

Screening Thermo- and Ethanol Tolerant Bacteria for Ethanol Fermentation

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Abstract The thermophilic bacteria receive considerably interest nowadays because of a current challenge of increasing global temperature. Particularly for ethanol production, the thermo-ethanogenic bacteria possess advantages due to lower contamination risk, cost saving in industrial scale, and the wide range of sugars utilization. In this study, 13 bacterial isolates obtained from the previous isolation study were tested for their fermentative capacity and ethanol tolerance at high temperatures. Five bacterial isolates HM2, M2, MC3, MR1 and RD were found to be tolerant up to 12% ethanol. Of which HM2, M2 and MR1 could ferment glucose well at 30, 35 and 40 °C, particularly isolates HM2 and MR1 could perform the fermentative capacity at 45 °C and even 50 °C. In the presence of 12, 16, and 20% w/v glucose, isolates HM2, M2, and MR1 showed the high fermentation rate by giving high gas production; however, the rate slightly decreased in the presence of 24% w/v glucose. The fermentative performance by these three isolates could happen at different pH levels of 4.0, 5.0 and 6.0. The favourable conditions of ethanol fermentation were found at 18.5% glucose, pH 5.0, and 33 °C for isolate HM2 and at 14% glucose, pH 5.5, and 40 °C for isolate MR1. The results of sequencing analysis of partial 16S rRNA gene showed that the gene sequences of the selected isolate HM2 shared 99% similarity with *Bacillus subtilis*.

Keywords: *Bacillus subtilis*, ethanol fermentation, ethanol tolerance, thermophilic bacteria, thermotolerance

1. Introduction

Recently, the natural energy resources (fossil fuel, petroleum and coal) have been estimated to run out over a few years. Most of the energy demands are met by nonrenewable energy sources, resulting in resource depletion, environmental deterioration and public health problems. Therefore, there is a demand to develop novel renewable energy harvesting technologies. Biofuels have gained increased interest in recent years due to environmental and economic reasons. Bioethanol as an alternative to fossil fuels has been extensively studied. Bacterial fermentation enables direct conversion of the cellulosic and hemi-cellulosic components of preferred delignified biomass to chemicals or fuels, without pretreatment to depolymerize the substrates. As a consequence of growth at high temperatures and unique macromolecular properties, obligate thermophilic bacteria can possess high metabolites, physically and chemically stable enzymes, and a higher end product to cell ratio than in metabolically similar mesophilic species. *Zymomonas mobilis*, one of popular thermophilic bacteria, has received research interests for ethanol fermentation at high temperatures [1,2,3]. Thermophilic processes are more stable, rapid, and facilitate reactant activity and product recovery [4,5]. Furthermore, the increase in global temperature in recent years has posed a serious challenge to industrial fermentation processing since abundant energy has to be spent for large cooling systems to

maintain an optimum fermentation temperature. Hence, exploring and application of useful thermophilic ethanogenic microorganisms including bacteria have attracted much interests [6,7,8,9] due to their profits.

2. Materials and Methods

2.1. Cultures and Media

Cultures: 13 bacterial strains were isolated from different kinds of samples collected in Vietnam, including fruits juices, flowers, fermented products, honey, bagasse and molasses and selected from the previous research at Biotechnology Research and Development Institute, Can Tho University, Vietnam (unpubl. data).

Media: MYPG agar, MYPG (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, and glucose 2%) and YPG (yeast extract 0.3%, peptone 0.5%, and glucose 20%).

2.2. Challenge Tests with Different Ethanol-Supplemented Levels

Pure ethanol was added to Durham tube containing 9 mL of YPG medium at levels of 0, 3, 6, 9 and 12% v/v. A volume of 1mL of bacterial suspension was inoculated into each Durham tube for the incubation at 30 °C. The fermentation rate was recorded during the incubation time, by measuring the gas production in Durham test tubes.

2.3. Screening for Growth and Fermentation at High Temperatures

Pre-culture bacteria (on MYPG broth for 24 hours at 30 °C) was prepared for the inoculation of 1mL of bacterial suspension into flasks containing 100mL of sterilized YPG medium. The initial bacterial density before incubation and the growing density during the incubation at 30, 35, 40, 45, and 50 °C were daily measured based on the optical absorbance at 600nm. Fermentation ability at high temperature was also determined by measuring the gas production in Durham test tubes during the incubation at 30, 35, 40, 45, and 50 °C.

2.4. Effects of Glucose Levels on the Fermentation

The fermentation ability of bacteria at different glucose concentrations (12, 16, 20, and 24% w/v) was examined by measuring the gas production in Durham test tubes and the bacterial growing density.

2.5. Effects of pH Levels on the Fermentation

The fermentation ability of bacteria at different pH levels (4.0, 4.5, 5.0, 5.5, and 6.0) was examined by measuring the gas production in Durham test tubes and the bacterial growing density.

2.6. Optimization of Fermentation Conditions

This experiment was set up in a factorial design with 3 factors, at 3 levels and each treatment had triplicates. The selected levels of temperature, glucose concentration, and pH were obtained from the previous experiments. The

fermentation ability of bacteria was determined by measuring the ethanol concentrations, using the analysis kit K-ETOH (Megazyme, Ireland).

2.7. Identification of Selected Target Bacteria

The selected target strain was identified by sequence analysis of partial 16S rRNA gene.

2.8. Statistical Analysis

Experimental data were statistically analyzed using Statgraphics Plus Version 5.0, Manugistics, Inc., Rockville, USA.

3. Results and Discussion

3.1. Tolerance Ability to Ethanol Supplemented in Medium

The ethanol fermentative capacity of all 13 bacterial isolates in glucose solution was screened by Durham test prior to ethanol challenge test. Only 7 isolates (HM2, M1, M2, MC3, MO, MR1, and RD) could perform their fermentative capacity and consequently were selected for further tests. In the challenge test, the fermentative capacity at different ethanol-supplemented levels including 0% v/v ethanol as a control was examined. The results of the gas production in Durham tubes during 6 days of fermentation were presented in [Table 1](#).

Table 1. Gas production (height of CO₂ in Durham tubes) during the fermentation at different ethanol-supplemented levels

Isolate	Ethanol (% v/v)	CO ₂ height (mm) in periods of time (day)					
		1	2	3	4	5	6
HM2	0	9,00	19,67	21,67	23,00	25,33	25,33 ^{bc}
M1		4,33	6,00	7,67	9,00	10,00	10,00 ^d
M2		7,33	17,67	21,33	23,00	24,67	24,67 ^{bc}
MC3		6,67	19,00	24,33	30,00	30,00	30,00 ^a
MO		4,00	6,33	7,33	9,00	10,00	10,00 ^d
MR1		4,00	18,33	25,00	25,67	26,67	26,67 ^b
RD		6,67	18,33	21,00	22,33	23,67	23,67 ^c
HM2	3	3,67	12,33	14,00	16,00	19,33	19,33 ^c
M2		9,33	22,00	26,00	28,67	30,00	30,00 ^a
MC3		5,67	12,33	17,67	19,00	20,00	20,00 ^c
MR1		5,33	19,00	24,67	27,33	30,00	30,00 ^a
RD		7,33	14,67	20,33	21,67	23,00	23,00 ^b
HM2	6	7,67	12,33	14,67	16,33	17,33	17,33 ^d
M2		6,00	13,67	17,00	17,33	18,33	18,33 ^{cd}
MC3		7,33	14,33	17,33	18,33	19,33	19,33 ^c
MR1		6,33	16,67	22,67	23,67	24,67	24,67 ^a
RD		7,33	13,33	17,00	18,33	21,00	21,00 ^b
HM2	9	5,33	12,33	17,00	18,33	19,67	19,67 ^a
M2		4,00	10,67	13,00	14,00	14,67	14,67 ^b
MC3		2,00	8,00	10,33	11,33	12,33	12,33 ^{cd}
MR1		1,33	8,67	10,67	11,67	12,67	12,67 ^c
RD		3,00	8,00	9,67	10,67	11,67	11,67 ^d
HM2	12	3,00	10,00	11,33	11,33	13,00	13,00 ^a
M2		2,00	8,67	11,00	11,67	12,67	12,67 ^a
MC3		2,00	6,67	7,33	7,67	9,00	9,00 ^b
MR1		2,00	9,00	10,33	10,67	12,67	12,67 ^a
RD		2,67	8,33	8,67	9,00	9,00	9,00 ^b

¹Values are means of triplicates; 2means with different subscripts within a column are statistically different at the 95% confidence level.

Five isolates HM2, M2, MC3, MR1 and RD performed the fermentative capacity by producing CO₂ in all treatments of adding ethanol at 3, 6, 9, and 12% ethanol whereas no gas production was found for 2 isolates M1 and MO at all different ethanol-supplemented levels. When exposed into ethanol challenge, HM2, M2, MC3,

MR1 and RD could ferment glucose after 1 day. In the second day, these 5 isolates quickly produced more CO₂ and kept the rate until the third day after fermentation. In most treatments, the gas production was gradually less after 4 days of fermentation and no increase of gas production was found after 6 days of fermentation. The

results showed that HM2, M2, MC3, MR1, and RD could ferment at 12% ethanol-supplemented level. Of which, 3 isolates HM2, M2, and MR1 having the significantly higher gas production compared to that of other 2 isolates MC3 and RD at 95% confidence level.

3.2. Screening for Growth and Fermentation at High Temperatures

Three isolates HM2, M2, and MR1 that were selected from the previous test due to their high ethanol tolerant ability were examined for all further tests. The results of gas production during the fermentation at different temperatures (Table 2) showed that isolates HM2 and MR1 were able to perform the fermentative capacity at temperatures up to 45°C and 50°C. Isolate M2 only gave the gas production at 30°C, 35°C, and 40°C.

Table 2. Gas production (height of CO₂ in Durham tubes) during the fermentation at different temperatures

Isolate	Temperature (°C)	CO ₂ height (mm) in periods of time (day)				
		1	2	3	4	5
HM2	30	24,00	41,33	43,33	45,00	45,00 ^a
M2		7,33	25,33	34,00	36,33	45,00 ^a
MR1		20,33	33,33	37,00	37,33	45,00 ^a
HM2	35	20,33	29,00	31,00	37,00	37,00 ^b
M2		8,33	32,00	39,00	45,00	45,00 ^a
MR1		19,67	26,00	29,00	34,00	34,00 ^c
HM2	40	24,00	31,33	34,33	36,00	36,00 ^b
M2		19,67	34,00	38,67	40,00	40,00 ^a
MR1		18,67	32,33	38,00	40,00	40,00 ^a
HM2	45	8,00	11,67	14,33	14,33	14,33 ^a
M2		0,00	0,00	0,00	0,00	0,00 ^c
MR1		6,33	9,33	10,00	11,00	11,00 ^b
HM2	50	2,00	3,00	3,67	3,67	3,67 ^a
M2		0,00	0,00	0,00	0,00	0,00 ^b
MR1		2,00	3,00	4,00	4,00	4,00 ^a

^aValues are means of triplicates; ²means with different subscripts within a column are statistically different at the 95% confidence level

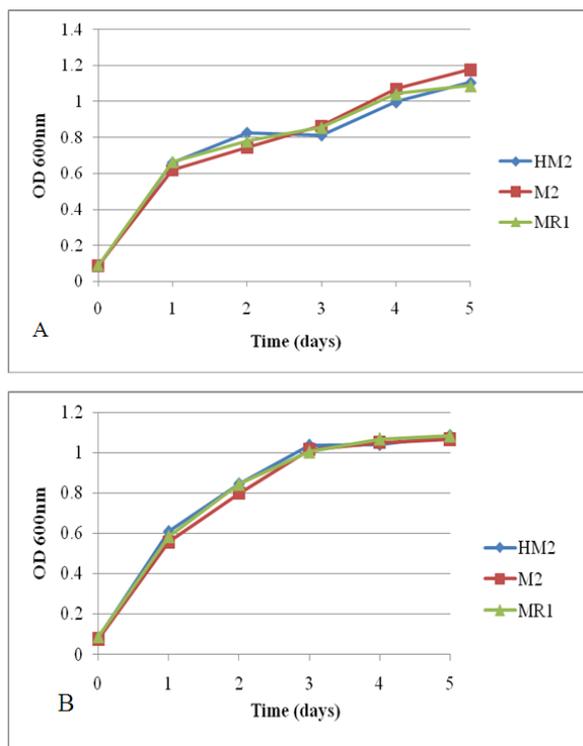


Figure 1. The growth performance of bacteria at 30 °C (A) and 35 °C (B)

For screening the bacterial growth at different temperatures, the results of growing density were described in Figure 1 and Figure 2. The gradually increasing growth density during 5 days of fermentation at 30°C and 35°C was identically found in all cases of 3 selected isolates HM2, M2, and MR1 (Figure 1).

For treatments at higher temperatures tested at 40 °C, 45 °C, and 50 °C the results of growing density varied differently (Figure 2). At 45 °C and 50 °C, isolate M2 gave the significantly lowest growing density compared to those in cases of HM2 and MR1. There was also a good correlation between the fermentative capacity and the growth. Isolates HM2 and MR1 could grow well at 45 °C and 50 °C and also could perform better their fermentative capacity. In other words, similar to the order of fermentation capacity, the less growth of M2 at 45 °C and 50 °C was distinguished.

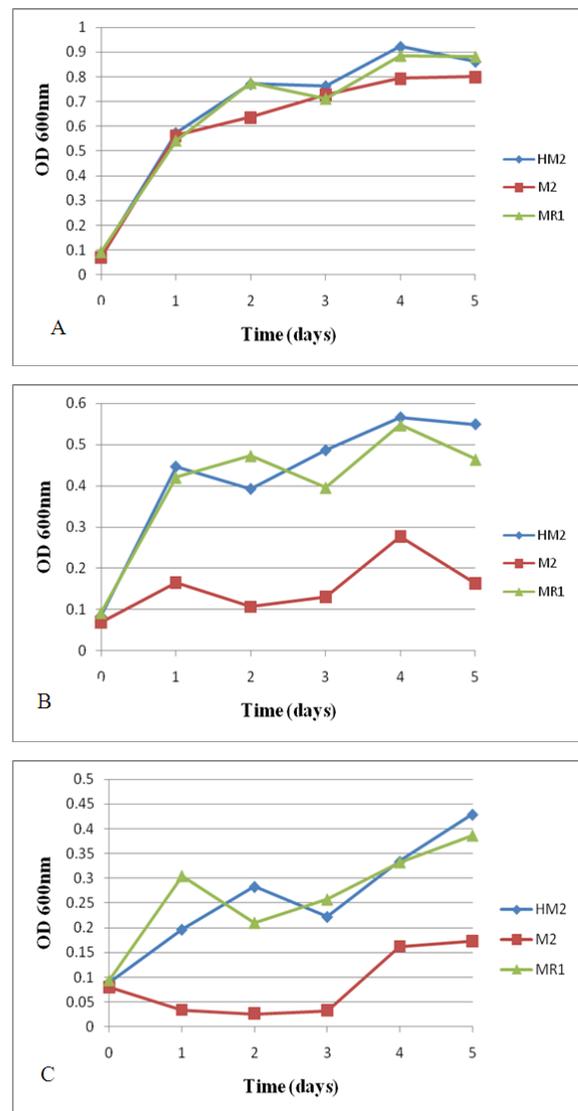


Figure 2. The growth performance of 3 bacterial isolates at 40 °C (A), 45 °C (B), and 50 °C (C)

3.3. Effects of Glucose Levels on the Fermentation

The results showed that all 3 tested isolates could ferment at different levels of glucose (12, 16, 20, and 24% w/v). The results of gas production and the growing

density after 8 days of fermentation were described in Figure 3 and Figure 4, respectively.

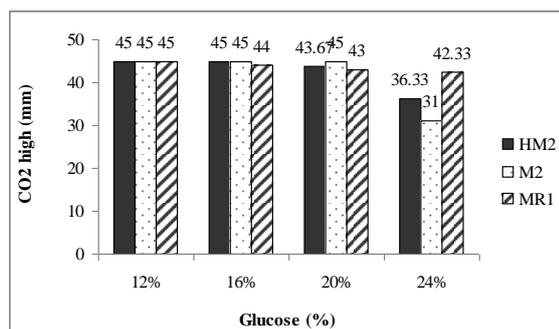


Figure 3. Gas production (height of CO₂ in Durham tubes) after 8 fermentation days at different initial glucose levels

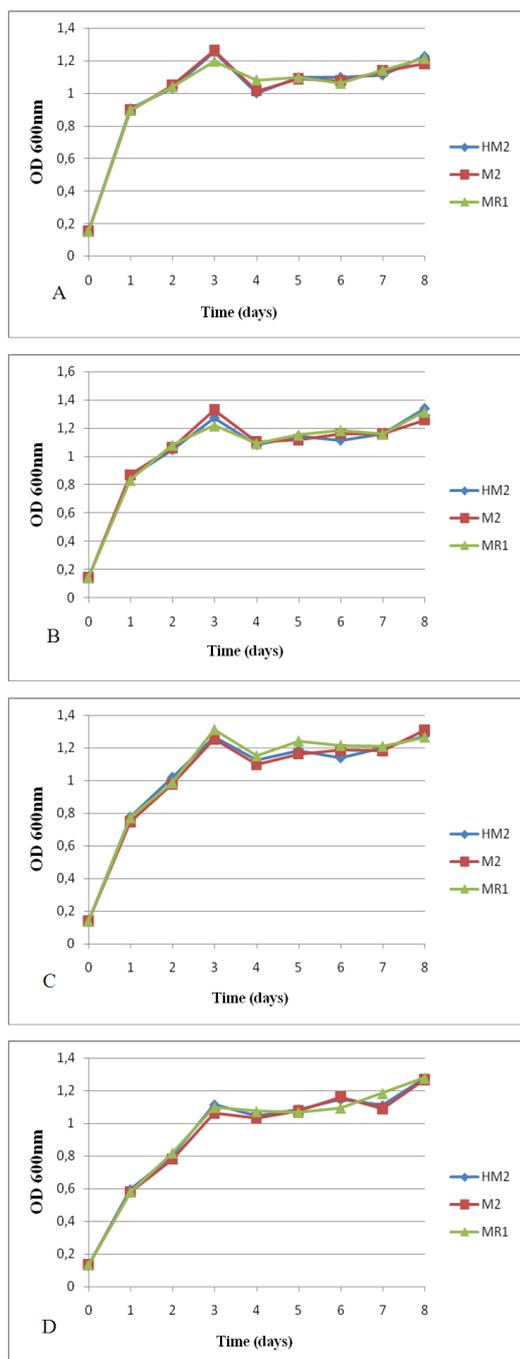


Figure 4. The growth of the bacteria at 12% (A), 16% (B), 20% (C), and 24% w/v (D) glucose during fermentation

In the same principle of growth performance by bacteria at different levels of glucose, the growing density rapidly increased after 1 day and reach the highest after 3 days, then slightly decreased and kept increasing again after 5 days until the last tested day (after 8 days).

3.4. Effects of pH Levels on the Fermentation

The results of gas production and growing density of 3 isolates HM2, M2 and MR1 during the fermentation were presented in pairs in Figure 5, Figure 6 and Figure 7, respectively. All tested isolates could be able to grow and ferment in a wide range of pH levels from 4.0 to 6.0.

To combine with all screening tests on ethanol tolerance, fermentative capacity at different temperatures and effect of pH levels, we found that 3 selected tested isolates HM2, M2, and MR1 could reach the same advantages somehow in comparison with a case of thermo-ethanogenic bacterial strain *Zymomonas mobilis* [1,10].

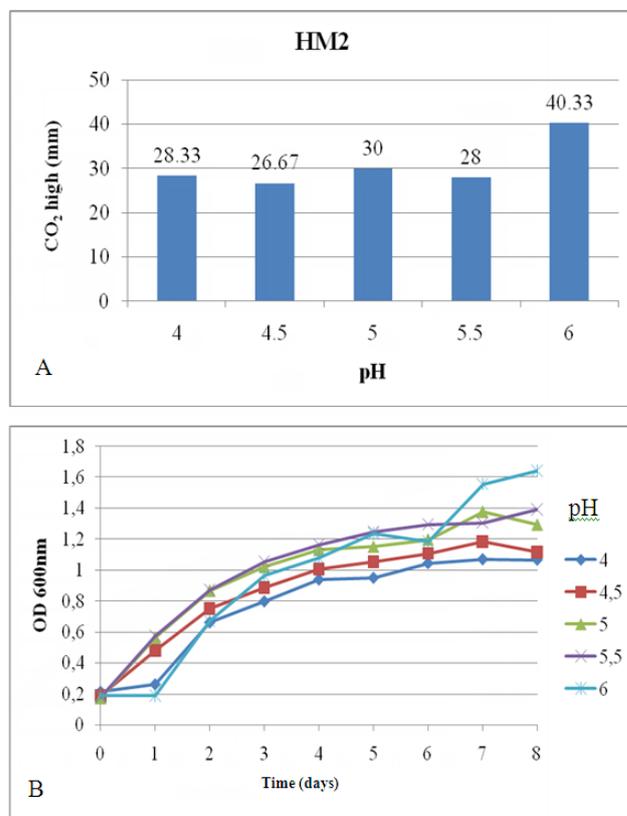


Figure 5. CO₂ produced (A) and the growth (B) of isolate HM2 after 8 days

3.5. Optimization of Fermentation Conditions

Because of the better growth ability and fermentative capacity reported from screening tests, 2 isolates HM2 and MR1 were selected for the study of optimization of factors affecting on the fermentation. The factors with different levels were designed as follows: glucose concentration (12, 16, and 20% w/v), temperature (30°C, 35°C, and 40°C), and pH level (4.0, 5.0, and 6.0). The fermentation ability of bacteria was determined by measuring the ethanol concentrations, using the analysis kit K-ETOH.

The results of glucose concentration effecting on the ethanol fermentation were described in Figure 8.

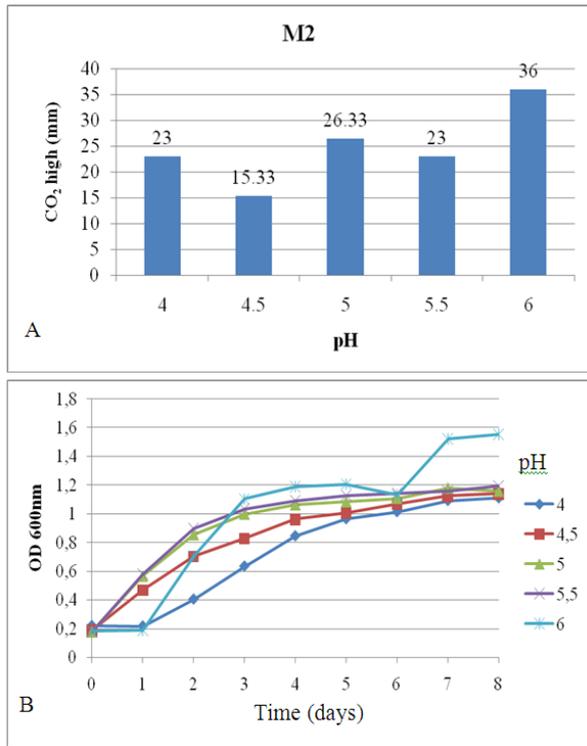


Figure 6. CO₂ produced (A) and the growth (B) of isolate M2 after 8 days

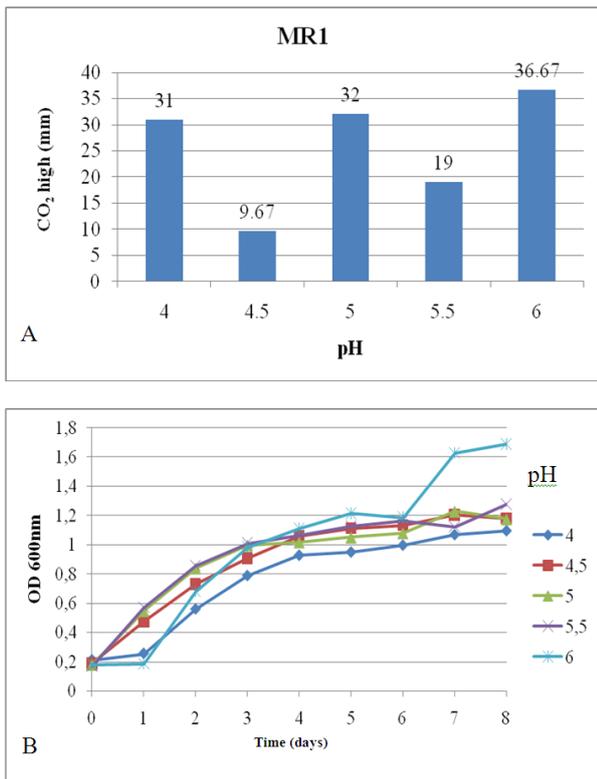
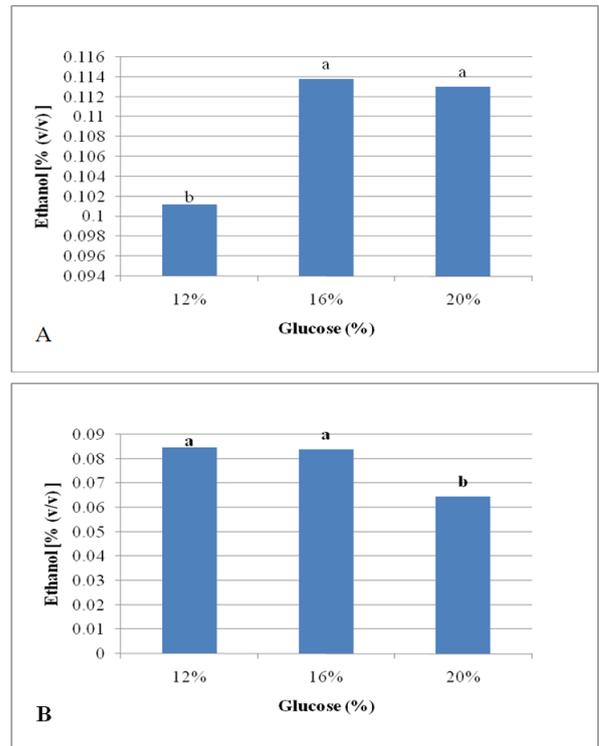


Figure 7. CO₂ produced (A) and the growth (B) of isolate MR1 after 8 days

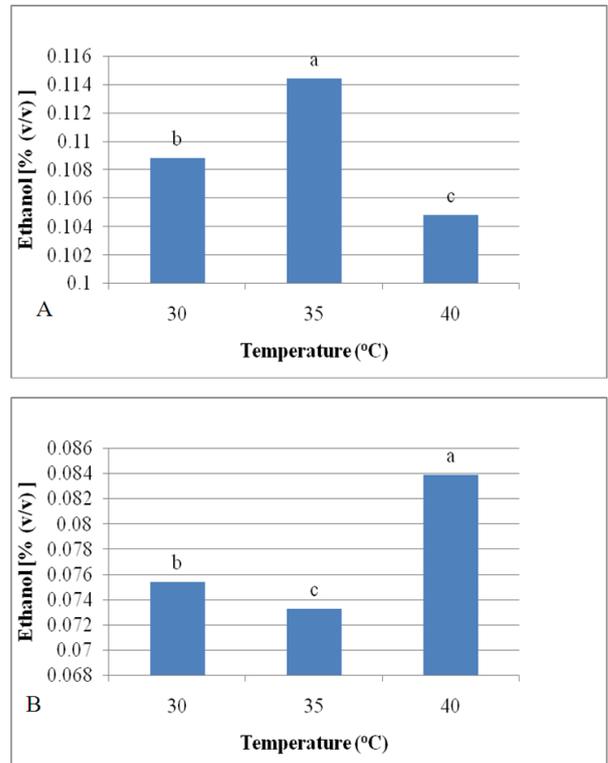
Both HM2 and MR1 gave the significantly highest ethanol concentration in a case at 16% w/v glucose. However, there were differences of the way to consume glucose and produce ethanol between these 2 isolates in cases at 12% w/v and 20% w/v glucose. MR1 could reach the highest ethanol production when fermented at 12% w/v glucose whereas HM2 required a bit more glucose concentration at 16% w/v glucose to reach the highest

ethanol production. When treated with 20% w/v glucose, an inhibition of ethanol produced was found in a case of MR1, but not for HM2. The results also indicated that the ethanol produced in the fermentation by HM2 was higher than ethanol produced by MR1.



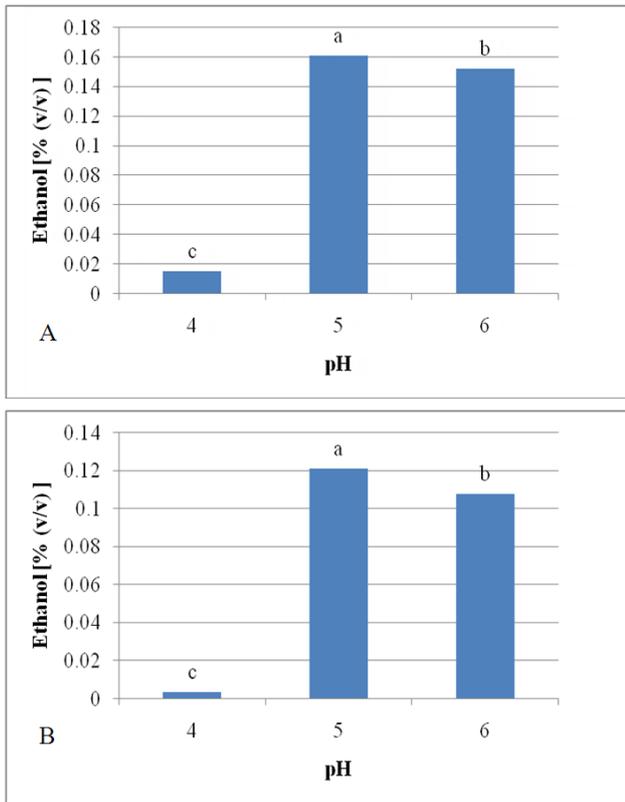
Different subtitles are statistically difference at 95% confidence level

Figure 8. Effect of glucose concentrations on ethanol fermentation by HM2 (A) and by MR1(B)



Different subtitles are statistically difference at 95% confidence level

Figure 9. Effect of temperature levels on ethanol fermentation by HM2 (A) and by MR1(B)



Different subscripts are statistically difference at 95% confidence level
Figure 10. Effect of pH on fermentation of HM2 (A) and MR1 (B)

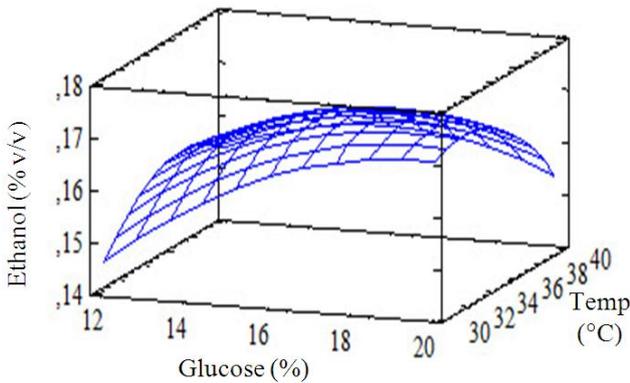


Figure 11. Surface plot of the optimum conditions for ethanol fermentation by HM2 (18.5% glucose, pH 5.0, and 33 °C)

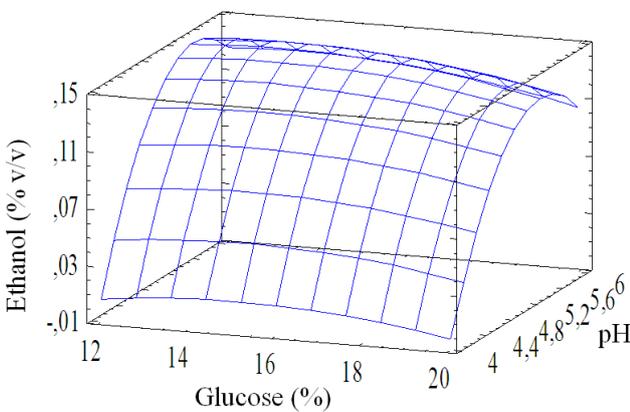


Figure 12. Surface plot of the optimum conditions for ethanol fermentation by MR1 (14% glucose, pH 5.5, and 40 °C)

The results of temperature levels effecting on the ethanol fermentation were described in [Figure 9](#). Both

HM2 and MR1 could still give the performance in the fermentation capacity at 40°C. HM2 gave the significantly highest ethanol production at 35°C whereas MR1 could ferment and produce highest ethanol in the fermentation at 40°C. However, again the ethanol produced in the fermentation by HM2 was higher than ethanol produced by MR1 in all treatments of temperature levels.

The results of pH levels effecting on the ethanol fermentation were described in [Figure 10](#). Both HM2 and MR1 could identically give the same performance of ethanol production at different pH levels as follows: significantly highest ethanol at pH 5.0, slightly less at pH 6 and almost no ethanol produced at pH 4.0.

Based on the results of statistical analysis and optimizing software, the surface plot of the optimum conditions for ethanol fermentation by HM2 and by MR1 were made and shown in [Figure 11](#) and [Figure 12](#), respectively.

3.6. Identification of Selected Target Bacteria

The selected target isolate HM2 was characterized based on the sequence 16S rRNA gene analysis and BLAST on NCBI website (<http://www.ncbi.nlm.nih.gov>). The result showed that the gene sequences of isolate HM2 shared 99% similarity with *Bacillus subtilis*.

4. Conclusions

Five bacterial isolates HM2, M2, MC3, MR1 and RD were be able to tolerate up to 12% ethanol and to show the fermentative capacity at 40 °C. The selected tested isolates HM2, M2, and MR1 could ferment in a range of glucose contents at 12, 16, and 20% w/v, and at pH levels of 4.0, 5.0 and 6.0. The favourable conditions of ethanol fermentation were found at 18.5% glucose, pH 5.0, and 33 °C for isolate HM2 and at 14% glucose, pH 5.5, and 40 °C for isolate MR1. The results of sequencing analysis of partial 16S rRNA gene showed that the gene sequences of the selected isolate HM2 shared 99% similarity with *Bacillus subtilis*.

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References

- [1] Panesar, P.S., Marwaha, S.S. and Kennedy, J.F, "Comparison of ethanol and temperature tolerance of *Zymomonas mobilis* strain in glucose and molasses medium," *Indian Journal of Biotechnology*, 6. 74-77. Jan.2007.
- [2] Zhang, K. and Feng, H, "Fermentation potentials of *Zymomonas mobilis* and its application in ethanol production from low-cost raw sweet potato," *African Journal of Biotechnology*, 9 (37). 6122-6128. Sept.2010.
- [3] Soleimani, S., Ghasemi, M.F. and Shokri, S, "Ethanol production by *Zymomonas mobilis* PTCC 1718 using low cost substrates," *African Journal of Microbiology Research*, 6 (4). 704-712. Jan.2012.
- [4] Sveinsdottir, M., Baldursson, S. and Orlygsson, J, "Ethanol production from monosugars and lignocellulosic biomass by thermophilic bacteria strains from Icelandic hot springs," *Icelandic Agricultural Sciences*, 22. 45-58. May 2009.

- [5] Chang, T. and Yao, S., "Thermophilic, lignocellulolytic bacteria for ethanol production: current state and perspectives," *Applied Microbiology and Biotechnology*, 92 (1). 13-27. Oct.2011.
- [6] Koskinen, E.P.P., Beck, R.S., Örylgsson, J., Puhakka, A.J., "Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas," *Biotechnology and Bioengineering*, 101 (4). 679-690. April 2004.
- [7] Taylor, P.M., Eley, L.K., Martin, S., Tuffin, I.M., Burton, G.S. and Cowan, A. D., "Thermophilic ethanogenesis: future prospects for second-generation bioethanol production," *Trends in Biotechnology*, 27 (7). 398-405. May 2009.
- [8] Yao, S., Mikkelsen, M.J., "Metabolic engineering to improve ethanol production in *Thermoanaerobacter mathranii*," *Applied Microbiology and Biotechnology*, 88 (1). 199-208. Sep.2010.
- [9] Auesukaree, C., Koedrith, P., Saenpayavai, P., Asvarak, T., Benjaphokee, S., Sugiyama, M., Kaneko, Y., Harashima, S., and Boonchird, C., "Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits," *Journal of Bioscience and Bioengineering*, 114 (2). 144-149. August 2012.
- [10] Cazetta, M.L., Celligoi, M.A.P.C., Buzato, J.B. and Scarmino, I.S., "Fermentation of molasses by *Zymomonas mobilis*: Effects of temperature and sugar concentration on ethanol production," *Bioresource Technology*, 98. 2824-2828. April 2007.