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Contributed Paper

Isolation of a High Potential Thermotolerant Strain of *Zymomonas mobilis* for Ethanol Production at High Temperature Using Ethyl Methane Sulfonate Mutagenesis

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ABSTRACT

The wild-type strain *Zymomonas mobilis* TISTR548 has a critical temperature for its growth and ethanol production at 38 °C. To obtain a high potential thermotolerant strain of this ethanologenic bacterium, this wild-type strain was subjected to chemical mutagenesis using ethyl methane sulfonate (EMS). After EMS treatment, the mutagenized cells were screened at temperatures ranging from 38 to 40 °C. The results showed that only six mutant strains, designated ZM EMS-52, ZM EMS-121, ZM EMS-143, ZM EMS-229, ZM EMS-263 and ZM EMS-297, could grow and produce a relatively high level of ethanol at 40 °C. Among these mutants, ZM EMS-229 displayed higher growth and ethanol production capacity than did the wild-type and other mutants at 37 and 40 °C. This mutant strain also showed greater resistance to high concentrations of ethanol, acetic acid and H_2O_2 at high temperature than did the wild-type. These findings suggested that the ZM EMS-229 was a good candidate for high-temperature ethanol fermentation.

Keywords: ethanol production, ethyl methane sulfonate (EMS), mutagenesis, thermotolerant microorganism, *Zymomonas mobilis*

1. INTRODUCTION

Z. mobilis, a Gram-negative ethanologenic bacterium, has been of considerable interest in recent years for high-temperature ethanol production [1-2], since it provides a high theoretical yield of ethanol from glucose and fructose (approximately 97% at 30 °C).

It is an osmo- and ethanol-tolerant bacterium that has shown a high specific rate of glucose uptake, a high ethanol fermentation rate, a relatively high ethanol tolerance, and a low biomass production [3]. This organism synthesizes ethanol via the Entner-Doudoroff (ED) pathway [4]. Its specific ethanol productivity is approximately 2.5-fold higher than that of *Saccharomyces* [5]. *Z. mobilis* generates a highly efficient glucose metabolic flux towards the product with low bacterial growth, converting 1 mol of hexose into 2 mol of ethanol and releasing 1 mol of ATP [6]. Similar to *Saccharomyces* and other ethanologenic organisms, the wild type *Z. mobilis* strains show a significant decrease in cell growth, cell viability and ethanol fermentation ability when the fermentation temperature is elevated from the optimum level (30 °C) [7-8]. Therefore, efforts have been made to overcome these types of problems, such as the supplementation of magnesium ions into the fermentation medium [7], and the use of chemical and physical mutagenesis, genetic and metabolic engineering and evolutionary adaptation to develop new strains of *Z. mobilis* that can grow and produce high levels of ethanol at high temperatures [9-14].

EMS, an alkylating agent, can induce point mutagenesis by both A-T to G-C and G-C to A-T transition mutations [15-17]. It has been used widely to generate mutant strains with some specific purposes, such as increasing glucose oxidase activity and citric acid production in *Aspergillus niger* [18-19], antibacterial activity in *A. oryzae* [20], antifungal activity and ethanol fermentation efficiency in *S. cerevisiae* [21-22], enhancing α-amylase production in *Bacillus licheniformis* [23], and producing extracellular polymeric substances (EPS) in *Halomonas xianhensi* [24]. Although there are many research studies on the application of EMS to induce mutation in several microorganisms, to the best of our knowledge, no study has been conducted using this potent mutagen to isolate and select thermotolerant strains of *Z. mobilis* to produce ethanol at high temperatures. In this study, thermotolerant strains of *Z. mobilis* were generated by chemical mutagenesis using EMS and their growth, ethanol production and stress resistance capacity under high temperature conditions were evaluated.

2. MATERIALS AND METHODS

2.1 Microorganism and Growth Conditions

Z. mobilis strain TISTR 548 was used as the type strain for EMS mutagenesis in this study. This strain was obtained from the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok. The wild-type and mutant strains of *Z. mobilis* were cultured at 30 °C on yeast extract-peptone-glucose (YPG) agar medium (3.0 g/L yeast extract, 5.0 g/L peptone, 30.0 g/L glucose and 15.0 g/L agar) and maintained at 4 °C prior to use.

2.2 Ethyl Methane Sulfonate (EMS) Treatment

EMS mutagenesis was performed as described by Mobini-Dehkordi et al. [22] with some modifications. Briefly, a single colony of *Z. mobilis* TISTR 548 was cultivated in 3 mL of YPG medium, incubated at 30 °C 100 rpm for 12 h, and transferred to fresh YPG medium at an initial optical density (OD) at 550 nm of 0.05. When the OD_{550} of the bacterial cells reached 0.7 - 0.8, the cells were collected using centrifugation at 5,000 rpm for 10 min, washed twice with sterile distilled water, and resuspended in 480 µL of sterile distilled water. Fifteen microliter of EMS (Sigma-Aldrich, USA) (40 mg/mL) were added to the cell suspension and

incubated at 30 °C 100 rpm for 20 min. The mutagenesis was stopped by the addition of 5% sterile sodium thiosulfate solution. An incubation temperature of 38 °C was used to isolate and screen for thermotolerant strains of *Z. mobilis* TISTR548, since this wild-type strain has a critical temperature for cell growth and ethanol production at this temperature.The EMS-treated cells were subjected to serial dilution, and an aliquot of cell suspension was inoculated onto a YPG agar plate and incubated at 38 °C to estimate cell viability.

2.3 Isolation and Screening of Mutant Strains

The mutant colonies that appeared on the YPG agar plates at 38 °C were randomly picked based on their morphological variation and maintained on YPG agar medium. These mutant colonies were repeatedly sub-cultured onto the YPG medium and incubated at a temperature ranging from 38 to 40 °C to confirm their stability. To isolate the thermotolerant strains resistant to high temperatures, the selected EMS mutagenized cells grown in YPG medium at 30 °C 100 rpm for 12 h were transferred to fresh YPG medium at an initial OD_{550} of 0.05 and statically incubated at 38, 39 and 40 °C for 24 h. The optical density at 550 nm of the bacterial cells was monitored using a spectrophotometer (Shimadzu, Japan).

2.4 Ethanol Fermentation Ability Test

The selected mutant strains were tested for their ability to produce ethanol. Each of the mutants was grown in the YPG medium at 30 °C 100 rpm for 12 h and transferred to a fresh YPG medium at an initial OD_{550} of 0.05. When the OD_{550} of the bacterial cells reached 0.7 - 0.8, 10% (v/v) of the inoculum was transferred to YPG medium containing 100 g/L of glucose and statically incubated at 30, 37 and 40 °C. During ethanol fermentation, samples were withdrawn periodically, and the ethanol concentration (*P, g/L*) was measured. A mutant strain that produced the highest ethanol concentration at high temperatures was chosen for further characterization.

2.5 Stress Resistance Analysis of the Selected Mutant

The selected mutant strain was tested for its resistance to various stress conditions, including ethanol, acetic acid and H_2O_2 stress. The experiment for ethanol stress was performed as described by Sootsuwan et al. [8]. Both wild-type and mutant cells were grown in YPG medium at 30 °C 100 rpm for 12 h and transferred to fresh YPG medium at an initial OD_{550} of 0.05. For ethanol stress, the cells were inoculated into YPG medium containing ethanol at a final concentration of 0 (control), 7, 10 and 13% (v/v) and statically incubated at 30 and 37 °C for 24 h. The method described by Liu et al. [14] was used to assess the acetic acid resistance ability of the wild-type and mutant strains. Cells of the wild-type and mutant grown in YPG medium at 30 °C 100 rpm for 12 h were transferred to fresh YPG medium containing acetic acid (Sigma-Aldrich, USA) at a final concentration of 0 (control), 150 and 200 mM and statically incubated at 30 and 37 °C for 24 h. The method described by Charoensuk et al. [25] was employed to measure H_2O_2 stress. The wild-type and mutant cells grown in YPG medium at 30 °C 100 rpm for 12 h were transferred to fresh YPG medium supplemented with 100 mM H_2O_2 (Wako, Japan) at a final concentration of 0 (control), 0.1, 0.2, 0.3 and 0.4 mM and statically incubated at 30 and 37 °C for 24 h. The optical density at 550 nm of the bacterial

cells grown under various stress conditions was monitored using a spectrophotometer (Shimadzu, Japan).

2.6 Cell Morphology Analysis

The wild-type and mutant strains of *Z. mobilis* were grown in YPG medium at 30 °C 100 rpm for 12 h and transferred to fresh YPG medium at an initial OD_{550} of 0.05. After incubation at 30 (control) and 37 °C for 18 h, the cells were harvested using centrifugation at 5,000 rpm for 5 min, washed twice, and resuspended in 100 µL of 0.85% saline solution. An aliquot of the cell suspension was placed on a glass slide, and the morphology of the bacterial cells was observed using a microscope (Nikon E600, Japan). To determine the cell size, 5 µL of the cell suspension was placed on a glass slide, and the bacterial cell size was measured under a microscope using PhotoRuler.

2.7 Analytical Methods

Growth of the bacterial cells was determined by measuring the OD at 550 nm using a spectrophotometer (Shimadzu, Japan). The concentration of glucose in the fermentation medium was measured using a high-performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) as described by Sanda et al. [26]. The ethanol concentration (*P, g/L*) was determined using gas chromatography (Shimadzu GC-14B, Kyoto, Japan) as described by Nuanpeng et al. [27]. All the experiments were independently performed twice, each with three replicates, and the results were expressed as the mean values ± SD. The Duncan's Multiple Range Test (DMRT) ($p = 0.05$) using the SPSS program for Windows was used to compare the mean from each treatment.

3. RESULTS AND DISCUSSION

3.1 Isolation of Mutants after EMS Treatment

The wild-type *Z. mobilis* TISTR548 is known as one of the highest potential ethanol producing bacteria [8]. However, its growth and ethanol production ability is restricted at a temperature of 38 °C. To obtain the thermotolerant strains that can grow and produce ethanol at a temperature higher than 38 °C, chemical mutagenesis using EMS was conducted. A total of 390 mutants were obtained from EMS-treated cells grown on YPG agar medium incubated at 38 °C. Based on their morphological variation, 299 mutants were randomly selected and their stability to grow at high temperatures was tested by repeated cultivation at a temperature range of 38 to 40 °C. Six mutant strains designated ZM EMS-52, ZM EMS-121, ZM EMS-143, ZM EMS-229, ZM EMS-263 and ZM EMS-297 were able to grow at 40 °C and exhibited genetically stability for over 20 generations. Therefore, these six mutants were chosen for further study.

The growth ability of the selected mutants at high temperatures was determined, and the results are summarized in Figure 1. The growth of all the mutant strains was higher than the wild-type at all the temperatures tested. When the temperature was increased from 38 to 39 or 40 °C, the growth of all the mutants, except ZM EMS-52, tended to decrease. Interestingly, at 38 and 39 °C, ZM EMS-121, ZM EMS-229 and ZM EMS-297 displayed higher growth than the other mutants. At 40 °C, the growth of the mutants was insignificant. However, a low growth of the wild-type was detected at 39 and 40 °C, similar to that reported by Thanonkeo et al. [7], Sootsuwan et al. [8] and Sreekumar and Basappa [9]. It has been previously reported that high-temperature or heat

stress has a negative impact on various cellular aspects, such as modifying plasma membrane fluidity, disrupting the cellular ionic homeostasis, and inhibiting the synthesis of membrane phospholipids, genetic materials (DNA and RNA) and proteins, which therefore leads to a reduction in cell growth, cell division, cell viability, and eventually leads to cell death [7-9, 28]. The molecular mechanisms related to high-temperature stress are complicated and are involved several thermotolerant genes that are essential for survival at a critical high temperature of microorganisms. In *Z. mobilis* TISTR548, nine conserved thermotolerant genes involved in general metabolism, membrane stabilization, transporter, protection and repair of macromolecules (DNA, RNA and proteins), cell division and transcriptional

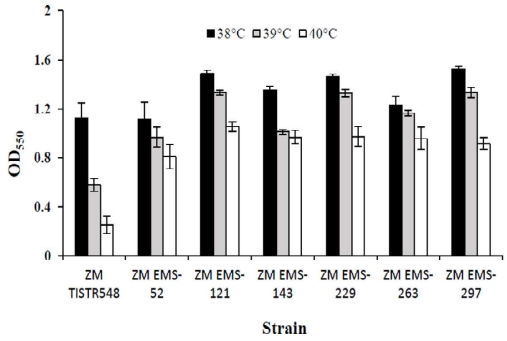

Figure 1. Growth of the wild-type *Z. mobilis* TISTR 548 and selected mutant strains after incubation at various temperatures. Cells were grown in YPG medium and incubated at 38, 39 and 40 °C. Black, gray and white columns represent the optical density of the bacterial cells at 38, 39 and 40 °C, respectively.

for survival of this organism at high temperature [25]. Therefore, further study to clarify the molecular mechanism involved in high-temperature tolerance in mutant strains is required.

3.2 Ethanol Fermentation Ability Test

The ethanol fermentation ability of the wild-type and selected mutants was evaluated at various temperatures using YPG medium containing 100 g/L of glucose as a carbon source, and the results are summarized in Figure 2. The maximum ethanol concentration produced by the wild-type was detected at 30 °C, and its fermentation ability was reduced when the incubation temperature was shifted from 30 °C to 37 and 40 °C. The ethanol production at 30 and 37 °C by each mutant strain was almost insignificant, with the exception of ZM EMS-229 and ZM EMS-297. At 40 °C, the ethanol concentrations produced by the mutants decreased significantly, similar to those observed in the wild-type. One plausible reason is that high-temperature causes the disruption of membrane integrity, resulting in the leakage of the cofactors and coenzymes required for enzyme activity in the ethanol production pathway [29]. The denaturation of cellular proteins at high-temperature has also been reported [30]. As shown in Figure 2, two mutant strains designated ZM EMS-121 and ZM EMS-229 displayed

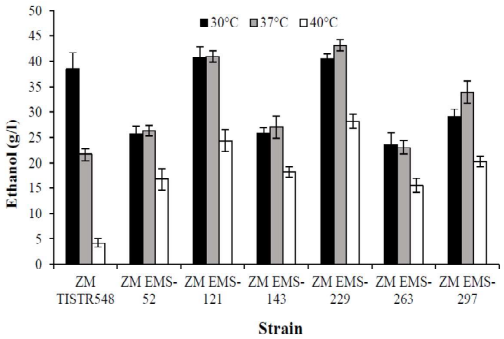

Figure 2. Ethanol production by the wild-type *Z. mobilis* TISTR548 and selected mutant strains at various temperatures using YPG medium containing 100 g/L glucose. Black, gray and white columns represent the ethanol production at 30, 37 and 40 °C, respectively.

greater ethanol fermentation capability than the wild-type and other mutants at all the temperatures tested. Since ZM EMS-229 produced higher ethanol concentration at 37 and 40 °C than ZM EMS-121, it was chosen for further experiments.

3.3 Stress Resistance Analysis of the Selected Mutant

The growth of the wild-type and ZM EMS-229 subjected to various stresses, including ethanol, acetic acid and H_2O_2 stress at 30 and 37 °C, was evaluated, and the results are illustrated in Figure 3 to Figure 5. Ethanol, a chaotropic compound, has been reported to cause a reduction in the growth and viability of microbial cells [7, 31]. In this study, the effect of ethanol stress on the growth of the wild-type and ZM EMS-229 was assessed. As shown in Figure 3, there was no significant different in the growth of the wild-type and ZM EMS-229 at 30 °C when cultured in YPG medium without ethanol supplementation. However, in the medium supplemented with 7, 10 and 13% (v/v) ethanol, ZM EMS-229 exhibited significantly higher growth than the wild-type. No growth of the wild-type was detected in the medium supplemented with 13% (v/v) ethanol. At 37 °C, the growth of the wild-type and ZM EMS-229 in the medium with or without ethanol supplementation was reduced compared with 30 °C. Interestingly, ZM EMS-229 displayed significantly higher growth than the wild-type in all conditions tested at 37 °C. These findings clearly indicated that ZM EMS-229 was more resistant to ethanol stress than the wild-type at both 30 and 37 °C. The present results were in good agreement with Sreekumar and Basappa [9] who demonstrated that the mutant strain of *Z. mobilis* designated ZMI_2 was more tolerance to high concentration of ethanol than the wild-type at 30 and 42 °C.

The ethanol response is complicated and involves many cellular metabolites, genes and proteins related to cellular processes, metabolism and stress responses. The synthesis of several metabolites correlated to lipid metabolism, such as glycerol, palmitic acid, stearic acid and hopanoids, has been reported in the ethanol-stressed *Z. mobilis*. The accumulation of lactate during ethanol stress has been detected in *Z. mobilis*. In addition, the genes involved in energy metabolism and stress response, such as beta-fructofuranosidase and chaperones are up-regulated upon ethanol

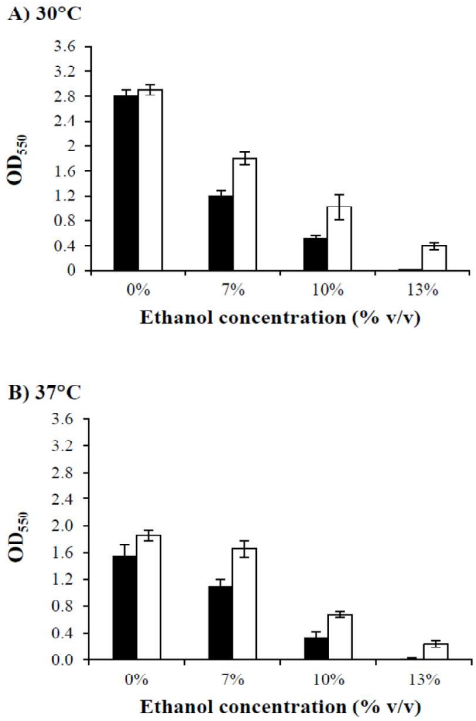

Figure 3. Effect of ethanol stress on the growth of *Z. mobilis* TISTR 548 and ZM EMS-229 in YPG medium at 30 and 37 °C. Cells were grown in YPG medium at 30 °C 100 rpm for 12 h, transferred to fresh YPG medium containing 0, 7, 10 and 13% (v/v) ethanol and statically incubated at 30 and 37 °C. Black and white columns represent the wild-type and ZM EMS-229, respectively.

stress [31]. In *S. cerevisiae*, over-expression of tryptophan permease or tryptophan biosynthetic genes increase the ethanol tolerance in this organism [32]. Based on this information, further investigation to clarify the ethanol response mechanism in ZM EMS-229 is merited.

In addition to furfural, 5-hydroxymethyl furfural and phenolic acid, acetic acid is also found to be one of the predominant inhibitors generated from acetylated hemicellulose during the pretreatment and hydrolysis of lignocellulosic biomass. It can inhibit microbial growth and fermentation activity during ethanol production using lignocellulosic hydrolysates, particularly at low pH values [33]. The concentration of acetic acid in the hydrolysate varies depending on the type of feedstock and the pretreatment process. Normally, it ranges from 16.7 to 258 mM [34]. In this study, the inhibitory effect of acetic acid at the concentrations of 150 and 200 mM on the growth of the wild-type and ZM EMS-229 was determined, and the results are illustrated in Figure 4. At 30 °C, the growth of the wild-type and ZM EMS-229 in the medium without acetic acid supplementation was insignificant. However, the wild-type showed significantly decreased growth when cultured in the medium supplemented with 150 mM acetic acid, and no growth of the wild-type was observed in the presence of 200 mM acetic acid. At high temperature (37 °C), acetic acid was more toxic to the *Z. mobilis* cells especially the wild-type strain. The growth of the wild-type was very low and completely inhibited in the medium supplemented with acetic acid at 150 and 200 mM, respectively. These results were consistent with those reported in *Z. mobilis* ZM481 (ATCC 31823) [14]. An experiment performed by Yang et al. [35] demonstrated that the hfq regulator contributed to acetate tolerance in *Z. mobilis*.

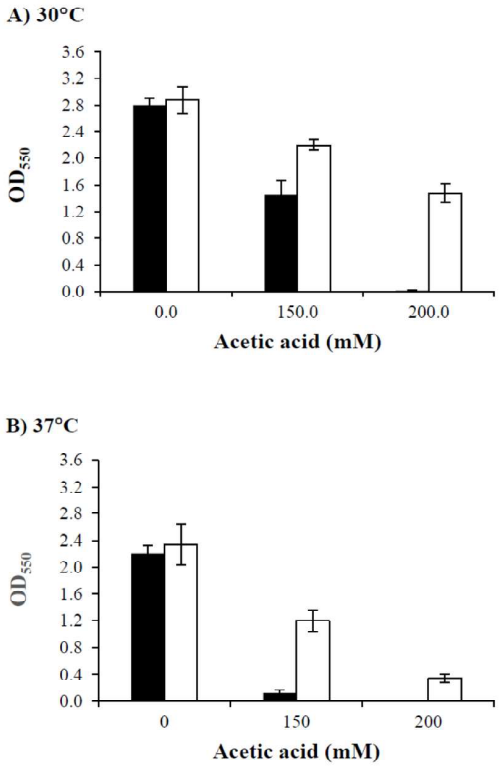

Figure 4. Effect of acetic acid stress on the growth of *Z. mobilis* TISTR 548 and ZM EMS-229 in YPG medium at 30 and 37 °C. Cells were grown in YPG medium at 30 °C 100 rpm for 12 h, transferred to fresh YPG medium containing 0, 150 and 200 mM acetic acid and statically incubated at 30 and 37 °C. Black and white columns represent the wild-type and ZM EMS-229, respectively.

Recently, Liu et al. [14] proposed that the putative terminator sequences of ZMO0117 encoding hydroxylamine reductase were involved in acetate tolerance. In *S. cerevisiae*, an acetic acid-responsive transcriptional activator, *HAA1*, correlated with the adaptation of organism to weak acid stress [36]. To elucidate the molecular mechanism involved in acetic acid tolerance in ZM EMS-229, further studies are merited.

The inhibitory effect of H_2O_2 on the growth of the wild-type and ZM EMS-229 at 30 and 37 °C at the concentrations of

0.1, 0.2, 0.3 and 0.4 mM was evaluated, and the results are shown in Figure 5. There were no significant differences in the growth of the wild-type and ZM EMS-229 at 30 and 37 °C when cultured in the medium containing 0, 0.1 and 0.2 mM of H_2O_2. When the H_2O_2 concentration in the medium was increased to 0.3 mM, a pronounced decreased in the growth of the wild-type was observed at both temperatures. ZM EMS-229 displayed significantly higher tolerance to H_2O_2 at 0.3 mM compared with the wild-type. The growth of the wild-type and ZM EMS-229 was almost prohibited in the medium containing 0.4 mM H_2O_2.

Oxidative stress by endogenous reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), can cause DNA mutation, damage biomolecules (such as membrane lipid and cellular proteins), decrease biological activity and consequently hamper cell growth, cell viability and lead to cell death [37-39]. ROS-scavenging enzymes, such as superoxide dismutase (Sod), catalase (Cat) and peroxidase (Prx) play important roles in ROS detoxification in most living organisms. The periplasmic cytochrome *c* peroxidase (CcP) is one of the peroxidase enzymes that contributes to H_2O_2 resistance in *Leptospirillum* sp. [39]. In *Z. mobilis*, several genes responsible for oxidative stress, such as *Zmsod* (encoding superoxide dismutase), *ZmahpC* (encoding alkyl hydroperoxide reductase), *ZmcytC* (encoding cytochrome c peroxidase), *ZMO1573* (encoding iron-dependent peroxidase) and *Zmcat* (encoding catalase), which are highly up-regulated under high temperature have been proposed to be involved in H_2O_2 stress protection [25]. Therefore, additional study to clarify the mechanism contributed to H_2O_2 resistance in ZM EMS-229 is merited.

3.4 Cell Morphology Analysis

The morphology of the wild-type and ZM EMS-229 cells at 30 and 37 °C was determined using a microscope with PhotoRuler, and the results are illustrated in Figure 6. At 30 °C, the wild-type and ZM EMS-229 displayed similar cell morphology. However, the wild-type cells became elongated at 37 °C, while the ZM EMS-229 cells exhibited a short cell length at this temperature. These results were consistent with those reported by Sreekumar and Basappa [9] and Charoensuk et al. [25]. An elongation of the wild-type cells at high temperature may be correlated with oxidative

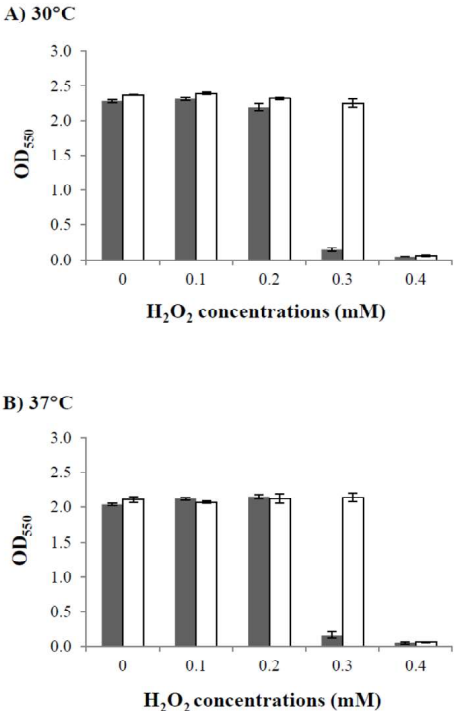

Figure 5. Effect of H_2O_2 stress on the growth of *Z. mobilis* TISTR 548 and ZM EMS-229 in YPG medium at 30 and 37 °C. Cells were grown in YPG medium at 30 °C 100 rpm for 12 h, transferred to fresh YPG medium containing 0, 0.1, 0.2, 0.3 and 0.4 mM H_2O_2 and statically incubated at 30 and 37 °C. Black and white columns represent the wild-type and ZM EMS-229, respectively.

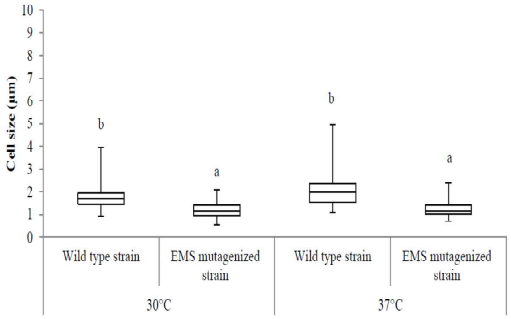

Figure 6. Cell size of the wild-type *Z. mobilis* TISTR 548 and ZM EMS-229 after incubation at 30 and 37 °C. The length of 100 cells per strain was measured using PhotoRuler. The top and the bottom of a bar show the maximum and minimum cell length, respectively. The line in the box indicates the average value of cell size. The values with different letters are significant different at $p < 0.05$ based on DMRT analysis.

stress. Hayashi et al. [40] demonstrated that an oxidative stress causes DNA damage, resulting in the inhibition of cell division, particularly under high temperature condition. Thus, the molecular mechanism to reduce the intracellular oxidative stress may be evolved in the mutant strain, and further investigation is needed to clarify this assumption.

3.5 Ethanol Production at High Temperature by ZM EMS-229

A comparative study on the ethanol production at a high temperature between the wild-type and ZM EMS-229 using YPG medium containing 10% (w/v) of glucose was investigated, and the results are illustrated in Figure 7. At 30 °C, the growth of bacterial cells, glucose utilization and ethanol production between the wild-type and ZM EMS-229 were not significantly different. The maximum ethanol concentrations produced by the wild-type and ZM EMS-229 were 4.25 and 4.12% (w/v), respectively, after 24 h of fermentation. In contrast, ZM EMS-229 exhibited better growth, glucose consumption and ethanol production than the wild-type at 40 °C. The growth and glucose consumption of ZM EMS229 were approximately 1.6 and 1.3-fold higher than the wild-type. The maximum ethanol concentration produced by ZM EMS-229 was 2.86% (w/v), which was approximately 2.8-fold higher than the wild-type. A high ethanol fermentation capacity of the mutant strain at high temperature may be related to the high activity of enzymes involved in the

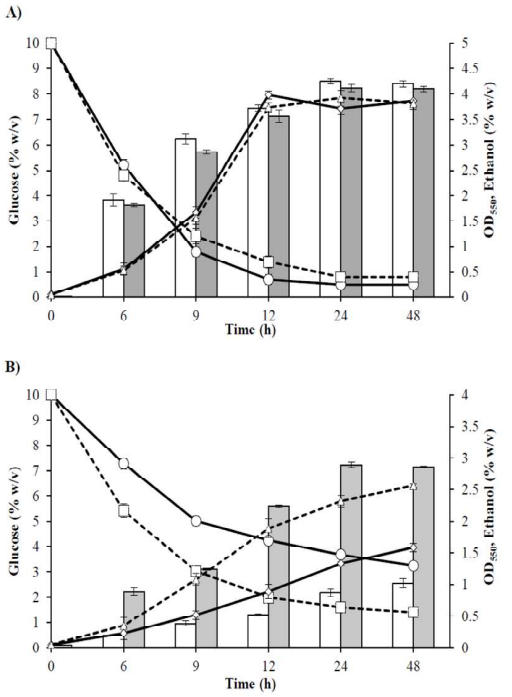

Figure 7. The ethanol production at 30 °C (A) and 40 °C (B) between the wild-type *Z. mobilis* TISTR 548 and ZM EMS-229 using YPG medium containing 10% (w/v) of glucose. Symbols: diamonds, circles and white columns represent the growth, glucose consumption and ethanol production by the wild-type, whereas triangles, squares and gray columns represent the growth, glucose consumption and ethanol production by ZM EMS-229, respectively.

ethanol production pathway. It has been previously reported that the activity of alcohol dehydrogenase in the mutant strain (ZMI_2) is higher than the wild-type (ZM4) at high temperature (42 °C) [9]. Further investigation on the activity of enzyme involved in the ethanol production pathway may be needed to clarify this phenomenon in ZM EMS-229.

4. CONCLUSIONS

The thermotolerant strain of *Z. mobilis* ZM EMS-229 was successfully generated by chemical mutagenesis using EMS. This mutant strain displayed higher growth and ethanol fermentation ability than the wild-type at 37 and 40 °C. The maximum ethanol concentrations produced by the ZM EMS-229 were 43.14 and 28.23 g/L, while those from the wild-type were 21.59 and 4.15 g/L at 37 and 40 °C, respectively. The mutant strain also exhibited greater tolerance to ethanol and acetic acid stress than the wild-type at normal growth conditions (30 °C) and high temperature. In addition, it was also tolerant to high concentrations of H_2O_2 compared with the wild-type. Based on these results, we propose that ZM EMS-229 is a good candidate for high-temperature ethanol production.

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