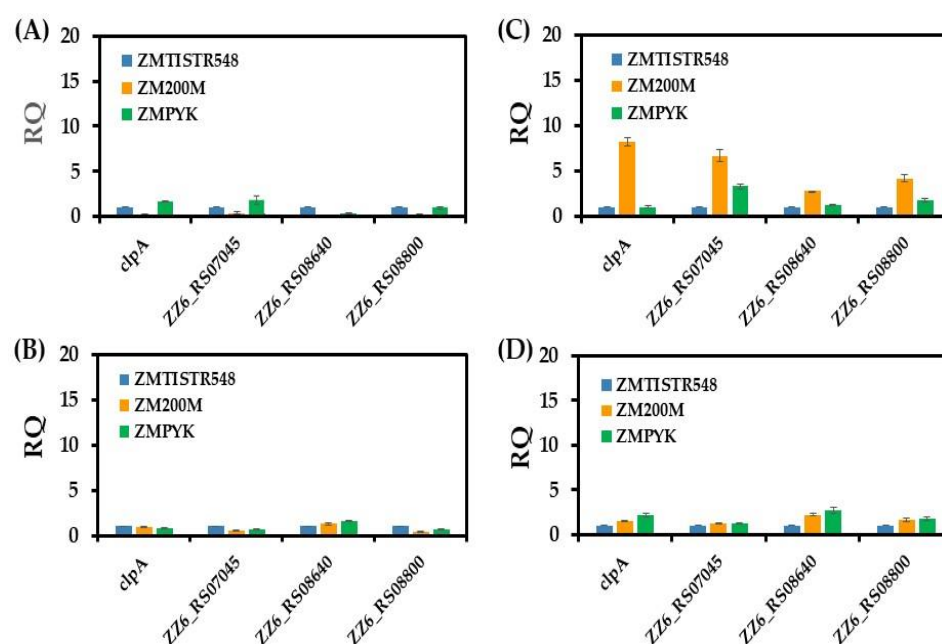


Figure 10. Relative expression level of genes involved in protein quality control of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

Beyond protein quality control, the adaptive response of *Z. mobilis* to thermal stress involves the coordinated induction of genes associated with programmed cell death and heat shock proteins (HSPs). As illustrated in Figure 11, the expression of most targeted genes remained low during exponential growth at 30 °C. However, a pronounced upregulation of the entericidin (EcnAB) toxin–antitoxin (TA) system was observed during the stationary phase, particularly in the thermotolerant strains. This outer membrane protein is composed of two small peptides, namely EcnA (44 kDa) and EcnB (48 kDa). The expression of the *ecnAB* gene is tightly regulated by the stationary-phase sigma factor encoded by *rpoS* and by stressful conditions, including osmotic stress and starvation [50,51]. EcnB

is a bacteriolytic toxin that disrupts membranes and induces cell lysis, whereas EcnA acts as an antidote by forming a complex with EcnB or by counteracting its effects at the membrane. We speculate that the induction of this TA system reflects a population-level survival strategy. The lysis of a subpopulation may release essential nutrients, thereby sustaining the metabolic viability of the remaining population under prolonged stress or nutrient scarcity.

In response to 40 °C thermal stress, nearly all targeted genes were significantly upregulated in the thermotolerant strains during the exponential phase. This was specifically characterized by the induction of HSPs with molecular weights of 40, 70, and 100 kDa. During the stationary phase, the thermotolerant strains maintained significantly higher expression of these genes relative to the wild type, most notably those encoding HSP100 and the putative GlsB/YeaQ/YmgE protein complex (ZZ6_RS00300). These molecular chaperones are essential for maintaining proteostasis, protein assembly, and membrane integrity when temperatures exceed optimal ranges [52,53]. Our findings align with previous reports in *Z. mobilis* ZM4, where primary chaperones such as DnaK, DnaJ, ClpB, and GroEL were significantly upregulated under severe thermal stress [48].

Notably, the gene ZZ6_RS00300 (encoding the GlsB/YeaQ/YmgE complex) showed exceptional upregulation in the thermotolerant strain PYK during the stationary phase at 40 °C. These protein complexes are recognized for their role in stabilizing the cytoplasmic membrane and preserving the electrochemical gradient required for ATP synthesis under diverse chemical and physical stresses. Similar survival mechanisms have been reported for *Bacillus subtilis* DM2 and *Bifidobacterium animalis* under petroleum and acid stress, respectively [54,55]. Thus, the overexpression of ZZ6_RS00300, alongside the classic chaperones, represents a critical specialized mechanism for enhancing thermotolerance and membrane stability in thermotolerant *Z. mobilis* under thermal stress.

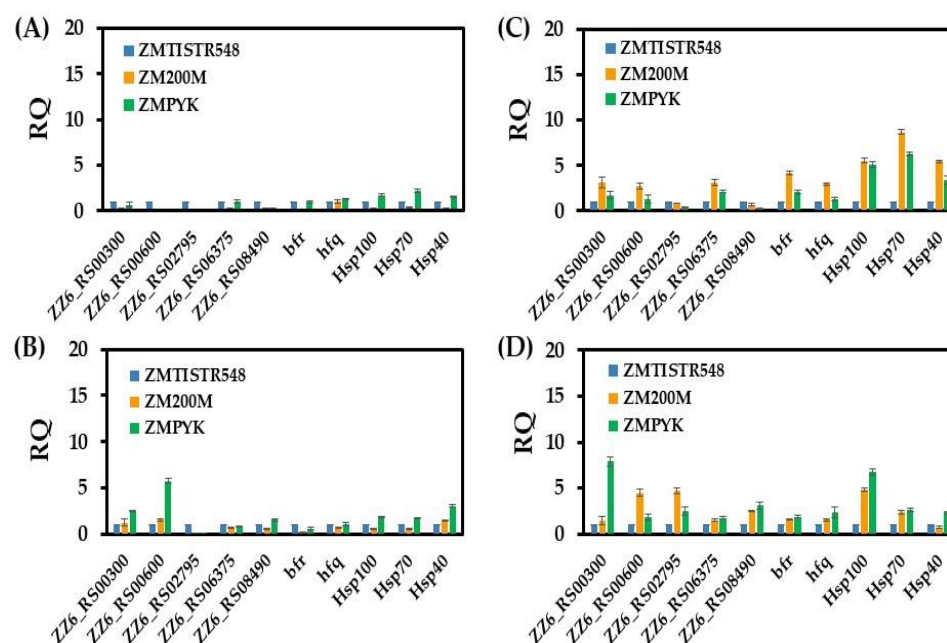


Figure 11. Relative expression level of genes involved in the general stress response of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

There are several open reading frames that potentially encode proteins involved in transcriptional regulation. For instance, the TetR family transcriptional regulator (ZMO0281, ZMO1547), the LysR family transcriptional regulator (ZMO0774), and the RpiR family transcriptional regulator (ZMO0190) [56] have all been identified. However,

as demonstrated in this study, the expression of these stress-responsive genes in thermotolerant strains did not differ significantly from that of the wild-type strain under heat stress. Interestingly, while transcriptional control appeared similar across strains, genes involved in translational regulation and RNA stability were significantly upregulated in the exponential-growth cells of thermotolerant strains at 40 °C (Figure 12). This induction was phase-specific, as expression levels returned to near-baseline in the stationary phase relative to the wild type.

Specifically, we identified the significant upregulation of TsaE (ZZ6_RS01140), the ATPase subunit of the Threonylcarbamoyltransferase complex, and DEAD/DEAH-box helicases (ZZ6_RS08660). TsaE is a crucial component of the TsaB/C/D/E enzymatic machinery responsible for the essential tRNA modification, which is required for accurate decoding of the genetic code during translation. TsaE's ATPase activity is vital for cell growth, particularly under ethanol and thermal stress, where translation fidelity is often compromised [57]. Simultaneously, the DEAD/DEAH-box helicases function as RNA chaperones, utilizing ATP to resolve misfolded RNA structures and prevent the formation of non-functional RNA aggregates. By ensuring that mRNA remains in a translationally competent conformation, these helicases facilitate translation initiation even under harsh conditions [58]. Such helicases have previously been linked to ethanol stress resistance in *Z. mobilis* [56], but their role in thermotolerance has remained largely uncharacterized. Based on these expression profiles, we speculate that the coordinated upregulation of *tsaE* and RNA helicases provides a specialized translational safeguard, ensuring proper RNA folding and efficient protein synthesis at elevated temperatures. This mechanism likely allows thermotolerant strains to maintain higher metabolic flux than the wild type under thermal stress.

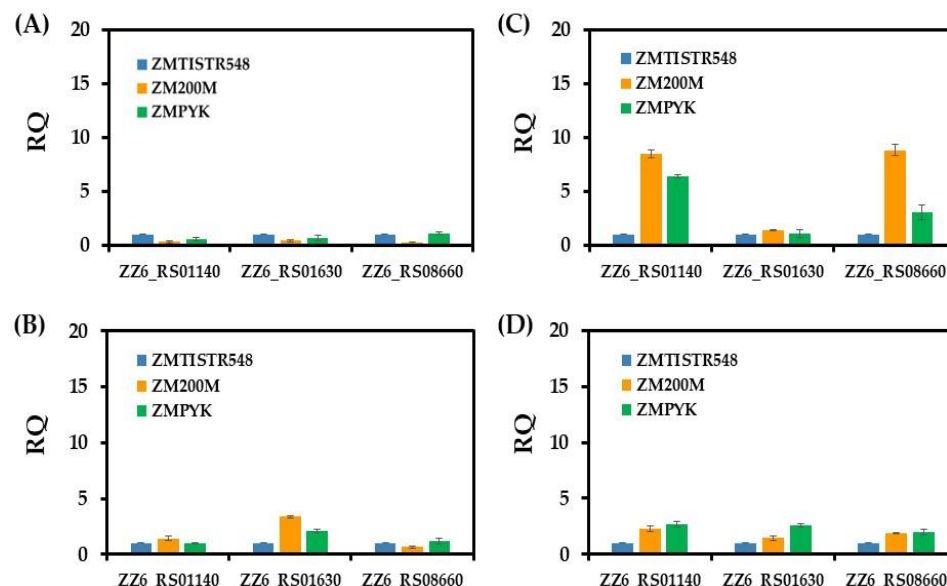


Figure 12. Relative expression level of genes involved in translation control of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

In the current study, a suite of genes encoding proteins involved in signal transduction and protein–protein interactions was significantly upregulated in the exponential-growth cells of thermotolerant strains under thermal stress (Figure 13). This group included a gene encoding a putative signaling intermediate in phalloidin-induced localization (SIMPL)-domain-containing protein (ZZ6_RS04870), two genes encoding SEL1-like repeat proteins (SLRs) (ZZ6_RS05750 and ZZ6_RS06260), and three genes encoding

tetratricopeptide repeat proteins (TPRs) (ZZ6_RS05545, ZZ6_RS05550, and ZZ6_RS07365). While the SIMPL domain-containing protein remains relatively uncharacterized and unclassified in *Z. mobilis*, its differential expression at 40 °C suggests a novel role in heat-responsive signaling. In contrast, the SLR proteins, a specialized subfamily of the TPR superfamily, are more established as mediators of intracellular signal transduction. Though better known in eukaryotes (e.g., *Caenorhabditis elegans*), SLR proteins have been identified in bacteria such as *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Rhizobium leguminosarum*, where they facilitate the assembly of multi-protein complexes [59].

Furthermore, the TPR proteins, characterized by their 34-amino-acid repeat motifs, act as critical molecular scaffolds. Under environmental stress, these proteins function as co-chaperones, working in tandem with high-molecular-weight HSPs to facilitate the folding or systematic degradation of denatured proteins [60]. The upregulation of TPR proteins has previously been noted in *Z. mobilis* ZM4 as a defense against phenolic aldehyde inhibitors [45]. Based on this evidence, we speculate that the simultaneous upregulation of SIMPL, SLR, and TPR genes enables the thermotolerant *Z. mobilis* 200M and PYK to build more robust proteostasis networks. By enhancing the interaction between signaling molecules and the chaperone machinery, these proteins effectively bridge the detection of thermal stress with a rapid, coordinated cellular response at 40 °C.

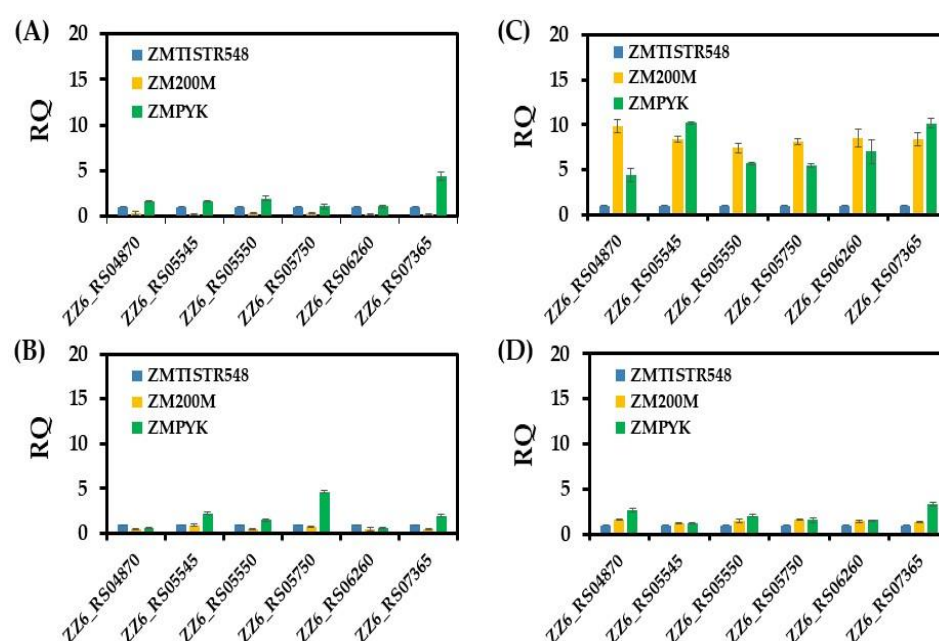


Figure 13. Relative expression level of genes involved in signal transduction and protein–protein interaction of *Zymomonas mobilis* wild-type and newly developed thermotolerant strains at 30 °C (A,B) and 40 °C (C,D) in the exponential (A,C) and stationary growth phase (B,D).

This study elucidates the comprehensive molecular reprogramming that enables the newly developed thermotolerant strains, *Z. mobilis* 200M and PYK, to maintain robust growth and high ethanol titers at the CHT of 40 °C. The novelty of this work lies in the identification of a synchronized, multi-layered systemic response, spanning ethanol biosynthesis, metabolic flux stabilization, and specialized proteostasis, that fundamentally distinguishes these thermotolerant strains from the wild type. Specifically, we demonstrate that their superior fermentative performance is underpinned by the sustained upregulation of the core ethanol-producing pathway (*adhA*, *adhB*, and *pdc*) during the exponential phase, ensuring high metabolic throughput even under thermal pressure. This is coupled with a unique phase-specific survival strategy during the stationary phase, involving the *ecnAB* toxin–antitoxin system for population-level resource management and

the GlsB/YeaQ/YmgE membrane-stabilizing family for preserving the electrochemical gradient against thermal leakiness.

Furthermore, the discovery of upregulated RNA chaperones (DEAD/DEAH-box helicases) and molecular scaffolding proteins (SLR and TPR superfamilies) reveals a sophisticated translational safeguard. These systems ensure translational fidelity and protein homeostasis by preventing RNA misfolding and facilitating the assembly of multi-protein repair complexes under thermal stress. By pinpointing these specific genetic markers, this study provides a definitive molecular basis for thermal resilience in *Z. mobilis*. These findings not only clarify the physiological basis of thermotolerance but also offer precise metabolic engineering targets for the development of next-generation, industrially robust ethanologenic biocatalysts.

4. Conclusions

The newly developed thermotolerant strains *Z. mobilis* 200M and *Z. mobilis* PYK exhibited improved growth and ethanol fermentation performance compared with the wild-type strain. The CHT for growth of *Z. mobilis* 200M and PYK was 2.0 and 2.5 °C higher than that of the wild type, respectively. Their morphologies, particularly cell shape and length, differed from those of the wild-type strain. Thermotolerant strains exhibited a regular rod-shaped morphology and shorter cell length under heat stress at 40 °C than the wild type. The ethanol fermentation efficiency of these newly thermotolerant strains was also significantly greater than that of the wild type. At 40 °C, the ethanol concentrations produced by thermotolerant strains ranged from 50.69 to 55.63 g/L and from 49.51 to 51.29 g/L at 24 and 48 h after fermentation, which was approximately 6.0- and 3.0-fold greater than those of the wild type, respectively. Notably, the thermotolerant strains exhibited superior multi-stress tolerance to heat, ethanol, acetic acid, formic acid, and H₂O₂ relative to the wild-type strain. Several genes encoding proteins involved in the ethanol production pathway, carbon and energy metabolism, transporters, protein quality control, general stress response, translation control, signal transduction pathway, and protein–protein interactions were significantly upregulated in the thermotolerant strains, particularly under elevated temperature. Based on these findings, we speculated that the higher growth and ethanol fermentation performance of the newly developed thermotolerant *Z. mobilis* 200M and PYK at 40 °C may be linked to the upregulation of these genes.

In summary, this study identified several candidate genes that may contribute to the improved growth and ethanol fermentation capacity in the newly developed thermotolerant *Z. mobilis* strains. While our findings clearly demonstrate the physiological and molecular advantages of these strains at 40 °C, further investigation is warranted to fully elucidate the adaptive mechanisms across a broader range of temperatures and stressor concentrations. Future research focusing on transcriptomic analysis and target mutagenesis under these wider stress gradients will provide deeper insights into the robust performance of these strains. Such knowledge will be instrumental in developing highly efficient microbial cell factories for sustainable second-generation bioethanol production.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biology15050404/s1>, Table S1: List of primers used in this study; Table S2: Specific growth rate of *Z. mobilis* wild-type and thermotolerant strains at various temperatures under the critical high temperature assay; Table S3: Specific growth rate of *Z. mobilis* wild-type and thermotolerant strains at various stress conditions.

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writing—original draft preparation, K.C., P.K., S.T. and P.T.; writing—review and editing, P.K., S.T., M.Y. and P.T.; visualization, K.C. and P.T.; supervision, P.T.; project administration, P.T.; funding acquisition, K.C. and P.T. All authors have read and agreed to the published version of the manuscript.

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