The potential of the newly isolated thermotolerant *Kluyveromyces marxianus* for high-temperature ethanol production using sweet sorghum juice

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Abstract

In this work, the newly isolated thermotolerant *Kluyveromyces marxianus* DBKKUY-103 exhibited a high ethanol fermentation efficiency at high temperatures using sweet sorghum juice (SSJ). The highest ethanol concentrations and productivities achieved under the optimum conditions using thermotolerant *K. marxianus* DBKKUY-103 were 85.16 g/l and 1.42 g/l.h at 37 °C and 83.46 g/l and 1.39 g/l.h at 40 °C, respectively. The expression levels of genes during ethanol fermentation at 40 °C were evaluated and the results found that the transcriptional levels of the *RAD10*, *RAD14*, *RAD33*, *RAD50*, *ATPH*, *ATP4*, *ATP16*, and *ATP20* genes were up-regulated compared with those at 30 °C, suggesting that the high growth and high ethanol production efficiencies of *K. marxianus* DBKKUY-103 during high-temperature ethanol production associated with the genes involved in DNA repair and ATP production.

Keywords DNA repair · Ethanol production · Gene expression · Sweet sorghum · *Kluyveromyces marxianus* · Thermotolerant yeast

Introduction

Bioethanol is one of the alternative energy sources largely produced from agricultural crops, such as maize, cassava, and sugarcane. However, these crops are mainly used for human and animal consumption as well as other industries, which leads to increased competition for these raw materials. Therefore, alternative renewable energy crops for industrial bioethanol production are essential, especially during the shortage of raw materials (Maeda et al. 2013). One of the most promising feedstocks for commercial bioethanol production is sweet sorghum (*Sorghum bicolor* L. Moench). The juice extracted from the plant stalks contains plenty of

sugars, such as sucrose, glucose, and fructose, which can be directly converted via biological fermentation process into ethanol (Nuanpeng et al. 2016; Techaparin et al. 2017a). Sweet sorghum exhibits several better characteristics over the other energy crops, e.g., drought and cold temperature tolerance, has a short period of growth (up to 4 months) and requires less water and fertilizer leading to a low cost of production (Gnansounou et al. 2005).

Apart from raw materials, the bioethanol production efficiency is also dependent on microbial activity, especially that of yeasts. Currently, the most well-known commercial yeast for bioethanol production is *Saccharomyces cerevisiae* since it can produce a high amount of ethanol and tolerate high sugar and high ethanol concentrations (Walker 1998). However, the temperature growth limits of this yeast species are usually reached when the temperature inside the bioreactor increases during ethanol fermentation. To solve this problem, spraying cool water on the fermentation vessel walls is often carried out, which leads to an increase in operating costs. Therefore, new fermentation technology, particularly high-temperature fermentation technology (HTFT) using a potentially high ethanol-producing thermotolerant yeast, may be needed. HTFT has several advantages, such as reducing the operating costs due to the energy used in the

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cooling system and reducing the contamination risk. It also increases the ethanol fermentation rate and provides the possibility of using continuous stripping for ethanol recovery (Limtong et al. 2007; Nuanpeng et al. 2016).

During ethanol fermentation, several factors, such as incubation temperature, pH, initial sugar concentration and nitrogen source, have been shown to influence ethanol production efficiency. With respect to the fermentation temperature, it is a key factor that is carefully controlled during ethanol production. The ethanol fermentation capacity depends on incubation temperature and that the ethanol concentration increases when the fermentation temperature increases (Charoensoparat et al. 2015). The pH is another one of the major factors affecting the ethanol yield. The high ethanol fermentation efficiency can be achieved by controlling the pH of the production medium during ethanol fermentation (Lin et al. 2012). Regarding the sugar concentration, it is also a major factor significantly affecting the growth of microorganisms and the production rate of ethanol. The high ethanol productivity and ethanol yield can be achieved using a high sugar concentration, but it requires a long period of fermentation and hence increases the operating cost (Nuanpeng et al. 2016). Nitrogen is another one of the main nutrients in the ethanol production medium. It is essential not only for yeast growth but also for influencing the rate of ethanol fermentation and ethanol tolerance. The optimum nitrogen source for production of ethanol is dependent on culture conditions and the yeast species used in the fermentation process (Pereira et al. 2010; Charoensoparat et al. 2015).

Apart from ethanol stress, high-temperature conditions or heat stress is well known as a stressful condition for microbial growth during the fermentation process. It inhibits glucose and amino acid transport systems, resulting in the inhibition of cell growth and division, reduction of cell survival and eventually cell death (Stanley et al. 2010). It can disrupt cellular ionic homeostasis, which adversely affects metabolic activities, resulting in the reduction of cells for performing the efficient conversion of sugars to ethanol. Furthermore, it can modify plasma membrane fluidity, inhibit nutrient uptake and the H⁺-ATPase, dissipating the proton-motive force (Walker 1998; Lei et al. 2007) and stimulate specific stress responses (Charoensoparat et al. 2015; Nuanpeng et al. 2016; Techaparin et al. 2017b). Although a set of genes involved in stress responses in yeast have been reported (Auesukaree et al. 2012; Lertwattanasakul et al. 2015; Charoensoparat et al. 2015; Nuanpeng et al. 2016; Techaparin et al. 2017b), the molecular mechanism supporting growth under heat and ethanol stresses during fermentation process is not fully understood. Based on a review of the literatures, little information is known about the molecular mechanism related to thermotolerance, especially the genes involved in DNA repair and ATP production under stressful

conditions in *K. marxianus* DBKKUY-103, one of the most newly isolated thermotolerant yeast strain with the potential capability for growth and ethanol fermentation at high temperatures of up to 45 °C (Charoensoparat et al. 2015). Therefore, the objective of this research was to evaluate the factors affecting the ethanol production efficiency of thermotolerant *K. marxianus* DBKKUY-103 using SSJ. Furthermore, the expression levels of genes encoding RAD2, RAD10, RAD14, RAD33, RAD50, ATPH, ATP4, ATP16, and ATP20 were also evaluated using quantitative RT-PCR (qRT-PCR) analysis.

Materials and methods

Raw material

In this work, SSJ was provided by the Faculty of Agriculture, Khon Kaen University, Thailand. It contained total soluble solids of 21.5°Brix, and total sugar concentrations of 194 g/l, in which sucrose (175.97 g/l) was the major sugar found in the SSJ, followed by glucose and fructose. The SSJ also contained some minerals essential for yeast growth and enzyme activity involved in glycolysis and ethanol production pathways, such as Fe (2.39 mg/l), Zn (1.67 mg/l), Mn (3.58 mg/l), and Cu (0.36 mg/l).

The SSJ extracted from the plant stalks was concentrated by evaporation until the total soluble solids reached a value of approximately 75°Brix. This material was kept at − 18 °C prior to being used.

Microorganism and inoculum preparation

A loopful of *K. marxianus* DBKKUY-103 cells was inoculated into 100 ml of yeast extract-malt extract (YM) medium (g/l; 3.0 yeast extract, 3.0 malt extract, 5.0 peptone and 10.0 glucose). The cultures were incubated in a controlled temperature incubator shaker (150 rpm) at 35 °C for 12 h (the mid-exponential growth phase). The active viable cells (10⁶ cells/ml) were then transferred into 100 ml of SSJ (100 g/l sugar concentration) and subsequently incubated at the same conditions. After 12 h of incubation, cells were collected by centrifugation, resuspended in 0.85% (w/v) NaCl and used as the starter cultures for next experiments.

Fermentation conditions for the ethanol production from SSJ by thermotolerant *K. marxianus* DBKKUY-103

In this study, the ethanol fermentation process was conducted in batch mode using a 500-ml Erlenmeyer flask containing 300 ml of the SSJ under shaking conditions at 150 rpm using 1 × 10⁷ cells/ml. The effects of fermentation

factors including incubation temperature, initial sugar concentration, pH, nitrogen sources and their concentrations on ethanol production by thermotolerant *K. marxianus* DBKKUY-103 using SSJ were evaluated. Various incubation temperatures (30, 37, 40 and 45 °C), the initial sugar concentrations of the SSJ (180, 230, 280 and 300 g/l), the initial pHs of the SSJ (4.0, 4.5, 5.0, 5.5 and 6.0), and nitrogen sources and their concentrations (NH_4NO_3 , $(\text{NH}_4)_2\text{SO}_4$ and $(\text{NH}_4)_2\text{HPO}_4$ at 0.25, 0.50, 0.75 and 1.00 g/l) on ethanol fermentation were assessed. The initial pHs of SSJ were adjusted using 1 M HCl or 1 M NaOH. The fermentation broths were collected at certain time interval, centrifuged and analyzed during ethanol fermentation. Data were analyzed and expressed as the mean values $\pm$ SD.

qRT-PCR analysis of gene expression during ethanol fermentation

The expression levels of genes involved in DNA repair (*RAD2*, *RAD10*, *RAD14*, *RAD33*, *RAD50*) and ATP production (*ATPH*, *ATP4*, *ATP16*, *ATP20*) in *K. marxianus* DBKKUY-103 during ethanol fermentation were determined using qRT-PCR. The yeast cells (1×10^7 cells/ml) were cultured under shaking condition (150 rpm) in 100 ml of SSJ containing 230 g/l sugar concentration at 30 and 40 °C for 12 h (the mid-exponential growth phase). Cells were collected by centrifugation and washed twice with a 0.85% NaCl solution. The total RNA was isolated from yeast cells using the Trizol reagent (Invitrogen), and its concentration was monitored using a Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). The qRT-PCR amplifications were independently carried out in triplicate in the Biorad-I-Cycler with the qPCRBIO SyGreen One-Step Lo-ROX (PCR Biosystems, London, UK) using the specific primers (Table 1). The reactions (final volume 20 μ l) were composed of 1 μ l RNA template (100 ng total RNA), 0.8 μ l of each forward and reverse primer, 1 μ l 20X RTase, 10 μ l 2X qPCRBIO Sygreen One-Step mix, and 6.4 μ l RNase-free water. The thermal cycling conditions were performed using a protocol described by Nuanpeng et al. (2016). The actin (*ACT*) gene and RNase-free water were used as an internal and a negative control, respectively. The data from the qRT-PCR were evaluated using the CFX Manager Software (Bio-Rad, Hercules, CA, USA) and expressed as the mean values $\pm$ SD. The relative gene expression was calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001).

Analytical methods

The total sugar concentrations and total soluble solids were analyzed using the phenol–sulfuric acid method (Mecozzi 2005) and a hand-held refractometer (Zoecklein et al. 1995), respectively. The viable yeast cells were monitored using a

Table 1 Primers used for qRT-PCR analysis in this study

Gene name	Primer name	Primer sequence (5' → 3')
<i>RAD2</i>	RAD2-F	GAAGTGGATACCAACCAAA
	RAD2-R	TAGCTCTTGTTCCTTGAGC
<i>RAD10</i>	RAD10-F	CTAGCCAATGTCAGGAAAAG
	RAD10-R	ATTAGAATCGTCATGCTGCT
<i>RAD14</i>	RAD14-F	CCAGCTTTCGAAAAAGATAA
	RAD14-R	TTAAAAGCAGGCATTCTTC
<i>RAD33</i>	RAD33-F	CCTAAGGAGTGGGTCTCTCT
	RAD33-R	GCCTTTTGTACGTCTTTCAC
<i>RAD50</i>	RAD50-F	AAAACAGACGAAGTCTCCAA
	RAD50-R	AACGTCTCGGATAATGCTAA
<i>ATPH</i>	ATPH-F	ATCCAAGCCACAGTCACTAC
	ATPH-R	TGTTAGCAACAGACAAGTCG
<i>ATP4</i>	ATP4-F	ACGCCATTTCTAACCAATTA
	ATP4-R	CAGATTTCGATTCTTTCCCTTG
<i>ATP16</i>	ATP16-F	TTGTAATCTGGCTCTCCAGT
	ATP16-R	GTGGAGTCTGGTTGAACAGT
<i>ATP20</i>	ATP20-F	TAAGGTGACCGCTGAACATAT
	ATP20-R	CATTTGTACACCAACAGCAC
<i>act</i>	Actin-F	CATTCAAGCCGTTTGTCTCT
	Actin-R	GGAAATCACTGCTTTGGCTC

protocol described by Zoecklein et al. (1995) with a methylene blue staining technique. The concentration of ethanol (P , g/l) was measured by gas chromatography (GC) (Shimadzu GC-14B, Kyoto, Japan) with a polyethylene glycol (PEG-20 M) packed column using a protocol described by Laopai-boon et al. (2009). The ethanol productivity (Q_p , g/l.h) and ethanol yield ($Y_{p/s}$, g/g) were calculated as described by Nuanpeng et al. (2016).

All ethanol fermentation experiments were independently carried out twice, each with two replicates, and the data are expressed as the mean values $\pm$ SD. Comparison of the mean from each treatment was done by the Duncan's multiple range test (DMRT) ($p = 0.05$) using the SPSS program for Windows.

Results and discussion

The effects of fermentation factors on ethanol production by *K. marxianus* DBKKUY-103 using SSJ

Based on a literature review, there are various factors affecting the growth and ethanol fermentation capability of yeast (Limtong et al. 2007; Charoensopharat et al. 2015; Nuanpeng et al. 2016; Techaparin et al. 2017a). Therefore, the effects of some of these major factors, i.e., the incubation temperature, the sugar concentration, the pH of the production medium and the nitrogen sources, on ethanol fermentation efficiency

of thermotolerant *K. marxianus* DBKKUY-103 using the SSJ as a raw material were investigated.

Temperature is one of the major important physical parameters that influence the yeast growth and ethanol production efficiency. It has been reported that most laboratory and industrial yeasts, except those from natural habitats, grow well at temperatures in the range of 20–30 °C (Walker 1998). Generally, the maximum temperature for growth is dependent not only on the yeast species but also on the growth conditions, such as carbon source, oxygen availability, media water potential and the presence of growth factors or ethanol in the fermentation broth (Gross and Watson 1996; Charoensopharat et al. 2015). Nuanpeng et al. (2016) demonstrated that ethanol productivity increases when the incubation temperature increases. In this study, the effect of incubation temperatures (30, 37, 40 and 45 °C) on ethanol production from SSJ (230 g/l sugar concentration) by *K. marxianus* DBKKUY-103 was evaluated. There were no significant differences in the ethanol concentrations, productivities, and yields at 30 and 37 °C. However, at 40 and 45 °C, the ethanol concentrations and productivities produced by *K. marxianus* DBKKUY-103 were significantly lower than those at 30 and 37 °C. No significant differences in the ethanol yields were observed at any temperature tested, suggesting that one unit of consumed sugar (1 g) was converted into almost the same amount of ethanol at all temperatures tested. The highest ethanol concentrations and productivities produced by *K. marxianus* DBKKUY-103 at 30 and 37 °C were 69.18, and 69.15 g/l, which were approximately 1.28- and 2.13-fold greater than those at 40 and 45 °C, respectively (Table 2). It should be noted from the present study that at incubation temperatures greater than 37 °C, the ethanol concentrations, productivities, and ethanol fermentation efficiencies were remarkably reduced. These results are consistent with those reported by Limtong et al. (2007), Tofighi et al. (2014), Charoensopharat et al. (2015) and Nuanpeng et al. (2016), who found a reduction in the ethanol production efficiency when the temperature increased to greater than 37 °C. This might be because high-temperature conditions retard the growth, viability and fermentation activity of the *K. marxianus* DBKKUY-103 cells, resulting in

a reduction of the ethanol production efficiency (Limtong et al. 2007; Nuanpeng et al. 2016). It was reported that high temperatures had negative effects on the plasma membrane, the cellular ionic homeostasis and the cellular proteins, which resulted in reductions in cell growth and metabolic activity and eventually to cell death (Walker 1994; Charoensopharat et al. 2015).

Considering the results of the ethanol production in this study, the concentrations, productivities and yields of ethanol at 30 and 37 °C did not show significant differences. Furthermore, an approximately 15 g/l and 0.05 g/l.h of ethanol concentration and productivity, respectively, at 40 °C was lower than that at 30 and 37 °C. In contrast, ethanol production at 45 °C was much lower than that at other temperatures. Therefore, based on these results, the incubation temperatures of 37 and 40 °C were chosen for further experiments.

Carbon sources, especially sugars, are a major structural element of yeast cells, in combination with hydrogen, oxygen, and nitrogen (Walker 1998). With respect to ethanol fermentation, the substrate consumption rate, the ethanol fermentation rate and the ethanol yield are also dependent on the sugar concentration in the raw material used in the fermentation process (Ozmichi and Kargi 2007; Charoensopharat et al. 2015; Nuanpeng et al. 2016). Although high sugar concentrations are not frequently used in the commercial bioethanol production because they may inhibit the cell growth and the enzymes involved in the metabolic process and reduce the viability of yeast cells, the conversion rate of substrate and the yield of ethanol (Ozmichi and Kargi 2007), ethanol fermentation using high sugar concentrations has also been described, e.g., the ethanol fermentation by *S. cerevisiae* NP01 using SSJ containing 280 g/l of total sugars (Laopaiboon et al. 2009), the ethanol fermentation by *K. marxianus* DBKKUY-102 using Jerusalem artichoke tuber juice containing 250 g/l of total sugars (Charoensopharat et al. 2015) and the ethanol fermentation by *S. cerevisiae* DBKKUY-53 using SSJ containing 250 g/l of total sugars (Nuanpeng et al. 2016). To clarify the influence of sugar concentration on ethanol fermentation performance at a high temperature by the thermotolerant *K. marxianus* DBKKUY-103, SSJ samples containing various

Table 2 Kinetic parameters of ethanol production from SSJ at various incubation temperatures using thermotolerant yeast *K. marxianus* DBKKUY-103

Temperature (°C)	P (g/l)	Q_p (g/l.h)	$Y_{p/s}$ (g/g)	E_y (%)	t (h)
30	69.18 ± 0.17 ^c	0.96 ± 0.01 ^c	0.48 ± 0.03 ^a	88.89 ± 0.91 ^b	66
37	69.15 ± 1.34 ^c	0.96 ± 0.02 ^c	0.49 ± 0.04 ^a	90.74 ± 0.94 ^c	66
40	54.40 ± 1.06 ^b	0.91 ± 0.02 ^b	0.48 ± 0.04 ^a	88.89 ± 0.95 ^b	60
45	32.63 ± 1.56 ^a	0.54 ± 0.03 ^a	0.47 ± 0.03 ^a	87.03 ± 1.14 ^a	60

Mean values with different letters in the same column are significantly different at $p < 0.05$ based on DMRT analysis

P ethanol concentration, Q_p volumetric ethanol productivity, $Y_{p/s}$ ethanol yield, E_y ethanol fermentation efficiency

sugar concentrations of 180, 230, 280 and 300 g/L were tested. There were significant differences in the ethanol concentrations, productivities, yields and ethanol fermentation efficiencies at 37 and 40 °C using SSJ at different sugar concentrations (Table 3). The maximum ethanol concentrations and productivities were achieved using 230 g/l sugar concentration, i.e., 65.13 g/l and 0.90 g/l.h at 37 °C, and 52.31 g/l and 0.87 g/l.h at 40 °C, respectively. It can be seen from the current work that increasing the concentration of sugar from 180 to 230 g/l enhanced the ethanol production efficiencies in terms of ethanol concentrations, productivities, and yields at both temperatures. When the concentration of sugar in the SSJ was increased from 230 to 280 g/l, slight decreases in the ethanol concentrations and productivities were observed. In contrast, the ethanol concentrations, productivities and yields were remarkably decreased at the sugar concentration of 300 g/l, and a large amount of sugar remained in the culture broth at both temperatures. This might be because high sugar concentration had negative effects on the morphology and viability of yeast cells due to a high osmotic pressure (Pratt-Marshall et al. 2003). It has been reported that a high concentration of sugar can cause cell disruption and prolong the completion of sugar utilization, leading to a reduction in cell number and a lower final ethanol concentration, productivity and yield (Ozmichi and Kargi 2007). The results of this study are consistent with those reported

by Charoensopharat et al. (2015), Nuanpeng et al. (2016) and Techaparin et al. (2017a). Based on the highest ethanol concentrations and productivities achieved in this work, the SSJ containing 230 g/l sugar concentration was selected for next experiments.

Another physical growth factor for yeast is the pH of the medium. Most yeasts, such as *S. cerevisiae*, *Kluyveromyces marxianus*, and *Pichia kudriavzevii*, grow very well at a pH between 4.5 and 6.5 (Walker 1998; Charoensopharat et al. 2015; Nuanpeng et al. 2016). The optimum pH for growth of yeast cells is normally dependent on several factors, e.g., the incubation temperature, the availability of oxygen and the yeast species (Narendranath and Power 2005). In addition, several enzymes involved in the metabolic process also work well under certain pH conditions. Thus, the pH of the fermentation medium influences not only on the yeast growth but also the enzyme activity, which is directly related to ethanol production efficiency. In this research, the effect of pH of the SSJ on the ethanol production at 37 and 40 °C by *K. marxianus* DBKKUY-103 was investigated. There were significant differences in the ethanol concentrations, productivities, and yields at both temperatures when SSJ at different pH values was used (Table 4). At 37 °C, the maximum ethanol concentration (71.20 g/l) and productivity (0.99 g/l.h) were achieved at pH 5.0, which were not significantly different from those at pH 4.0 and 4.5. The ethanol concentrations

Table 3 Kinetic parameters of ethanol production from SSJ at various initial sugar concentrations using thermotolerant yeast *K. marxianus* DBKKUY-103 at 37 and 40 °C

Sugar concentration (g/l)	37 °C				40 °C			
	<i>P</i> (g/l)	<i>Q_p</i> (g/l.h)	<i>Y_{p/s}</i> (g/g)	<i>E_y</i> (%)	<i>P</i> (g/l)	<i>Q_p</i> (g/l.h)	<i>Y_{p/s}</i> (g/g)	<i>E_y</i> (%)
180	63.41 ± 0.06 ^b	0.88 ± 0.03 ^b	0.46 ± 0.01 ^b	85.19 ± 0.32 ^b	51.49 ± 0.24 ^b	0.72 ± 0.02 ^a	0.41 ± 0.02 ^b	75.93 ± 0.44 ^b
230	65.13 ± 1.11 ^d	0.90 ± 0.01 ^b	0.50 ± 0.01 ^c	92.59 ± 1.01 ^c	52.31 ± 1.11 ^b	0.87 ± 0.03 ^b	0.49 ± 0.01 ^c	90.74 ± 1.24 ^c
280	64.02 ± 0.14 ^c	0.89 ± 0.01 ^b	0.51 ± 0.02 ^c	94.44 ± 0.89 ^c	51.39 ± 0.73 ^b	0.86 ± 0.01 ^b	0.48 ± 0.03 ^c	88.89 ± 1.18 ^c
300	59.74 ± 1.23 ^a	0.83 ± 0.01 ^a	0.41 ± 0.04 ^a	75.93 ± 1.35 ^a	43.73 ± 0.61 ^a	0.73 ± 0.01 ^a	0.34 ± 0.01 ^a	62.96 ± 1.10 ^a

Mean values with different letters in the same column are significantly different at $p < 0.05$ based on DMRT analysis

P ethanol concentration, *Q_p* volumetric ethanol productivity, *Y_{p/s}* ethanol yield, *E_y* ethanol fermentation efficiency

Table 4 Kinetic parameters of ethanol production from SSJ at various initial pH values using thermotolerant yeast *K. marxianus* DBKKUY-103 at 37 and 40 °C

pH	37 °C				40 °C			
	<i>P</i> (g/l)	<i>Q_p</i> (g/l.h)	<i>Y_{p/s}</i> (g/g)	<i>E_y</i> (%)	<i>P</i> (g/l)	<i>Q_p</i> (g/l.h)	<i>Y_{p/s}</i> (g/g)	<i>E_y</i> (%)
4.0	69.52 ± 0.11 ^b	0.97 ± 0.01 ^b	0.51 ± 0.01 ^b	94.45 ± 0.65 ^b	57.79 ± 0.50 ^a	1.20 ± 0.01 ^b	0.50 ± 0.02 ^b	92.52 ± 0.81 ^b
4.5	69.20 ± 1.01 ^b	0.96 ± 0.01 ^b	0.52 ± 0.01 ^b	96.30 ± 1.41 ^b	59.01 ± 1.01 ^{ab}	1.23 ± 0.02 ^{bc}	0.48 ± 0.04 ^a	88.87 ± 1.51 ^a
5.0	71.20 ± 1.56 ^b	0.99 ± 0.02 ^b	0.52 ± 0.01 ^b	96.30 ± 1.12 ^b	60.00 ± 0.18 ^b	1.25 ± 0.02 ^c	0.48 ± 0.02 ^a	88.88 ± 0.26 ^a
5.5	65.57 ± 0.89 ^a	0.91 ± 0.01 ^a	0.50 ± 0.01 ^a	92.59 ± 1.01 ^a	61.46 ± 0.11 ^c	1.28 ± 0.02 ^c	0.48 ± 0.02 ^a	88.89 ± 0.05 ^a
6.0	64.46 ± 0.11 ^a	0.90 ± 0.01 ^a	0.52 ± 0.02 ^b	96.29 ± 1.17 ^b	58.07 ± 0.34 ^a	0.81 ± 0.02 ^a	0.48 ± 0.03 ^a	88.85 ± 0.03 ^a

Mean values with different letters in the same column are significantly different at $p < 0.05$ based on DMRT analysis

P ethanol concentration, *Q_p* volumetric ethanol productivity, *Y_{p/s}* ethanol yield, *E_y* ethanol fermentation efficiency

and productivities dramatically decreased when pH of the SSJ increased from 5.0 to 5.5 or 6.0, which is in good agreement with that reported by Ercan et al. (2013) and Nuanpeng et al. (2016). However, at 40 °C, the highest ethanol concentration (61.46 g/l) and productivity (1.28 g/l.h) were achieved at pH 5.5, similar to that reported by Hashem et al. (2013). Increasing the initial pH of SSJ from 5.5 to 6.0 led to a reduction in the ethanol concentration and productivity, similar to that observed at 37 °C. This might be attributed to the enzyme activity involved in the glucose-to-pyruvate (GP) and pyruvate-to-ethanol (PE) pathway (Narendranath and Power 2005). In the current study, pH of the fermentation broth was reduced from the original to the final values of approximately 4.0–4.5 during ethanol fermentation. This might be attributed to the accumulation of H⁺ ions excreted from the yeast cells. Generally, the concentration of H⁺ in the fermentation broth can change the total charge of the plasma membrane affecting the permeability of some essential nutrients into yeast cells (Hashem et al. 2013). When the extracellular pH is higher or lower than the optimum pH, yeast cells need to provide energy to either pump H⁺ ions in or out to keep up the optimal intracellular pH (Thomas et al. 2002). The initial pH of the SSJ used in this study was 5.04. In order to reduce the cost of chemical used for adjusting the pH of SSJ, therefore, a pH value of 5.0 was chosen for further experiments.

Besides the carbon source, nitrogen is also one of the essential nutrients in the ethanol production medium. It has been reported that the ethanol fermentation rate and ethanol tolerance are also dependent on the nitrogen source. The nitrogen source can also suppress the formation of

byproducts and enhance the ethanol yield (Yue et al. 2012; Hashem et al. 2013). Several kinds of nitrogen have been used, but inorganic nitrogen sources in the form of NH₄NO₃, (NH₄)₂SO₄, or (NH₄)₂HPO₄ are most widely used for yeast growth and ethanol fermentation on a commercial scale. In this study, the effect of nitrogen sources at various concentrations on ethanol production by thermotolerant *K. marxianus* DBKKUY-103 using SSJ was tested. There were significant differences in the ethanol concentrations and productivities when the SSJ was supplemented with nitrogen sources at different concentrations. At 37 °C, supplementation with NH₄NO₃, (NH₄)₂SO₄ and (NH₄)₂HPO₄ in the SSJ at different concentrations significantly enhanced the ethanol production efficiency in terms of ethanol concentration and productivity, compared with the control condition without nitrogen supplementation. The highest ethanol concentration (85.16 g/l) and productivity (1.42 g/l.h) were achieved when the SSJ was supplemented with (NH₄)₂HPO₄ at 0.75 g/l, which were approximately 1.25- and 1.51-fold greater than those of the control condition, respectively (Table 5). Ethanol production conducted at 40 °C also provided similar results, i.e., supplementation with nitrogen into the SSJ tended to significantly increase the ethanol concentration and productivity, except for that of (NH₄)₂SO₄-supplemented medium (Table 6). It was suggested from this study that (NH₄)₂SO₄ was a poor nitrogen source for ethanol fermentation at a high temperature (40 °C) using thermotolerant *K. marxianus* DBKKUY-103. These findings are in good agreement with those reported by Limtong et al. (2007) and Yue et al. (2012). It can be proposed from this finding that (NH₄)₂SO₄ was not completely taken up by *K. marxianus*

Table 5 Kinetic parameters of ethanol production from SSJ supplementation with various nitrogen sources using thermotolerant yeast *K. marxianus* DBKKUY-103 at 37 °C

Nitrogen source	Nitrogen concentration (g/l)	Kinetic parameters			
		<i>P</i> (g/l)	<i>Q_p</i> (g/l.h)	<i>Y_{p/s}</i> (g/g)	<i>E_y</i> (%)
Control	0.00	68.16 ± 1.45 ^a	0.94 ± 0.02 ^a	0.48 ± 0.51 ^a	90.75 ± 1.21 ^{bc}
NH ₄ NO ₃	0.25	73.11 ± 1.28 ^{abc}	1.02 ± 0.02 ^{ab}	0.49 ± 0.02 ^{abc}	91.48 ± 1.16 ^{bc}
	0.50	74.69 ± 0.17 ^{bcd}	1.04 ± 0.00 ^{bc}	0.49 ± 0.01 ^{abc}	89.84 ± 1.85 ^{ab}
	0.75	76.31 ± 1.01 ^{cd}	1.27 ± 0.02 ^g	0.49 ± 0.02 ^{abc}	90.59 ± 1.19 ^{bc}
	1.00	77.03 ± 0.11 ^{cd}	1.28 ± 0.00 ^g	0.50 ± 0.01 ^{cde}	92.02 ± 0.13 ^{cd}
(NH ₄) ₂ SO ₄	0.25	70.59 ± 1.28 ^{ab}	0.98 ± 0.02 ^{ab}	0.48 ± 0.02 ^{ab}	88.32 ± 1.61 ^a
	0.50	71.89 ± 1.79 ^{abc}	1.00 ± 0.02 ^{ab}	0.48 ± 0.01 ^{ab}	88.55 ± 1.22 ^a
	0.75	73.98 ± 0.28 ^{bc}	1.23 ± 0.00 ^{fg}	0.49 ± 0.01 ^{abcd}	91.34 ± 0.34 ^{bc}
	1.00	75.01 ± 0.50 ^{bcd}	1.25 ± 0.01 ^{fg}	0.50 ± 0.01 ^{bcd}	92.61 ± 0.62 ^{cd}
(NH ₄) ₂ HPO ₄	0.25	79.43 ± 1.28 ^{de}	1.32 ± 0.02 ^{cd}	0.51 ± 0.01 ^e	94.96 ± 1.54 ^{df}
	0.50	82.75 ± 1.86 ^{ef}	1.38 ± 0.07 ^{de}	0.51 ± 0.01 ^e	93.70 ± 1.67 ^{def}
	0.75	85.16 ± 0.67 ^f	1.42 ± 0.01 ^h	0.52 ± 0.01 ^e	95.58 ± 0.75 ^f
	1.00	82.40 ± 1.25 ^{ef}	1.37 ± 0.09 ^{ef}	0.51 ± 0.03 ^{de}	93.57 ± 1.53 ^{de}

Mean values with different letters in the same column are significantly different at *p* < 0.05 based on DMRT analysis

P ethanol concentration, *Q_p* volumetric ethanol productivity, *Y_{p/s}* ethanol yield, *E_y* ethanol fermentation efficiency

Table 6 Kinetic parameters of ethanol production from SSJ supplementation with various nitrogen sources using thermotolerant yeast *K. marxianus* DBKKUY-103 at 40 °C

Nitrogen sources	Nitrogen concentration (g/l)	Kinetic parameters			
		P (g/l)	Q_p (g/l.h)	$Y_{p/s}$ (g/g)	E_y (%)
Control	0.00	56.16 ± 1.25 ^b	0.94 ± 0.05 ^a	0.48 ± 0.51 ^{ab}	85.32 ± 1.21 ^a
NH ₄ NO ₃	0.25	61.50 ± 0.39 ^{cd}	1.28 ± 0.01 ^{de}	0.49 ± 0.01 ^{ab}	91.11 ± 0.58 ^d
	0.50	63.56 ± 0.06 ^{de}	1.32 ± 0.00 ^{ef}	0.49 ± 0.01 ^{ab}	91.59 ± 0.58 ^d
	0.75	64.19 ± 0.06 ^e	1.34 ± 0.00 ^{efg}	0.49 ± 0.02 ^{ab}	91.44 ± 0.08 ^d
	1.00	64.94 ± 0.34 ^e	1.35 ± 0.01 ^{fg}	0.49 ± 0.01 ^{bc}	90.09 ± 0.97 ^d
(NH ₄) ₂ SO ₄	0.25	51.63 ± 0.17 ^a	1.08 ± 0.00 ^b	0.47 ± 0.01 ^{ab}	86.52 ± 0.83 ^{ab}
	0.50	54.87 ± 0.39 ^b	1.14 ± 0.01 ^c	0.47 ± 0.01 ^{ab}	87.71 ± 1.40 ^{cd}
	0.75	56.05 ± 1.73 ^b	1.17 ± 0.02 ^c	0.47 ± 0.05 ^a	86.50 ± 1.67 ^{ab}
	1.00	60.12 ± 1.58 ^c	1.25 ± 0.05 ^d	0.48 ± 0.06 ^{ab}	88.38 ± 0.70 ^d
(NH ₄) ₂ HPO ₄	0.25	78.25 ± 0.06 ^f	1.63 ± 0.00 ^h	0.51 ± 0.02 ^c	95.05 ± 1.27 ^{ef}
	0.50	81.05 ± 0.78 ^g	1.69 ± 0.02 ⁱ	0.51 ± 0.01 ^c	94.45 ± 0.07 ^e
	0.75	82.36 ± 0.39 ^g	1.72 ± 0.01 ⁱ	0.51 ± 0.01 ^c	95.44 ± 0.45 ^{ef}
	1.00	83.46 ± 0.06 ^g	1.39 ± 0.01 ^g	0.52 ± 0.01 ^c	96.60 ± 0.62 ^f

Mean values with different letters in the same column are significantly different at $p < 0.05$ based on DMRT analysis

P ethanol concentration, Q_p volumetric ethanol productivity, $Y_{p/s}$ ethanol yield, E_y ethanol fermentation efficiency

DBKKUY-103 under high-temperature ethanol production. It has been described that the nitrogen uptake is controlled by the nitrogen catabolite repression (NCR) mechanism, which enables yeast cells to select the best nitrogen source for their growth under different conditions (ter Schure et al. 2000). Basically, the NCR is located in the plasma membrane, and its mechanism is affected by several factors, such as heat, ethanol and osmotic stresses. Because heat and ethanol stresses have been reported to cause modifications in the plasma membrane, the NCR system may be affected by these stresses, resulting in the impairment of (NH₄)₂SO₄ uptake (Beltran et al. 2005, 2007). From the results observed in this study, (NH₄)₂HPO₄ at 0.75 g/l was the best nitrogen source for ethanol production by *K. marxianus* DBKKUY-103 using SSJ; therefore, it was selected for further study.

The ethanol concentration and productivity achieved in this study using *K. marxianus* DBKKUY-103 are comparable with the values reported in the literature using different yeast strains and substrates. For example, Abdel-Fattah et al. (2000) reported the highest ethanol concentration and productivity of 80.6 g/l and 2.88 g/l.h, respectively, at 43 °C by *K. marxianus* WR12 using sugarcane molasses containing 180 g/l sugar concentration. Limtong et al. (2007) reported the highest ethanol concentrations and productivities of 87.0 g/l and 1.45 g/l.h at 37 °C, and 67.8 g/l and 1.13 g/l.h at 40 °C, respectively, by *K. marxianus* DMKU 3-1042 using sugarcane juice containing 220 g/l sugar concentration. Nuanpeng et al. (2016) reported the highest ethanol concentrations and productivities of 106.82 g/l and 2.23 g/l.h at 37 °C and 85.01 g/l and 2.83 g/l.h at 40 °C, respectively, by *S. cerevisiae* DBKKU Y-53 using SSJ containing 250 g/l

sugar concentration. Recently, Techaparin et al. (2017a) reported the maximum ethanol concentration and productivity of 89.32 g/l and 2.48 g/l.h at 40 °C, respectively, by *S. cerevisiae* KKU-VN8 using SSJ containing 238.52 g/l sugar concentration. Based on the results of this study, *K. marxianus* DBKKUY-103 is a good candidate for high-temperature ethanol production using SSJ as a raw material.

Gene expression analysis during ethanol fermentation using qRT-PCR

The molecular mechanisms conferring thermotolerance in yeast are sophisticated and are regulated by several genes involved in various cellular aspects, such as those genes involved in heat shock proteins biosynthesis, protein degradation processes, RNA processing and modification, DNA replication and repair, carbohydrate, amino acid, lipid and inorganic ion transport and metabolism, ATP production and cell wall/membrane biogenesis (Lertwattanasakul et al. 2015). Although a set of genes responsible for thermotolerance in yeast cells under stressful environments have been described, most studies have focused on the genes involved in the heat-shock response, ethanol, glycogen and trehalose metabolism and protein degradation process (Auesukaree et al. 2012; Kim et al. 2013; Charoensopharat et al. 2015; Nuanpeng et al. 2016; Techaparin et al. 2017b). To the best of our knowledge, little is known about the differential expression of genes involved in the DNA repair and ATP production, especially in the newly isolated thermotolerant *K. marxianus* DBKKUY-103 during high-temperature ethanol fermentation. In this study, the expression levels

of *RAD2*, *RAD10*, *RAD14*, *RAD33*, *RAD50*, *ATPH*, *ATP4*, *ATP16*, and *ATP20* in the mid-exponential growth phase of *K. marxianus* DBKKUY-103 grown in SSJ at 30 and 40 °C were assessed by qRT-PCR. As found in the current study, all genes, except for *RAD2*, were up-regulated at 40 °C, compared with 30 °C (Fig. 1), suggesting that the expression of these genes could be activated by heat stress. Among the *RAD* genes, the expression level of *RAD10* gene was significantly higher than *RAD14*, *RAD33*, and *RAD50*. This finding clearly indicated that the *RAD10*, *RAD14*, *RAD33*, and *RAD50* genes were transcriptionally up-regulated not only by UV radiation but also by a high-temperature treatment. It has been reported in *S. cerevisiae* that the excision repair of DNA damaged by stressful conditions is a complex biochemical process and is regulated by various genes including *RAD2*, *RAD10*, *RAD14*, *RAD33*, and *RAD50*. Disruption of the *RAD2* and *RAD10* genes negates the incision of damaged DNA, while mutations in the *RAD14* affect the proficiency of excision repair. Genetic and biochemical studies using these mutations revealed that the *RAD2*, *RAD10*, and *RAD50* gene products, all of which possess endonuclease activity, are essential for the incision step of excision repair of UV-damaged DNA and mitotic recombination, one of the nucleotide excision repair (NER) mechanisms in yeast (Tomkinson et al. 1993; Wang et al. 1997). In addition, the *RAD14* protein is also necessary for the incision step of NER, whereas the *RAD33* protein is required for the repair of RNA polymerase I-transcribed rDNA and RNA polymerase II-transcribed DNA regions, which may help in stabilizing the DNA repair proteins *RAD4* and *RAD34* (Friedberg et al. 1995). Although the DNA repair pathway in yeast is conserved and is a unique process dependent on the stress conditions and the yeast species, based on the overexpression of the *RAD10*, *RAD14*, *RAD33*, and *RAD50* genes observed in this study, these gene products may play

a crucial role in the DNA repair process in the thermotolerant *K. marxianus* DBKKUY-103 under high-temperature stress, which resulted in the high growth and high ethanol production efficiency during ethanol fermentation at 40 °C. However, to clarify this hypothesis, further study on gene disruption and molecular characterization of the disrupted strain is needed.

With respect to the *ATPH*, *ATP4*, *ATP16*, and *ATP20* genes involved in ATP production, their expression levels were also up-regulated at 40 °C compared with 30 °C. The highest expression level was observed in the *ATP16* gene compared with other genes tested. This finding suggested that there is a requirement for ATP under heat stress, which has been reported to be important for the new synthesis of heat-shock proteins, and the function of the ATP-dependent chaperones and proteases. The results of this work are in agreement with Soini et al. (2005), who found a strong transient increase in respiration and a rapid increase in ATP in *Escherichia coli* upon the temperature increases from 37 to 48 °C or 35 to 55 °C.

Previous studies have also been reported in which heat stress can modify the plasma membrane proteins, induce the plasma membrane-association of Hsp30 and stimulate the activity of the plasma membrane H⁺-ATPase (Piper 1995). Furthermore, the fluidity of the cell membrane also increases upon an increase in temperature. All these physiological changes lead to an additional ATP requirement. Heat stress can also cause numerous changes at all possible levels from transcription to protein modification and enzyme activity involved in the metabolic processes, all of which involve an increased need for ATP by the yeast cells under heat stress (Mensonides et al. 2014).

Conclusions

As shown in this work, a newly isolated thermotolerant *K. marxianus* DBKKUY-103 exhibited a high ethanol production efficiency at 37 and 40 °C using SSJ as substrate. The SSJ containing 230 g/l sugar concentration with an initial pH of 5.0 and supplementation with 0.75 g/l of (NH₄)₂HPO₄ was the best conditions for ethanol production by *K. marxianus* DBKKUY-103. The highest ethanol concentrations and productivities achieved using *K. marxianus* DBKKUY-103 were 85.16 g/l and 1.42 g/l.h at 37 °C and 83.46 g/l and 1.39 g/l.h at 40 °C, respectively, which are comparable to those values reported in the literature for other thermotolerant yeast strains. The current study also demonstrated that the high growth and ethanol fermentation efficiencies of *K. marxianus* DBKKUY-103 during high-temperature ethanol production correlated with the expression of the genes involved in DNA repair and ATP production.

Fig. 1 Transcription levels of genes encoding *RAD2*, *RAD10*, *RAD14*, *RAD33*, *RAD50*, *ATPH*, *ATP4*, *ATP16* and *ATP20* in the mid-exponential growth phase of the thermotolerant *K. marxianus* DBKKUY-103 during ethanol fermentation at 40 °C. Values presented are the mean $\pm$ SD

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Authors contributions W. Pilap carried out the experiments and analyzed the data. S. Thanonkeo participated in the data analysis of the raw material (SSJ). P. Klanrit contributed to the design of the gene expression analysis and participated in drafting the manuscript. P. Thanonkeo contributed to the design of the experiments, conducted the experiments, analyzed the data and revised the manuscript. All of the authors read, corrected and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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