Ethanol Production by Thermotolerant Yeast using Sugarcane Molasses as Substrate

Kanlapaphruek Faichanthuek[1,2], Sirinda Yunchalard[2]
[1]Graduate School, Khon Kaen University, Nai-Muang, Muang District, Khon Kaen
[2]Department of Biotechnology, Faculty of Technology, Khon Kaen University, Nai-Muang, Muang District, Khon Kaen

Abstract

In this study, reference strain *Saccharomyces cerevisiae* TISTR 5088 and thermotolerant strains MT-02, MT-08 and MT-11 were grown in sugarcane molasses and YM broth containing 15% (w/v) glucose at temperatures 30, 37, and 40°C in order to test each strain's capacity for ethanol production in those two different substrate mediums. The results indicated that MT-08 proved to be the most efficient ethanol producing strain when compared to the others. When fermented at 30°C for 48 hr. in sugarcane molasses, MT-08 proved to produce ethanol better than the reference strain could under the same conditions. However, when fermented for the same amount of time in the same substrate medium, it was found that MT-08 could not produce ethanol as well as the reference strain could at temperatures 37 and 40°C.

Keywords: thermotolerant yeast / ethanol production / sugarcane molasses

Introduction

Ethanol, so-called bio-ethanol, is one of the organic solvents with the potential of being used as bio-fuels, an alternative energy source to fossil fuels (Basso et al., 2008). It has brought attention as it is a renewable energy, which means that it can be replenished from any appropriate renewable substrate available under solar energy and simple production process thru fermentation using ethanol producing organisms. In order for bio-ethanol to replace fossil fuels such as gasoline, the cost of production must be lowered so that it attracts as a cheaper energy source. There are two main factors in lowering production cost. The first one being the choice of using cheaper renewable resources such as waste that is obtainable from agricultural and/or industrial sectors. The second being the reduction of energy input by reducing cooling costs during fermentation. *Saccharomyces cerevisiae* is a common yeast strain used for industrial ethanol production. For most *S. cerevisiae* strains, the optimal temperature is in the range between 25°C to 33°C (Rosenberger et al., 2002) The use of thermotolerant yeasts presents many advantages in terms of reduction in cooling costs during fermentation and distillation costs, faster fermentation rates, and also helps to decrease the contamination during fermentation (Abdel-Banat et al., 2010). Hence, improvement of the thermotolerance of these yeast strains would be beneficial to obtain a relatively cheaper bio-ethanol apart from using cheaper renewable raw material. In Thailand, three types of raw materials regarded as having high potentials to be used for ethanol production such as cassava, molasses, and sugarcane. Sugarcane molasses is an abundant agro-industrial by-product often used in alcohol distilleries due to the presence of fermentative sugars, being an

optimal carbon source for the microorganism metabolism (Jiménez et al., 2004). The Thai government has a policy to encourage bio-ethanol production from cheaper available waste like molasses from sugar plant, taking advantage of the available supply, simple conversion process as well as existing sugar-based distillery infrastructure (Nguyen & Gheewala, 2008). The aim of this study was to produce ethanol under higher temperature using thermotolerant *S. cerevisiae* strains capable of using sugarcane molasses as substrate for a cheaper ethanol production.

Methodology

Microorganisms and Microbiological Media

S. cerevisiae TISTR 5088 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). This yeast was stored on YM agar [0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 0.5% (w/v) peptone, 1% (w/v) glucose, and 1.5% (w/v) agar] at 4 °C. Thermotolerant microbial strains used in this study were *S. cerevisiae* strains MT-02, MT-08, and MT-11. These thermo tolerant yeast strains were obtained from the stock culture of the Department of Biotechnology, Faculty of Technology, Khon Kaen University. YM broth containing (w/v): 0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose was prepared by adding 100 ml of distilled water prior to sterilization. Two fermentation media used in this study were as follows: the first one was YM broth + 15% (w/v) glucose and the second one was sugarcane molasses broth. The first fermentation medium was prepared by supplementing YM broth with 15% (w/v) glucose and sterilized prior to using as fermentation medium. The second fermentation medium was prepared using sugarcane molasses obtained from MITR PHU VIENG, the Sugar Plant in Khon Kaen province as raw material. Sugarcane molasses was obtained from MITR PHU VEANG Sugar Plant in Khon Kaen province and adjusted to contain 190 g/L of sugars by diluting 146.15 ml of sugarcane molasses with an initial sugar concentration at 650 g/L with 353.85 ml of distilled water prior to using as fermentation medium. The prepared sugarcane molasses was sterilized and used as fermentation broth without addition of any nitrogen source.

Ethanol Production in YM broth containing 15% (w/v) glucose

Each of these yeast strains was grown in 50 ml YM broth at 30°C in an incubator shaker at 150 rpm for 18 hr. Then, 5×10^7 yeast cells were transferred into YM broth containing 15% (w/v) glucose and was incubated at 30, 37, and 40°C for 48 hr. Samples were collected at 48 hr of fermentation for analyses of both total residual sugars and ethanol concentration.

Ethanol Production in Sugarcane Molasses Medium

Each yeast strain was cultivated in 50 ml YM broth at 30°C in an incubator shaker at 150 rpm for 18 hr. Then, 5×10^7 yeast cells of each strain were transferred into sugarcane molasses containing 190 g/L of sugars and was incubated at 30, 37, and 40°C for 48 hr. Samples were collected at 48 hr to analyze for both total residual sugars and ethanol concentration.

Analytical Methods

Yeast cells and the fermentation broth was separated using centrifugation at 8,000 rpm for 10 min. The supernatant was used to determine for total residual sugars using the phenol sulfuric acid method (Mecozzi, 2005). The ethanol concentration (*P*, g•L^{-1}) was analyzed using gas chromatography (Shimadzu GC-14B, Kyoto, Japan) thru a polyethylene glycol (PEG-20M) packed column with a flame ionization detector. N_2 was used as carrier gas, and 2-propanol was used as an internal standard (Laopaiboon et al., 2007). The ethanol yield (*Yp/s*) was calculated as the actual ethanol produced and was expressed as g ethanol per g glucose utilized (g¨ g• L^{-1}). The volumetric ethanol productivity (*Qp*,g• L^{-1}•hr) was calculated using the following equations: *Qp* = *P*/t, where *P* is the ethanol concentration (g•L^{-1}), and t is the fermentation time (hr) giving the greatest ethanol concentration

Results

Ethanol Production in YM broth containing 15% (w/v) glucose [YM broth+15% (w/v) glucose]

The ethanol concentrations *(P)* and productivity rates (*Qp*) using YM broth + 15% (w/v) glucose as substrate for 48 hrs. at 30, 37, and 40°C among the yeast strains including three thermotolerant strains MT-02, MT-08, and MT-11 and *S. cerevisiae* TISTR 5088, the reference strain, were collectively listed in Figure 1 and Table 1. At 30°C among the three thermotolerant yeast strains were at 22.32 (g•L^{-1}) and 0.46 (g•L^{-1}•hr.); 27.00 (g•L^{-1}) and 0.56 (g•L^{-1}•hr.); 22.19 (g•L-1) and 0.46 (g•L^{-1}•hr.), respectively. At 37°C among the three thermotolerant yeast strains were at 26.33 (g•L^{-1}) and 0.55 (g•L^{-1}•hr.); 26.73 (g•L^{-1}) and 0.56 (g•L^{-1}•hr.); 25.60 (g•L^{-1}) and 0.53 (g•L^{-1}•hr.), respectively. Also, at 40°C among the three yeast strains were at 17.17 (g•L^{-1}) and 0.36 (g•L^{-1}•hr.); 22.84 (g•L^{-1}) and 0.48 (g•L^{-1}•hr.); 20.48 (g• L^{-1}) and 0.43 (g•L^{-1}•hr.), correspondingly. Whereas the reference strain's ethanol concentrations and productivity rates at 30, 37, 40°C were founded at 23.47 (g•L^{-1}) and 0.49 (g•L^{-1}•hr.); 25.32 (g•L^{-1}) and 0.53 (g•L^{-1}•hr.); 19.37 (g•L^{-1}) and 0.40 (g•L^{-1}•hr.), accordingly. For the sugar consumption and specific ethanol yield ($Y_{p/s}$) for thermotolerant yeast strains at 30°C were at 35.80% and 0.46 ; 46.30% and 0.43; 34.06% and 0.48, respectively. At 37°C, sugar consumption and specific ethanol yield for thermotolerant yeast strains were at 47.25% and 0.41; 42.10% and 0.46; 37.46% and 0.50, respectively. At 40°C, sugar consumption and specific ethanol yield for thermotolerant yeast strains were at 31.74% and 0.40; 37.07% and 0.45; 37.46% and 0.40, respectively. Whereas, the reference strain's sugar consumption and specific ethanol yield at 30, 37, 40°C were found at 41.67% and 0.41; 44.01% and 0.42; 33.91% and 0.42, respectively. It was found that thermotolerant yeast strains MT-08 showed the highest ethanol concentration and productivity among the three thermotolerant yeast strains, especially at 40°C.

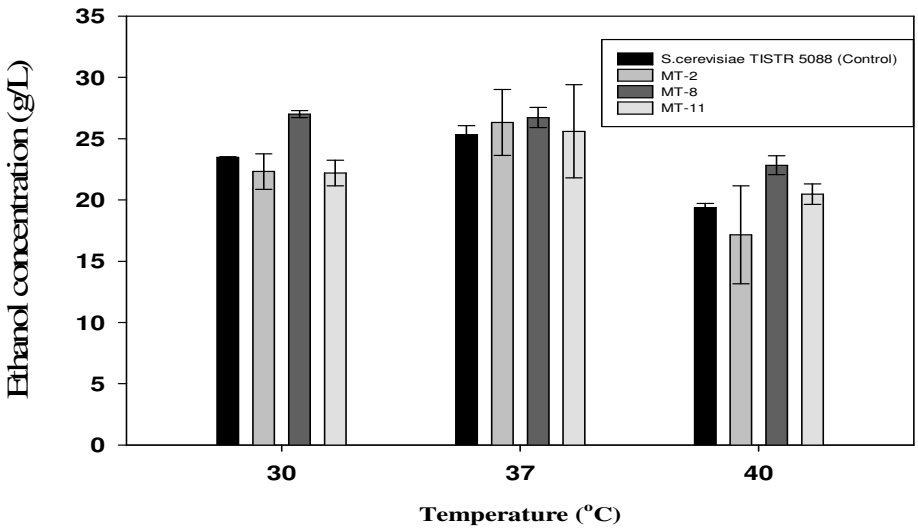

Figure 1: Ethanol production of *S.cerevisiae* TISTR 5088 (reference strain) and thermotolerant yeast strains (MT-02, MT-08, MT-11) in YM broth + 15% (w/v) glucose at various high temperatures for 48 hr.

Table 1: Kinetic parameters of ethanol production from YM broth + 15% (w/v) glucose at various temperatures by *S. cerevisiae* strain TISTR 5088 and thermotolerant yeast strains: MT-02, MT-08, and MT-11 for 48 hr.

Yeast Strain	*T*(°C)	sugar consumption	*P* (g·L^{-1})	Q_p (g·L^{-1}·hr)	$Y_{p/s}$
S cerevisiae TISTR 5088		41.67	23.47 ±	0.49	0.41
MT-02	30	35.80	0.06	0.46	0.46
MT-08		46.30	22.32 ±	0.56	0.43
MT-11		34.06	0.29	0.46	0.48
			27.00 ±		
			1.45		
			22.19 ±		
			1.05		
S cerevisiae TISTR 5088		44.01	25.32 ±	0.53	0.42
MT-02	37	47.25	0.75	0.55	0.41
MT-08		42.10	26.33 ±	0.56	0.46
MT-11		37.46	0.82	0.53	0.50
			26.73 ±		
			2.69		
			25.60 ±		
			3.80		
S cerevisiae TISTR 5088		33.91	19.37 ±	0.40	0.42
MT-02	40	31.74	0.35	0.36	0.40
MT-08		37.07	17.17 ±	0.48	0.45
MT-11		37.46	0.77	0.43	0.40
			22.84 ±		
			3.99		
			20.48 ±		
			0.83		

T: temperature (°C); *P* : ethanol concentration (g•L^{-1}); *Qp*: volumetric ethanol productivity (g•L-1•hr.)
$Y_{p/s}$: ethanol yield

Ethanol production in sugarcane molasses containing 190g/L of total sugar

Thermotolerant yeast strain MT-08 was further subjected for ethanol production by using sugarcane molasses containing 190 g/L of sugars (referred as to glucose equivalent) as substrate at 30, 37, and 40°C for 48 hr. in comparison with the reference strain TISTR 5088. The ethanol concentration (*P*) and productivity rate (*Q*) using sugarcane molasses as substrate for 48 hr. at 30, 37, and 40°C. For MT-08 strain were found at 60.32 (g•L^{-1}) and 1.31 (g•L^{-1}•hr.); 51.71(g•L^{-1}) and 1.06 (g•L^{-1}•hr.); 34.95(g•L^{-1}) and 0.73(g•L^{-1}•hr.); respectively. Whereas, the reference strain's ethanol concentration and productivity rates at 30, 37, and

40°C were shown at 52.76 (g•L^{-1}) and 1.15(g•L^{-1}•hr.); 60.91 (g• L^{-1}) and 1.30 (g•L^{-1}•hr.); 49.64 (g•L^{-1}) and 1.03 (g•L^{-1}•hr.), respectively. As for the sugar consumption and specific ethanol yield for MT-08 at 30, 37, 40°C, the data shows them at 63.91% and 0.46; 71.40% and 0.42; 43.03% and 0.39, respectively. Whereas, the reference strain's sugar consumption and specific ethanol yield at 30, 37, 40°C were shown at 57.42% and 0.48; 65.62% and 0.44; 65.35% and 0.42, respectively. It was found that MT-08 could produce ethanol better than the reference strain when fermented for 48 hr at 30°C. However, at temperatures 37 and 40°C, it was found that MT-08 could not produce ethanol as well as the reference strain could for the same amount of fermentation time.

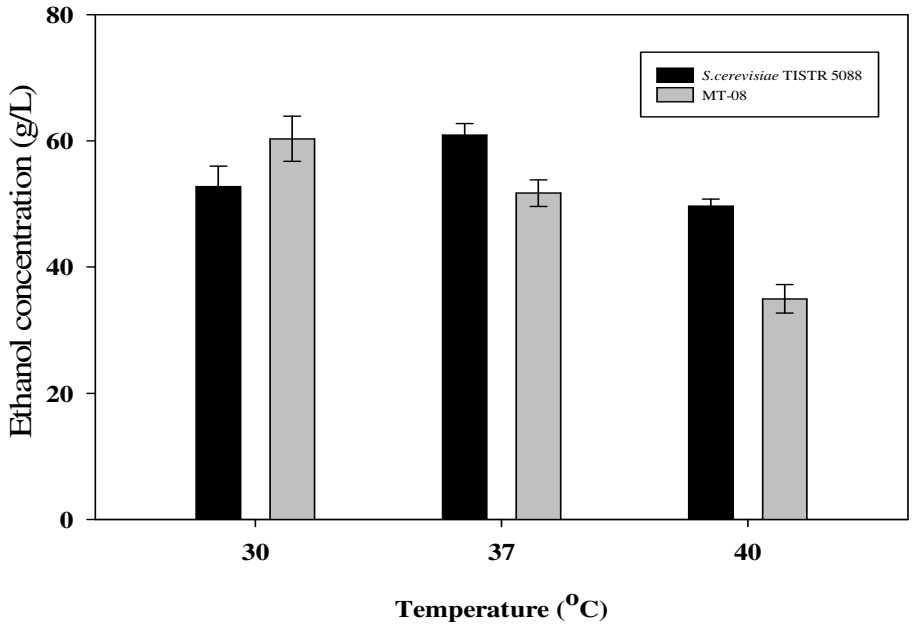

Figure 2: Ethanol production of *S. cerevisiae* strain TISTR 5088 and strain MT-08 in sugarcane molasses containing 190 g/L of sugar at various temperatures

Table 2: Kinetic parameters of ethanol production from Sugarcane molasses containing 190 g/L of total sugar with various temperatures for 48 hr. by *S.cerevisiae* strain TISTR 5088 and strain MT-08

Yeast Strain	*T* (°C)	%sugar consumption	*P* ($g \cdot L^{-1}$)	Q_p ($g \cdot L \cdot hr$)	$Y_{p/s}$
S cerevisiae TISTR 5088	30	57.42	52.76 ± 3.20	1.15	0.48
	37	65.62	60.91 ± 1.85	1.30	0.44
	40	65.35	49.64 ± 1.12	1.03	0.42
MT-08	30	63.91	60.32 ± 3.56	1.31	0.46
	37	71.40	51.71 ± 2.09	1.06	0.42
	40	43.03	34.95 ± 2.26	0.73	0.39

T: temperature (°C); *P* : ethanol concentration ($g \cdot L^{-1}$); *Qp*: volumetric ethanol productivity
(g•L-1•hr.) $Y_{p/s}$: ethanol yield

Discussion and conclusion

Ethanol production in YM broth containing 15% (w/v) Glucose

All these three strains, MT-02, MT-08, and MT-11, were screened for their ethanol production so as to cover all the possibilities to get the yeast strain that could produce a higher yield of ethanol at 30, 37, and 40°C in YM broth + 15% (w/v) glucose. From this experiment, MT-08 exhibited the highest percentage of sugar consumption during ethanol production at 30°C, as shown in Figure 1 and Table 1. This suggests that the yeast made use of the sugar that was consumed; therefore, increasing the quantity of ethanol. Moreover, the sugar consumption coincides with the ethanol productivity rate or Q_p, which indicates which of the strains could produce ethanol the fastest taking into account the duration of fermentation. The ethanol yield or $Y_{p/s}$ indicates the amount of sugar that was used to convert into ethanol. Mostly, the yeast converted glucose into ethanol rather than producing other metabolites, making ethanol the main product. It was found that all strains could produce ethanol at their best at 37°C, which corresponds to Limthong (2005) who stated that the appropriate temperature for ethanol fermentation is between 5-10°C higher than the appropriate temperature for growth depending on the strain of yeast. When the temperature increased from 25 to 38°C, the yeast's metabolism shifted more towards fermentation resulting from the oxidation enzyme's delayed response to the reaction, causing an accumulation of pyruvates and ethanol. At 40°C, it was found that all yeast strains in the experiment had a way of utilizing glucose that corresponds to the research of Torija et al.

(2003) that suggested that when the temperature increases, yeasts will start producing other types of metabolites such as glycerol, succinate, and acetaldehyde, which will cause a decrease in ethanol production at the same time. However, ethanol productivity from all of the strains in YM broth + 15% (w/v) or 150 g/L glucose was found to be lower than other previous studies such as the study by Kiran et al. (2000) who found that *S. cerevisiae* strain VS3 could produce ethanol in YM broth+15% (w/v) glucose at 30 and 40°C at 75 and 60 g/L, respectively. One of the possible reasons to this is probably due to having meager quantity of starter cells. Sridee et al. (2011) reported that the appropriate amount of yeast cells for ethanol production to be from approximately between 5×10^7 to 1×10^8 cells/mL in order to enable the yeast to utilize the nutrients and sugar in the raw material leading to a higher ethanol production rate. However, in this study, only 1×10^7 cells/mL of yeast cells was used prior to fermentation, which was not enough to achieve the desired level of ethanol. Furthermore, this experiment demonstrates that all three strains, including the reference and thermotolerant strains, could produce ethanol well at 37°C, which corresponds with Limtong, S. (2005) that stated that the appropriate temperature for ethanol fermentation is anywhere between 5-10°C higher than the appropriate temperature for yeast growth, depending on the strain of yeast. Also, when the temperature changes from 25°C and increases to 38°C, the yeasts' metabolism deviates towards fermentation due to the enzyme's delayed response to the reaction, causing an accumulation of pyruvates and ethanol. However, at 40°C, it was found that MT-08 could produce ethanol better than all of the other strains, including the reference strain, exhibiting Q_p and $Y_{p/}$ values at 0.48 g·L·hr and 0.45 respectively. Therefore, MT-08 was selected to be further subjected in the subsequent experiment, which will compare the ethanol production capacity of MT-08 and the reference strain.

Ethanol production in sugarcane molasses containing sugar 190 g/L

When using sugarcane molasses as fermentation medium, both MT-08 and *S.crevisiae* TISTR 5088 were able to produce 5 times as much ethanol when compare with results from the previous experiment using YM broth +15% (w/v) Glucose at 48h, as shown in Figure 2 and Table 2. This was mainly due to the amount of starter culture equivalent to 5×10^7 cells, which refers to the research of Sridee et al. (2011) that suggests that an abundant amount of starter cultures will enable the yeast to utilize nutrients and sugar in the raw material and produce products faster than starting off with only a small amount of starter cultures. At 30°C, MT-08 could produce ethanol better than the reference strain. However, at 37 and 40°C, it was found that MT-08 produce ethanol less than the reference strain. One of the factors that caused MT-08 to not be able to produce ethanol in sugarcane molasses as well as the reference strain in the sugarcane molasses media were the lack of supplementary nitrogen sources such as urea or ammonium sulfate in the sugarcane molasses medium. On the contrary, YM broth used in the previous experiment contained nutrients such as Bacto-yeast extract, Bacto-malt extract, and Bacto-peptone, all of which are nitrogen sources that make it more complete than the sugarcane molasses medium used in this experiment. Furthermore, the high density of sugar in the sugarcane molasses, as high as 190 g/L, had slowed down MT-08's ability to produce ethanol. This supports the research of Ozmichi and Kargi, (2007) that suggested high sugar density causes high osmotic pressure lengthening the adaptation period of yeast in order to uptake the sugar into the cell to produce ethanol. Therefore, when

attempting to produce ethanol in sugarcane molasses, it is appropriate to add nutrients such as nitrogen sources since nitrogen is an essential element in the growth of yeast. This was supporting research done by Nuanpeng et al. (2016) who experimented by adding different sources of nitrogen, which were yeast extract, urea, and ammonium sulfate and founded that the yeasts could produce ethanol better than those in the fermentation medium without supplementary nitrogen source. In conclusion, the use of sugar is directly related to ethanol concentration. This means that the more sugar is consumed during fermentation, the more ethanol is produced. Temperature also plays an important role in the relationship when increased. MT-08 demonstrated poorer capacity than the reference strain in producing ethanol in the events of this study; therefore, the search continues for a strain that can produce ethanol at temperatures higher than 40°C.

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