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Overexpression of the lon protease and its impact on heat and ethanol stress tolerance in *Z. mobilis*

Yupaporn Phannarangsee[1], Haruthairat Kitwetcharoen[1], Sudarat Thanonkeo[2], Preekamol Klanrit[1,3], Mamoru Yamada[4,5], and Pornthap Thanonkeo[1,3,*]

[1]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen, Thailand
[2]Walai Rukhavej Botanical Research Institute (WRBRI), Mahasarakham University, Maha Sarakham, Thailand.
[3]Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Khon Kaen University, Khon Kaen, Thailand.
[4]Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan.
[5]Research Center for Thermotolerant Microbial Resources, Yamaguchi University, Yamaguchi, Japan.
[*]Corresponding author: portha@kku.ac.th

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Abstract

The Lon protease plays a crucial role in bacterial genome stability and cellular homeostasis under various stress conditions. In this study, the *lon* gene encoding the Lon protease in *Zymomonas mobilis* TISTR548 was cloned and characterized, and its overexpression in *Z. mobilis* cells was evaluated. The *lon* gene had an open reading frame of 2,427 bp, encoding 809 amino acid residues with a relative molecular weight of 89 kDa and a predicted isoelectric point (pI) of 6.42. The Lon protease of *Z. mobilis* shared several conserved structural and functional features with other Lon proteases. Overexpression of the *lon* gene improved bacterial cell growth under heat stress at 40.5°C and ethanol toxicity at 10% (v/v). Furthermore, it helped bacterial cells maintain a regular rod shape under stressful conditions, suggesting a role in the cell division mechanism. These findings are valuable for future efforts to improve *Z. mobilis* strains for enhanced tolerance to heat, ethanol, and other stressful conditions. The successful cloning, expression, and characterization of the Lon protease gene provide insights into the role of this important protein in the stress response and cell division of *Z. mobilis*.

Keywords: Ethanologenic fermentative bacteria, Genetic engineering, Molecular cloning, Stress-responsive proteins, *Zymomonas mobilis*

1. Introduction

Bacterial cells employ various proteases to maintain protein quality control and regulate stress responses. Among the four major cytosolic proteases in bacteria - Lon, ClpXP, ClpAP, and HslUV [1], the Lon protease stands out for its crucial role in maintaining genome stability and protein homeostasis under various stress conditions. Under stress conditions, different proteases exhibit specific responses; for instance, HslUV levels increase during heat shock [2] and participate in the SOS response triggered by DNA damage [3, 4]. Similarly, ClpXP responds to various stresses, including starvation [5], heat shock, and oxidative stress [6], while ClpAP regulates bacterial pathways and responds to proteotoxic stress induced by pH shifts or elevated temperatures [7]. The Lon protease, first identified as an ATP-dependent protease, is highly conserved across prokaryotes and eukaryotes, demonstrating its fundamental importance in cellular function. Studies have shown that Lon protease regulates DNA replication through selective protein degradation, particularly during stress conditions such as heat shock or nutrient deprivation [8–11]. In *Actinobacillus pleuropneumoniae*, LonA protease contributes significantly to osmotic and oxidative stress tolerance [12].

Zymomonas mobilis, a gram-negative bacterium, has emerged as a promising alternative to traditional yeast in industrial ethanol production, owing to its remarkable characteristics. These include high ethanol conversion efficiency, exceptional ethanol tolerance of up to 10%, and the ability to ferment in challenging conditions such

as low pH and high concentrations of lignocellulosic inhibitors. Unlike conventional ethanologenic fermenting yeast, *Saccharomyces cerevisiae*, *Z. mobilis* can efficiently process glucose, fructose, and sucrose through the Entner-Doudoroff (ED) pathway, converting these sugars into ethanol and carbon dioxide with relatively high theoretical yields. *Z. mobilis* demonstrates remarkable tolerance to environmental stresses, particularly thermal and ethanol stress, through the production of specialized stress proteins. These proteins represent a biological adaptation that enhances cell growth and survival under adverse conditions. However, both thermal and ethanol stress can disrupt cellular ionic balance, potentially leading to reduced metabolic activity and cell death [13]. Although several studies have investigated *Z. mobilis*' response to these stresses, the molecular mechanisms underlying its stress resistance remain poorly understood, especially the biological role of Lon protease.

This study aimed to investigate the role of the *lon* gene in *Z. mobilis* stress tolerance through overexpression experiments. The primary objective was to determine whether elevated levels of Lon protease enhance the organism's resistance to various environmental stresses, particularly focusing on thermal and ethanol stress conditions. Understanding the function of Lon protease in *Z. mobilis* could provide valuable insights for improving strain tolerance and fermentation efficiency in industrial applications.

2. Materials and methods

2.1 Bacterial strains and growth conditions

This study employed two bacterial strains for different experimental purposes. The primary strain, *Z. mobilis* TISTR548, obtained from the Thailand Institution of Science and Technological Research, served as the source of the *lon* gene. This strain was maintained in yeast extract peptone glucose (YPG) medium, containing 0.3% yeast extract, 0.5% peptone, and 3% glucose. Two bacterial systems were utilized, including *E. coli* DH5α for gene cloning and *Z. mobilis* TISTR548 for gene expression. The *E. coli* strains were cultured in Luria-Bertani (LB) medium, comprising 1% tryptone, 1% sodium chloride, and 0.5% yeast extract. Gene expression was facilitated using the pZA22 shuttle vector [14], which was maintained on LB agar plates supplemented with chloramphenicol (50 µg/mL).

For experimental cultures, bacterial cells were grown at 30°C until reaching the mid-exponential phase (OD_{550} 0.5–1.0), then inoculated into a liquid medium and cultured under static conditions. Bacterial growth was monitored spectrophotometrically using a Genesys 20 spectrophotometer (Thermoscientific, Germany) at OD_{550}. When necessary, the culture medium was supplemented with ampicillin at a final concentration of 100 µg/mL. For long-term storage and preservation, all bacterial strains were maintained in 15% glycerol at −80°C.

2.2 Genomic DNA isolation, PCR amplification, and Molecular Cloning of Z. mobilis lon gene

The genomic DNA (gDNA) was isolated from *Z. mobilis* TISTR548 using a modified phenol/chloroform method described by Sambrook et al. [15]. The extracted gDNA was either used immediately for downstream applications or stored at −20°C. PCR amplification was performed using specific primers (Table 1) designed based on the *lon* gene of *Z. mobilis* ATCC 29191 in the GenBank database (CP003704.1). All primers used in this study were synthesized at Macrogen (Korea). The PCR reaction mixture (25 µL) composed of 1 µL *ExTaq* DNA polymerase (Takara, Japan), 2.5 µL 10× buffer, 2.5 µL dNTPs (2.5 mmol), 1 µL each of forward and reverse primers (10 mM), 1 µL DNA template (10 ng), and 16 µL nuclease-free water. The amplification protocol consisted of initial denaturation at 94°C for 10 minutes, followed by 35 cycles of denaturation (94°C, 50 seconds), primer-specific annealing temperature (1 minute), and extension (72°C, 2 minutes).

The amplified PCR products were separated by 1% agarose gel electrophoresis, and the target DNA bands were excised and purified using a GF-1 AmbiClean (Gel & PCR) purification Kit (Vivantis, Malaysia). The purified products (60 ng) were then ligated into the pGEM-T Easy vector (50 ng) (Promega, USA) using T4 DNA Ligase and incubated at 25°C for 2 h according to the manufacturer's protocol. Competent *E. coli* DH5α cells were prepared using the $CaCl_2$ method. Briefly, cells were cultured in LB medium at 37°C with shaking at 200 rpm for 12-16 h. One milliliter of the bacterial culture was transferred to a fresh LB medium and further incubated under the same conditions for 3-4 h. Cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4°C and resuspended in ice-cold 0.1 M $CaCl_2$ solution. After 30 minutes of incubation on ice, cells were again centrifuged under the same conditions. The resulting cell pellets were resuspended in 0.1 M $CaCl_2$ containing 15% glycerol.

The ligation products were then transformed into the competent *E. coli* DH5α cells using chemical transformation, followed by heat shock at 42°C for 45 seconds [16]. The recombinant colonies were identified through blue-white screening on LB agar plates supplemented with ampicillin (100 µg/mL), isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.1 M), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (20 mg/mL). Plasmid DNA was extracted from selected recombinants using a GF1 plasmid DNA extraction kit (Vivantis, Malaysia), and the presence and sequence accuracy of the *lon* gene were confirmed by Sanger sequencing (Macrogen, Korea).

Table 1 List of primers used for gene cloning and expression in this study.

Primer name	Sequence (5' → 3')	References
pZA-5	GATCCTCTACGCCGGACGCATCGTG	[17]
pZA-3	GATCCACAGGACGGGTGTGGTCGCC	[17]
pZA-Ppdc-5	AGTCAGGCACCGTGTACGGGGCTATCCTTCAAAAGAAG	[17]
pZA-Ppdc-3	GCCGCCGCCTTCCATTGCTTACTCCATATATTCAAAACAC	[17]
lon 5-pGEM	ATGAAAGAAACCCTTCCCGTTTT	This study
lon 3-pGEM	TCAATGATGCACAGGACTGACAC	This study
lon-pZA-5	TATATGGAGTAAGCA ATGAAAGAAACCCTTCCCGTTTT	This study
lon-pZA-3	GCCGCCGCCTTCCAT TCAATGATGCACAGGACTGACAC	This study

2.3 Construction of expression vector and gene transformation

The construction of the expression system involved multiple steps of DNA amplification and cloning. Initially, the pZA22 shuttle vector was amplified to generate a linearized form, while the *Z. mobilis pdc* promoter (*Ppdc*) and *lon* gene were amplified from *Z. mobilis* gDNA using specific primers. All DNA amplifications were performed using high-fidelity Q5 polymerase (New England Biolabs, England). The *Ppdc* promoter was selected for driving *lon* gene expression [17], and all PCR products were purified using a GF-1 AmbiClean Gel & PCR Kit (Vivantis, Malaysia).

The expression construct was assembled by fusing the *lon* gene and *Ppdc* to the linearized pZA22 shuttle vector using an In-Fusion Snap Assembly cloning kit (Takara Bio, USA). The fusion reaction was performed at 50°C for 15 minutes, followed by immediate ice treatment. The resulting construct was transformed into *E. coli* DH5α using the chemical and heat shock method as previously described [16]. Transformants were selected on LB medium containing chloramphenicol (50 μg/mL) and incubated overnight at 37°C. Successful recombination was verified through colony PCR using specific primers, and the recombinant plasmids were isolated using a GF-1 Plasmid DNA Extraction Kit (Vivantis, Malaysia). The sequence accuracy was confirmed by Sanger sequencing (Macrogen, Korea).

Competent *Z. mobilis* were prepared by culturing cells in RM liquid medium supplemented with 2% (w/v) glucose at 30°C with shaking at 200 rpm until the culture reached an optical density of 0.4 at 550 nm. Cells were harvested by centrifugation at 4,000 rpm for 10 minutes at 4°C, washed with 10% (v/v) glycerol, and resuspended in the same solution. The resulting competent cells were then used as hosts for subsequent transformation experiments.

For gene expression in *Z. mobilis*, the verified recombinant plasmid was introduced into competent cells through electroporation using a Gene Pulser (Bio-Rad, CA, United States) with specific parameters (0.1-cm electrode gap, 1800V, 200W, 25mF) as described by Okamoto and Nakamura [18]. Transformants were selected on rich medium (RM) agar plates containing 2% w/v glucose and chloramphenicol (100 μg/mL). The presence of the recombinant construct was confirmed by colony PCR. The stability of the selected recombinants was evaluated through ten generations of subculturing on RM agar with and without chloramphenicol. Successfully verified recombinant strains were preserved at −80°C for long-term storage.

2.4 Characterization of Z. mobilis recombinant strains

2.4.1 Cell morphology analysis

Wild-type and recombinant strains of *Z. mobilis* were individually cultured in liquid YPG medium at 30°C for 24 hours. Each culture was then transferred to fresh YPG medium at an initial OD550 of 0.05 and incubated at either 30°C or 40.5°C for 18 hours. The cells were harvested by centrifugation at 5,000 rpm for 5 minutes, washed twice with PBS (pH 7.4), and resuspended in 100 μL of the same buffer. Cell morphology was examined by placing 5 μL of cell suspension onto a glass slide and observing under a microscope (ZEISS Primostar 3, Germany) using Zeiss Labscope software.

2.4.2 Growth of Z. mobilis recombinant strains under heat stress

Z. mobilis wild-type and recombinant strain overexpressing *lon* gene were first cultivated in YPG medium at 30°C with continuous shaking at 200 rpm until reaching the mid-exponential growth phase (OD550 ≈ 0.4-0.6). For heat stress experiments, the cultures were transferred into fresh YPG medium with an initial OD550 of 0.05 and incubated at either optimal temperature (30°C) or elevated temperature (40.5°C). Cell growth was monitored by measuring optical density at 550 nm at regular intervals over a 48-hour period.

2.4.3 Growth of Z. mobilis recombinant strains under ethanol stress

Z. mobilis wild-type and recombinant strain overexpressing *lon* gene were first cultivated in YPG medium at 30°C with continuous shaking at 200 rpm until reaching the mid-exponential growth phase (OD550 ≈ 0.4-0.6). For ethanol stress experiments, the cultures were transferred into fresh YPG medium containing 0% and 10% (v/v) absolute ethanol with an initial OD550 of 0.05 and subsequently incubated at 30°C. Cell growth was monitored by measuring optical density at 550 nm at regular intervals over a 48-hour period.

2.5 Statistical analysis

All experiments were performed in triplicate with two independent biological replicates. Data are presented as means ± standard deviations (SD). Statistical significance was determined based on a one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons using the IBM SPSS Statistics 28 for Windows (IBM Corporation, Armonk, NY, USA). Differences were considered statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1 Molecular cloning and characterization of Z. mobilis lon gene

The open reading frame (ORF) encoding Lon protease was amplified from *Z. mobilis* TISTR548 genomic DNA by PCR. The amplified product of approximately 2.5 kb was visualized by agarose gel electrophoresis, purified, and cloned into the pGEM T-Easy vector. After the transformation of the recombinant plasmid into *E. coli* DH5α, the recombinant strains were screened and selected. Sequencing analysis of the recombinant plasmid DNA isolated from the selected recombinant strain revealed that a complete nucleotide sequence of *Z. mobilis lon* gene was 2,427 bp long, encoding a polypeptide of 809 amino acid residues. The predicted molecular weight and the isoelectric point (pI) of the deduced polypeptide were approximately 89 kDa and 6.42, respectively. The accession number of the *lon* gene of *Z. mobilis* TISTR548 was PQ564446.

Sequence homology analysis based on the BLAST algorithm from NCBI revealed that the nucleotide sequence of the *lon* gene from *Z. mobilis* TISTR548 shared a high identity with other bacterial *lon* genes: 99.96% with *Z. mobilis* ATCC29191, 94.68% with *Z. mobilis* CP4, 94.64% with *Z. mobilis* ZM4 (Table 2). Furthermore, it also showed 70.11% identity with *Sphingomonas* sp., 65.78% with *E. coli* K12, and 62.89% with *Bacillus subtilis* strain 168. This finding suggests that the gene encoding Lon protease likely has conserved structural and functional features.

Table 2 Homology analysis of *Z. mobilis* Lon protease nucleotide sequences with those in the GenBank database.

Accession no.	Description	Identity (%)
CP003704.1	*Zymomonas mobilis* subsp. *mobilis* ATCC 29191, complete genome	99.96%
CP006818.1	*Zymomonas mobilis* subsp. *mobilis* CP4 (NRRL B-14023), complete genome	94.68%
CP035711.1	*Zymomonas mobilis* subsp. *mobilis* ZM4 (ATCC 31821), complete genome	94.64%

Multiple sequence alignment analysis was performed on the conserved domain sequences of full-length Lon proteases from six protein family members (Figure 1). Domain analysis using NCBI CDD and InterPro (EBI, UK) revealed that the *Z. mobilis* Lon protease, like those in most microorganisms, contains three highly conserved functional domains. The N-terminal domain (NTD) functions as a substrate recognition module, primarily targeting damaged or misfolded proteins that accumulate under stress conditions. This domain identifies specific degradation tags on proteins, facilitating their selection for degradation and helping manage protein damage during stress [19, 20]. The central hexameric AAA+ (A) domain possesses unfolding activity and serves as the ATPase domain. By hydrolyzing ATP, it provides energy for protein unfolding and translocation into the proteolytic core, which is crucial for maintaining protein quality under stress conditions such as heat or oxidative stress [21, 22]. The C-terminal serine protease (P) domain executes the proteolytic function by cleaving substrates into smaller peptides. This degradation of damaged proteins is essential for cellular homeostasis, particularly during stress when misfolded proteins can become toxic [19, 23]. Structural studies using cryo-electron microscopy (cryo-EM) have revealed significant structural conservation between bacterial Lon proteases and their human mitochondrial counterpart, LONP1, suggesting similar operational mechanisms [24].

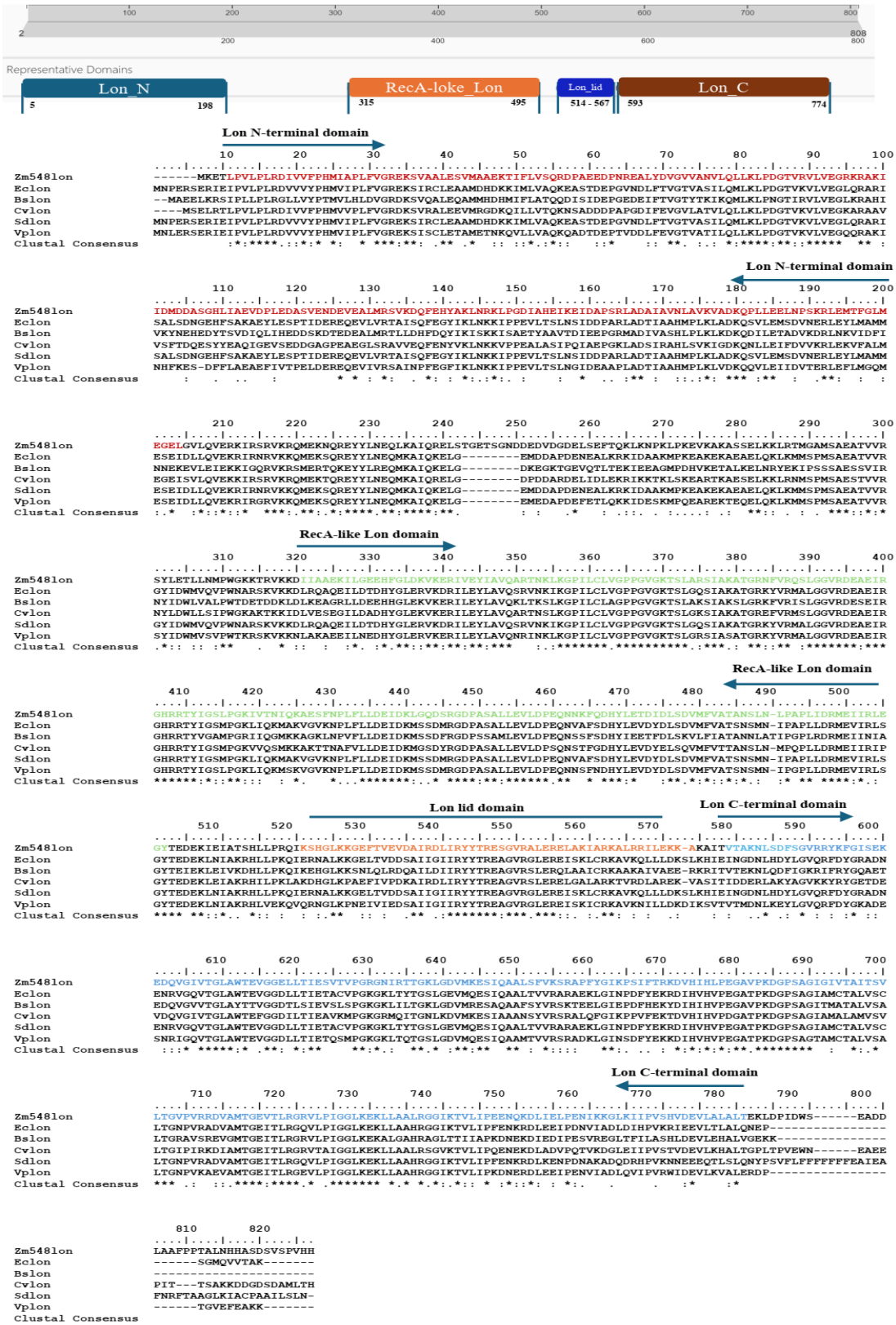

Figure 1 Multiple sequence alignments of the conserved domains sequence of full-length Lon proteases and 6 protein sequences of Lon protease gene family members: Zmlon, Eclon, Bslon, Cvlon, Sdlon, and Vplon. Lon proteases are from *Zymomonas mobilis*, *Escherichia coli* (Ec), *Bacillus subtilis* (Bs), *Caulobacter vibrioides* (Cv), *Shigella dysenteriae* (Sd), *Vibrio parahaemolyticus* (Vp). The alignment was performed using ClustalW in the BioEdit software. The conserved domains were identified in the sequence of the Lon protease protein using NCBI CDD, and InterPro (EBI, UK).

3.2 Construction of expression vector and overexpression of lon gene in Z. mobilis

The *Z. mobilis lon* gene was successfully cloned under the control of the *pdc* promoter in the expression vector pZA22 using the Infusion Snap Assembly Cloning Kit (Takara Bio, USA). Following electroporation transformation into *Z. mobilis*, twenty-one transformants were randomly selected for analysis. Colony PCR using specific primers for the *lon* and *pdc* promoter infusion genes revealed an approximately 3.0 kb DNA band in all selected transformants, confirming the successful integration of these genes into the genome of the *Z. mobilis* TISTR548 wild-type strain. The recombinant strains were then screened for their ability to grow at 40.5°C, a temperature critical for *Z. mobilis* wild-type strain survival. After 24 hours of cultivation, several transformants demonstrated notable growth, with one recombinant strain, designated Zm-pZA22-lon-9, exhibiting superior growth characteristics compared to other strains (Figure 2). This strain maintained genetic stability through repeated cultivation cycles in YPG medium, both with and without chloramphenicol supplementation, and was consequently selected for further experimentation.

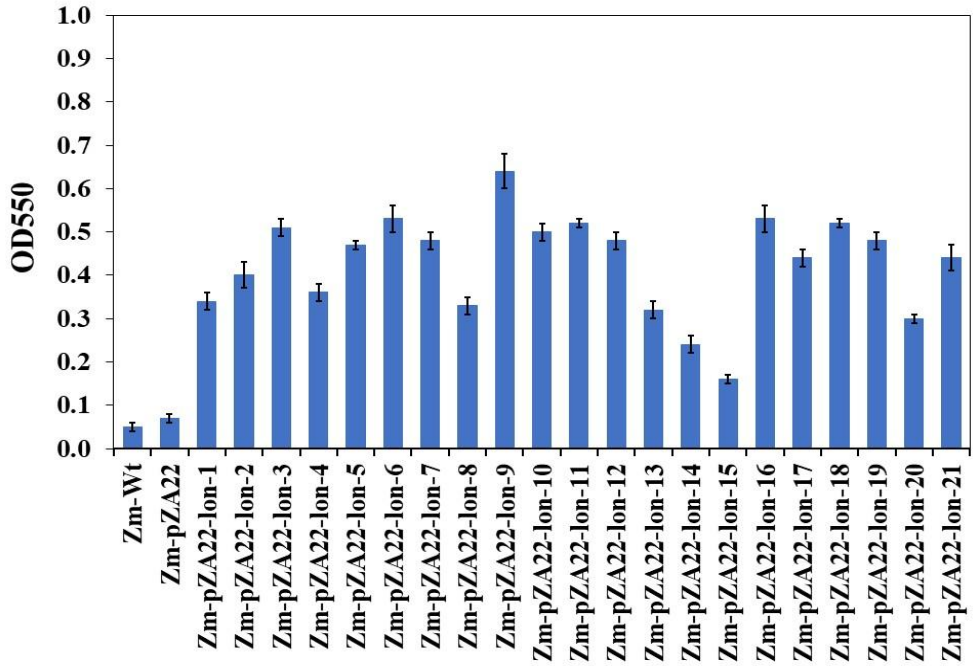

Figure 2 Growth of *Z. mobilis* recombinant strains in YPG medium under thermal stress at 40.5 °C for 24 hours. Zm-Wt, *Z. mobilis* TISTR548 wild-type; Zm-pZA22, *Z. mobilis* transformant strain harboring pZA22 empty plasmid; Zm-pZA-lon, *Z. mobilis* recombinant strain overexpressing *lon* gene; number 1 to 21, isolate number of the recombinant strains.

3.3 Effect of heat stress on growth and cell morphology of recombinant Z. mobilis

Temperature elevation during ethanol fermentation, caused by microbial metabolic activity, adversely affects cell growth, development, and fermentation efficiency. This thermal stress can disrupt cellular homeostasis and denaturation of several macromolecules, such as proteins and nucleic acids, leading to reduced metabolic activity and eventual cell death [13, 25, 26]. The Lon protease has been identified as a crucial factor in cellular stress response, helping cells maintain homeostasis by degrading damaged or misfolded proteins that accumulate under various stress conditions, including heat and salt stress [27, 28]. Studies in *Bacillus subtilis* have demonstrated upregulation of *lon* protease expression under heat shock conditions [29].

Given the limited understanding of Lon protease's biological role in *Z. mobilis* under heat stress, this study investigated the effects of elevated temperature (40.5°C) on growth and cell morphology in a *Z. mobilis* recombinant strain overexpressing the *lon* gene. At the optimal growth temperature of 30 °C, no significant growth differences were observed among the *Z. mobilis* wild-type (Zm-Wt), transformant strain harboring pZA22 empty plasmid (Zm-pZA22), and the *lon*-overexpressing recombinant strain (Zm-pZA22-lon-9) (Figure 3A). However, at 40.5°C, while all strains showed reduced growth, the Zm-pZA22-lon-9 strain maintained significantly higher growth rates compared to Zm-Wt and Zm-pZA22 strains, which showed negligible growth (Figure 3B). This enhanced thermotolerance in the recombinant strain aligns with previous research findings. For example, Barkad et al. [30] reported improved thermotolerance at 40°C and 45°C in recombinant *B. thuringiensis* serovar *israelensis* (Bti) strains overexpressing the *lon* gene, further supporting the gene's crucial role in bacterial thermotolerance.

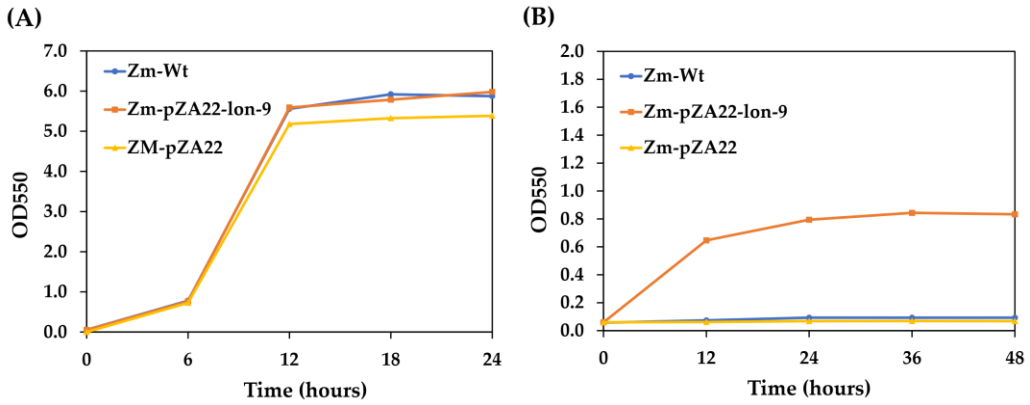

Figure 3 Growth of *Z. mobilis* at 30 °C (A) and 40.5 °C (B) after cultivation in YPG medium. Zm-Wt, *Z. mobilis* TISTR548 wild-type; Zm-pZA22, *Z. mobilis* transformant strain harboring pZA22 empty plasmid; Zm-pZA-lon-9, *Z. mobilis* recombinant strain overexpressing *lon* gene.

Cell morphology under heat stress conditions was examined for all bacterial strains (Figure 4). All strains maintained a characteristic rod-shaped morphology at the optimal growth temperature of 30 °C (Figure 4A). However, when exposed to 40.5 °C, distinct morphological differences emerged: the *Z. mobilis* wild-type and the strain harboring the empty pZA22 plasmid (Zm-pZA22) developed elongated cells, while the *lon*-overexpressing recombinant strain (Zm-pZA22-lon-9) maintained shorter, rod-shaped cells (Figure 4B). The observation of elongated cells under heat stress aligns with previous studies [25, 31], where this morphological change was attributed to heat-induced effects on bacterial DNA molecules. According to Charoensuk et al. [31] and Hayashi et al. [32], heat stress inhibits cell division processes, resulting in cell elongation. The maintenance of normal cell morphology in the *lon*-overexpressing recombinant strain under heat stress suggests that Lon protease may play a role in stabilizing proteins associated with cell division mechanisms.

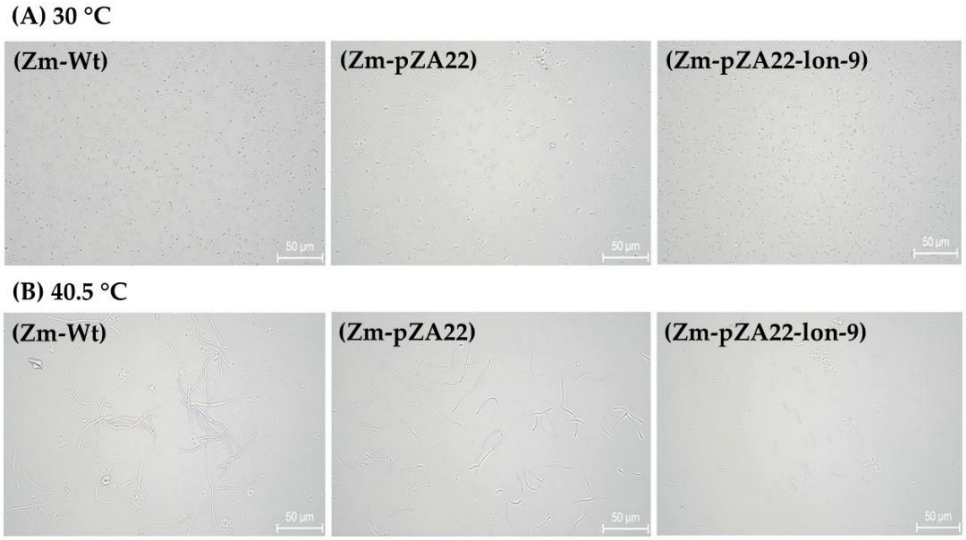

Figure 4 Morphology of *Z. mobilis* cells at 30°C (A) and 40.5°C (B) after cultivation in YPG medium for 18 hours. The images were taken at 100× magnitudes under the microscope. Zm-Wt, *Z. mobilis* TISTR548 wild-type; Zm-pZA22, *Z. mobilis* transformant strain harboring pZA22 empty plasmid; Zm-pZA-lon-9, *Z. mobilis* recombinant strain overexpressing *lon* gene.

3.4 Effect of ethanol stress on cell morphology and growth of recombinant Z. mobilis

Ethanol, similar to heat stress, is a significant environmental factor that poses multiple challenges to bacterial cells. High ethanol concentrations can severely impact cellular functions through various mechanisms, including the modification of plasma membrane fluidity [33] and disruption of cellular ionic homeostasis. Additionally,

ethanol exposure can lead to the denaturation of essential macromolecules, including proteins, DNA, and RNA, ultimately compromising cell viability and fermentation efficiency [26].

Although various stress-responsive proteins have been identified in cellular ethanol stress responses, the specific role of Lon protease in *Z. mobilis* under ethanol stress conditions has remained largely unexplored. Our study reveals that the *Z. mobilis* recombinant strain overexpressing the *lon* gene demonstrates significantly enhanced growth compared to both the wild-type strain and the transformant harboring the pZA22 empty plasmid when exposed to 10% (v/v) ethanol (Figure 5). Moreover, our morphological analysis shows that ethanol toxicity induces bacterial cell elongation, with the wild-type and pZA22 empty plasmid transformant exhibiting notably longer cells compared to the Zm-pZA22-lon-9 recombinant strain under 10% (v/v) ethanol stress (Figure 6). These observations suggest that high ethanol concentrations negatively impact bacterial cell division, similar to the effects observed under heat stress. The overexpression of the *Z. mobilis lon* gene appears to mitigate these negative effects, possibly by stabilizing proteins involved in cell division mechanisms.

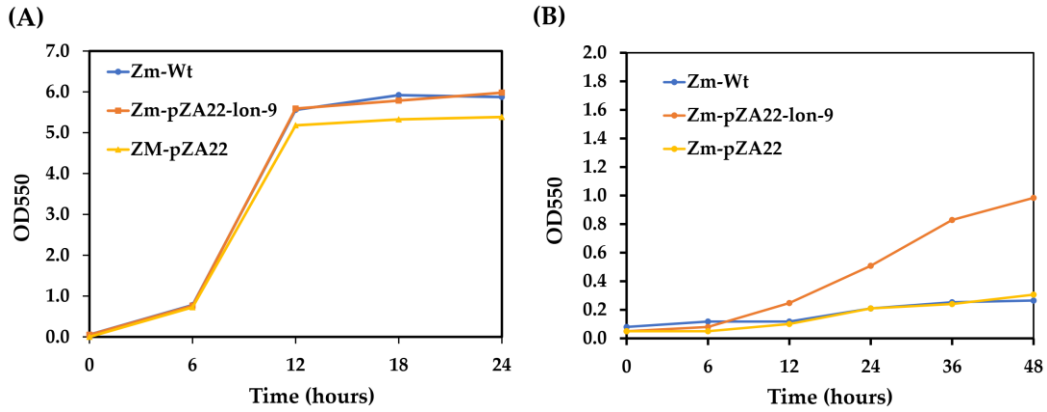

Figure 5 Growth of *Z. mobilis* in YPG medium supplemented with 0% (A) and 10% (v/v) ethanol (B) at 30°C. Zm-Wt, *Z. mobilis* TISTR548 wild-type; Zm-pZA22, *Z. mobilis* transformant strain harboring pZA22 empty plasmid; Zm-pZA-lon-9, *Z. mobilis* recombinant strain overexpressing *lon* gene.

The dual protective role of Lon protease against both heat and ethanol stress holds particular significance for industrial applications, as these stresses frequently occur simultaneously during fermentation processes. The Lon protease likely maintains cellular protein quality control and homeostasis by degrading damaged or denatured proteins that accumulate under ethanol stress. Given its role in bioethanol production, this protective mechanism is especially crucial in *Z. mobilis*, where cells must maintain functionality under progressively increasing ethanol concentrations.

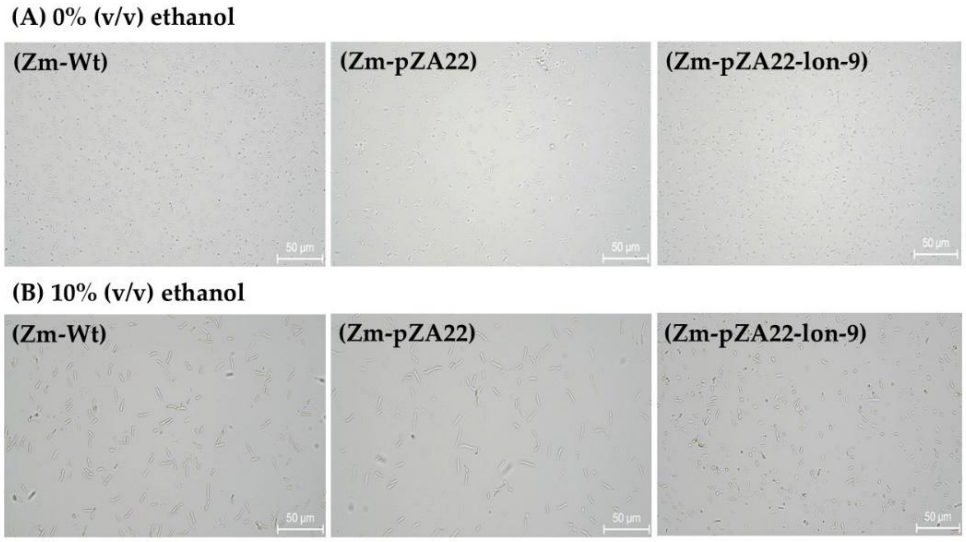

Figure 6 Morphology of *Z. mobilis* cells under ethanol stress at 0% (A) and 10% (v/v) (B) after cultivation in YPG medium at 30 °C for 18 hours. The images were taken at 100× magnitudes under the microscope. Zm-Wt, *Z. mobilis* TISTR548 wild-type; Zm-pZA22, *Z. mobilis* transformant strain harboring pZA22 empty plasmid; Zm-pZA-lon-9, *Z. mobilis* recombinant strain overexpressing *lon* gene.

These findings substantially advance our understanding of cellular stress response mechanisms and suggest promising strategies for developing more robust industrial strains with enhanced multiple stress tolerance. Future research focusing on the specific molecular pathways and protein targets of Lon protease under heat and ethanol stress conditions could provide valuable insights for strain improvement strategies. Additionally, investigating the potential synergistic effects of heat and ethanol stress on Lon protease activity could further optimize *Z. mobilis* strains for industrial bioethanol production.

4. Conclusion

The Lon protease gene from *Z. mobilis* was successfully cloned and expressed. The gene is 2,427 bp long and encodes a protein of 809 amino acids. Sequence analysis revealed that the Lon protease from *Z. mobilis* shares a high sequence identity with Lon proteases from other organisms in the GenBank database. Interestingly, overexpression of the *lon* gene enhanced the growth of *Z. mobilis* under heat and ethanol stress conditions. Furthermore, the overexpression of this gene also helped maintain the regular cell morphology of the recombinant strain, compared to the wild-type strain and the strain harboring the empty plasmid. This suggests that the Lon protease may play a role in cell division mechanisms in bacterial cells. Overall, the results presented in this study provide valuable insights into the function and importance of the Lon protease in *Z. mobilis*, which can inform future strain engineering and optimization efforts for this industrially relevant microorganism.

5. Acknowledgements

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6. Conflicts of interest

The authors declare no conflicts of interest.

7. References

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