Ethanol Production from Sweet Sorghum Juice at High Temperatures Using a Newly Isolated Thermotolerant Yeast Saccharomyces cerevisiae DBKKU Y-53

Authors:

Sunan Nuanpeng, Sudarat Thanonkeo, Mamoru Yamada, Pornthap Thanonkeo

Date Submitted: 2018-11-27

Keywords: gene expression, thermotolerant yeast, sweet sorghum, Saccharomyces cerevisiae, real-time RT-PCR, high temperature fermentation

Abstract:

Ethanol production at elevated temperatures requires high potential thermotolerant ethanol-producing yeast. In this study, nine isolates of thermotolerant yeasts capable of growth and ethanol production at high temperatures were successfully isolated. Among these isolates, the newly isolated thermotolerant yeast strain, which was designated as Saccharomyces cerevisiae DBKKU Y-53, exhibited great potential for ethanol production from sweet sorghum juice (SSJ) at high temperatures. The maximum ethanol concentrations produced by this newly isolated thermotolerant yeast at 37 °C and 40 °C under the optimum cultural condition were 106.82 g·L?1 and 85.01 g·L?1, respectively, which are greater than values reported in the literatures. It should be noted from this study with SSJ at a sugar concentration of 250 g·L?1 and an initial pH of 5.5 without nitrogen supplementation can be used directly as substrate for ethanol production at high temperatures by thermotolerant yeast S. cerevisiae DBKKU Y-53. Gene expression analysis using real-time RT-PCR clearly indicated that growth and ethanol fermentation activities of the thermotolerant yeast S. cerevisiae DBKKU Y-53 at a high temperature (40 °C) were not only restricted to the expression of genes involved in the heat-shock response, but also to those genes involved in ATP production, trehalose and glycogen metabolism, and protein degradation processes were also involved.

Record Type: Published Article

Submitted To: LAPSE (Living Archive for Process Systems Engineering)

Citation (overall record, always the latest version):	LAPSE:2018.0988
Citation (this specific file, latest version):	LAPSE:2018.0988-1
Citation (this specific file, this version):	LAPSE:2018.0988-1v1

DOI of Published Version: https://doi.org/10.3390/en9040253

energies

MDPI

Article

Ethanol Production from Sweet Sorghum Juice at High Temperatures Using a Newly Isolated Thermotolerant Yeast *Saccharomyces cerevisiae* DBKKU Y-53

Sunan Nuanpeng [1,2], Sudarat Thanonkeo [3], Mamoru Yamada [4] and Pornthap Thanonkeo [2,5,*]

1 Graduate School, Khon Kaen University, Khon Kaen 40002, Thailand; nuanpeng2523@gmail.com
2 Department of Biotechnology, Faculty of Technology, Khon Kaen University, Khon Kaen 40002, Thailand
3 Walai Rukhavej Botanical Research Institute, Mahasarakham University, Maha Sarakham 44150, Thailand; sthanonkeo@gmail.com
4 Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-8515, Japan; m-yamada@yamaguchi-u.ac.jp
5 Fermantation Research Center for Value Added Agricultural Products, Khon Kaen University, Khon Kaen 40002, Thailand
* Correspondence: portha@kku.ac.th; Tel.: +66-81-9743340; Fax: +66-43-362-121

Academic Editor: Tariq Al-Shemmeri
Received: 21 February 2016; Accepted: 15 March 2016; Published: 31 March 2016

Abstract: Ethanol production at elevated temperatures requires high potential thermotolerant ethanol-producing yeast. In this study, nine isolates of thermotolerant yeasts capable of growth and ethanol production at high temperatures were successfully isolated. Among these isolates, the newly isolated thermotolerant yeast strain, which was designated as *Saccharomyces cerevisiae* DBKKU Y-53, exhibited great potential for ethanol production from sweet sorghum juice (SSJ) at high temperatures. The maximum ethanol concentrations produced by this newly isolated thermotolerant yeast at 37 °C and 40 °C under the optimum cultural condition were 106.82 g· L^{-1} and 85.01 g· L^{-1}, respectively, which are greater than values reported in the literatures. It should be noted from this study with SSJ at a sugar concentration of 250 g· L^{-1} and an initial pH of 5.5 without nitrogen supplementation can be used directly as substrate for ethanol production at high temperatures by thermotolerant yeast *S. cerevisiae* DBKKU Y-53. Gene expression analysis using real-time RT-PCR clearly indicated that growth and ethanol fermentation activities of the thermotolerant yeast *S. cerevisiae* DBKKU Y-53 at a high temperature (40 °C) were not only restricted to the expression of genes involved in the heat-shock response, but also to those genes involved in ATP production, trehalose and glycogen metabolism, and protein degradation processes were also involved.

Keywords: high temperature fermentation; real-time RT-PCR; *Saccharomyces cerevisiae*; sweet sorghum; thermotolerant yeast; gene expression

1. Introduction

Bioethanol is a clean, renewable, environmental friendly source of fuel energy that can be produced from different feedstocks and conversion technologies. It is one of the most promising substitutes for fossil energy and has high potential to replace petroleum-based fossil fuels [1–3]. Bioethanol can be used directly or blended with gasoline (known as "gasohol") to power engines without modification. Development of bioethanol is not only addressing climate change due to the burning of petroleum-based fuels but is also of great significance in protecting national energy security and promoting rural economic growth [3,4].

Approximately 60% of global bioethanol is produced from sugar crops, the remaining 40% is produced from starchy grains [5]. Sweet sorghum (*Sorghum bicolor* L. Moench) is one of the most promising sugar crops for industrial bioethanol production. It is a C4 plant, is similar to sugarcane, and belongs to the grass family. Due to a high photosynthetic efficiency, it is known for high carbon assimilation and the ability to store high levels of extractable sugars in its stalks [6]. Sweet sorghum is one of the most drought-resistant agricultural crops and can be cultivated in nearly all temperate and tropical climate areas in both irrigated and non-irrigated lands [7,8]. Unlike sugarcane, sweet sorghum has a short growing period (3–4 months). Therefore, it can be planted two or three times a year. Its stalks contain both soluble carbohydrates (such as sucrose, glucose, fructose) and insoluble carbohydrates (such as cellulose and hemicellulose) that can be converted into fuel ethanol using a biological fermentation process [9,10]. With respect to the production cost, sweet sorghum has lower production cost than that of sugarcane and sugar beets because it requires less fertilizer. Therefore, considering the potential of sweet sorghum for industry, it is an ideal crop for commercial ethanol production [11]. Although sweet sorghum is considered as an important food resource in some countries, such as India, China, which uses sweet sorghum juice (SSJ) to produce syrup, the Thai government promotes sweet sorghum to be used as an energy crop for large-scale ethanol production together with sugarcane and cassava.

Fermentation at high temperature is a key requirement for effective bioethanol production in tropical countries where average daytime temperatures are usually high throughout the year. The advantages of fermentation at high temperatures are not only an increased the rate of fermentation but also a decreased risk of contamination by mesophilic microorganisms, such as *Williopsis* sp., *Candida* sp., *Zygosaccharomyces* sp., a reduced cost of the cooling system, and the possible use of simultaneous saccharification and fermentation (SSF) when coupled with a continuous stripping system for ethanol recovery. Utilization of a high potential thermotolerant yeast strain is a key to success in ethanol production at high temperatures [12,13]. There are several reports in the literature on the ethanol production at high temperatures using the thermotolerant yeast *Kluyveromyces marxianus* [13–18]; however, very few reports have considered the thermotolerant yeast *Saccharomyces cerevisiae* [12,19].

Under stressful conditions, such as heat, ethanol, or osmotic stress, several stress-responsive genes including those encoding for the heat shock proteins (HSPs), enzymes involved in protein degradation, such as ubiquitin ligase, and proteins involved in trehalose and glycogen metabolism in yeast have been reported to be stimulated [20,21]. HSPs play a key role as molecular chaperones by either stabilizing new proteins to ensure correct folding or refolding of proteins to the proper conformation, or degrading misfolded proteins that are damaged by stress conditions. HSPs also help transport proteins across membranes within the cell [22,23]. Trehalose, which is one of the compatible solutes synthesized during adverse environmental conditions, has been reported to protect the cell by replacing water at the surfaces of macromolecules, which holds proteins and membranes in their native conformation [24,25]. Glycogen, which is a reserve carbohydrate in *S. cerevisiae*, has also been reported to be involved with tolerance towards several stresses [26,27]. Although a number of genes responsible for the prevention of protein denaturation in yeast cells have been reported, the molecular mechanism conferring thermotolerance during ethanol fermentation at high temperatures is not fully understood.

In this study, the isolation and screening of highly efficient thermotolerant yeast strains capable of producing high levels of ethanol at high temperatures from SSJ were carried out. The optimum condition for ethanol production for the selected thermotolerant yeast strain was also investigated. Furthermore, to gain a better understanding of the molecular mechanism by which yeast cells adapt to adverse environmental conditions and acquire thermotolerance during high temperature ethanol fermentation, the expression of genes encoding the HSP26, HSP70, HSP90, HSP104, pyruvate kinase, trehalose-6-phosphate synthase, neutral trehalase, glycogen synthase, and ubiquitin ligase was evaluated. This work is the first to demonstrate the physiological changes related to the expression of genes involved in heat-shock response, ATP production, trehalose and glycogen metabolism, and

protein degradation in the newly isolated thermotolerant yeast *S. cerevisiae* DBKKU Y-53 during ethanol fermentation at high temperatures.

2. Experimental Section

2.1. Isolation of Thermotolerant Yeast Strains

Samples including sugarcane juice, SSJ, rotten fruits, soils from sugarcane and sweet sorghum plantations collected from the Khon Kaen, Udon Thani, Nakhon Ratchasima, Maha Sarakham, Kalasin, Chaiyaphum, and Roi Et provinces of Thailand were used for the isolation of thermotolerant yeasts using the enrichment method described by Limtong *et al.* [13]. YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose) supplemented with 4% (v/v) ethanol was used as a selective medium in this study. After incubation at 35 °C for 3 days on a rotary incubator shaker at 100 rpm, the enriched cultures were then spread on YM agar supplemented with 4% (v/v) ethanol and subsequently incubated at 35 °C. Pure cultures were collected and maintained on YM agar at 4 °C for short-term storage and in 50% (v/v) glycerol at −20 °C for long-term storage.

2.2. Screening of Thermotolerant Yeast for Ethanol Fermentation at High Temperatures

SSJ containing 100 g·L^{-1} total sugars was used as substrate for screening thermotolerant ethanol-producing yeast strains. The ethanol production capability was tested by culturing the isolated thermotolerant yeast strains in 16 × 160 mm test tube containing 10 mL SSJ, which were then incubated at high temperatures (37 °C to 50 °C) on a rotary incubator shaker at 150 rpm. The Durham tube was placed inside the test tube, and strains that produced high levels of CO_2 in the Durham tube were selected for further study.

2.3. Identification of The Selected Thermotolerant Yeast Strains

Identification of the yeast strains was carried out using morphological and the D1/D2 domain of the 26S rDNA gene sequencing analysis [28]. Genomic DNA was isolated from the yeast cells using the method described by Harju *et al.* [29]. Amplification of the D1/D2 domain of the 26S rDNA gene was carried out using the specific primers NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG) and NL-4 (5′-GGT CCG TGT TTC AAG ACG G) [30] with the genomic DNA isolated from yeast cells as the template. After the PCR reaction, the amplified product was separated on a 1.0% agarose gel and purified using Invisorb® Fragment CleanUp Kit (Invitek GmbH, Berlin, Germany). All procedures for DNA amplification and purification were carried out according to the manufacturer's instructions. DNA sequencing was performed in the First BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor Darul Ehsan, Malaysia). The sequences of the D1/D2 domain of the 26S rDNA gene were analyzed using GENETYX (Software Development, Tokyo, Japan), whereas homology searching was performed using the FASTA and BLAST programs in the GenBank and DDBJ databases. Phylogenetic analysis was performed using MEGA5 [31], and the tree topologies were analyzed using bootstrap analysis based on the neighbor-joining method [32].

2.4. Comparative Study of Ethanol Production by the Selected Thermotolerant Yeast Strains

The ethanol fermentation efficiencies of the selected thermotolerant yeast strains were compared using SSJ with an initial sugar concentration of 220 g·L^{-1} as substrate. Batch ethanol fermentation was performed in a 500-mL air-locked flask with a final working volume of 400 mL, and the initial cell concentration was 10^6 cells·mL^{-1}. Fermentation was carried out at 30 °C, 37 °C, 40 °C, 42 °C and 45 °C under static conditions. During ethanol fermentation, samples were withdrawn at certain time intervals and analyzed. All experiments were performed in duplicated and repeated twice, and the average values ± SD are presented.

2.5. Factors Influencing Ethanol Production by the Selected Thermotolerant Yeast

The SSJ was sterilized at 110 °C for 28 min and used as raw material for ethanol production using the selected thermotolerant yeast. In this study, *S. cerevisiae* SC90, which is commonly used in industrial ethanol production in Thailand, was used as a reference strain. The batch ethanol fermentation was carried out in 500-mL Erlenmeyer flasks equipped with an air-lock with a final working volume of 400 mL. The effects of pH (4.0, 4.5, 5.0, 5.5, 6.0), initial cell concentration (1×10^6, 1×10^7, 1×10^8 cells·mL^{-1}), initial sugar concentration (200, 250, 300 g·L^{-1}), and nitrogen source (yeast extract, urea, $(NH_4)_2SO_4$) at various concentrations on ethanol production at high temperatures (37 °C and 40 °C) by the selected thermotolerant yeast were investigated. During ethanol fermentation, samples were withdrawn at certain time intervals and analyzed. All experiments were performed in duplicated and were repeated twice. The average values ± SD are presented.

2.6. Ethanol Production in a 2-L Bioreactor

The ethanol production potential of the selected thermotolerant yeast strain was evaluated in a 2-L bioreactor (Biostat®B, B. Braun Biotech, Melsungen, Germany) with a working volume of 1.5 L. The fermentation conditions at the bioreactor scale were selected from the results obtained from the flask-scale trials. The fermentation was carried out at high temperatures (37 °C and 40 °C) with an agitation speed of 100 rpm, and samples were periodically collected and analyzed during ethanol fermentation.

2.7. Real-Time RT-PCR Analysis of Gene Expression during Ethanol Fermentation

The expressions of genes encoding HSP26 (*hsp26*), HSP70 (*hsp70*), HSP90 (*hsp90*), HSP104 (*hsp104*), pyruvate kinase (*cdc*), trehalose-6-phosphate synthase (*tps*), neutral trehalase (*nth*), glycogen synthase (*gsy*), ubiquitin ligase (*rsp*) during ethanol fermentation were evaluated using real-time RT-PCR. Cells were grown in SSJ containing 220 g·L^{-1} total sugars at 40 °C until growth reached the exponential phase, and then the cells were harvested by centrifugation at 4 °C and 5000 rpm for 5 min. Total RNA was extracted from the yeast cells using Trizol reagent. The concentration of RNA was measured and adjusted by Nanodrop (Nanodrop Technologies, Wilmington, DE, USA). The real-time RT-PCR amplifications were performed using the Biorad-I-Cycler with the qPCRBIO SyGreen One-Step Lo-ROX (PCRBIOSYSTEMS, London, UK). The reactions (final volume 20 µL) were composed of 1 µL RNA template (100 ng RNA), 0.8 µL of each forward and reverse primer, 1 µL 20X RTase, 10 µL 2X qPCRBIO Sygreen One-Step mix, and 6.4 µL RNase-free water. The thermal cycling conditions for gene amplification were initial denaturation at 95 °C for 2 min, followed by 40 cycles each of denaturation at 95 °C for 5 s and annealing at 55 °C for 30 s. The primers used in this study are listed in Table 1. The actin gene (*act*) was used as an internal control. As a negative control, RNase-free water was used instead of the RNA template. All experiments were independently carried out in triplicate, and data from real-time RT-PCR analysis were determined using the CFX Manager Software (Bio-Rad, Hercules, CA, USA). Calculation of relative gene expression was performed using the comparative critical threshold ($\Delta\Delta C_T$) method in which the amount of the target genes was adjusted to the reference gene (*act* gene) [33].

Table 1. Primer used in this study.

Gene Name	Primer Name	Sequence (5′→3′)
hsp26	HSP26-F	AAGGCGGCTTAAGAGGCTAC
	HSP26-R	TGTTGTCTGCATCCACACCT
hsp70	SSA4-F	CGGTTCCAGCCTATTTCAAC
	SSA4-R	TGTCTGAGCAGACGAAGACAG
hsp90	HSP82-F	TTCAAACGACTGGGAAGACC
	HSP82-R	AGCAGCCCTGTTTTGGGTAT
hsp104	HSP104-F	CATATGGAACGTGACTTATCATCTGA
	HSP104-R	ACGGCATTGGAAACAGCTTT
cdc	CDC19-F	TGCTTTGAGAAAGGCTGGTT
	CDC19-R	AAAGCTGGCAAATCGACATC
tps	TPS1-F	TGTCTTCCGTGCAAAGAGTG
	TPS1-R	CTTGTGCATGAAATGGATGG
nth	NTH1-F	CCGTACGAGGACTATGAGTGTTT
	NTH1-R	GCAATTTCGCCTACGTTGTT
gsy	GSY1-F	ACGACTGTGTCGCAAATCACT
	GSY1-R	TGCGGTGACCTCATTAACAG
rsp	RSP5-F	CCTTCTGGCCATACTGCATC
	RSP5-R	CCACCTCCCACTTGAACTGT

2.8. Analytical Methods

The yeast cell numbers and total soluble solids of the fermentation broth were determined using a direct counting method using hemacytometer and a hand-held refractometer, respectively [34]. The fermentation broth was centrifuged at 13,000 rpm for 10 min. The supernatant was then determined for total residual sugars using the phenol sulfuric acid method [35]. The ethanol concentration (P, g·L^{-1}) was analyzed using gas chromatography (Shimadzu GC-14B, Kyoto, Japan) using a polyethylene glycol (PEG-20M) packed column with a flame ionization detector. N_2 was used as a carrier gas, and 2-propanol was used as an internal standard. The ethanol yield (Yp/s) was calculated as the actual ethanol produced and was expressed as g ethanol per g glucose utilized (g·g^{-1}). The volumetric ethanol productivity (Qp, g·L^{-1}·h^{-1}) was calculated using the following equations: $Qp = P/t$, where P is the ethanol concentration (g·L^{-1}), and t is the fermentation time (h) giving the greatest ethanol concentration.

3. Results and Discussion

3.1. Isolation and Selection of Thermotolerant Yeast Strains

Natural habitats are a major source of useful microorganisms for biorefinery production [36]. In this study, soil and plant samples from different locations in Northeastern Thailand were collected, and the isolation of thermotolerant yeasts was performed using the enrichment culture technique as described by Limtong *et al.* [13]. The utilization of this technique under selection pressures has been widely used to isolate several thermotolerant yeast strains [13,17]. It has been reported that the effects of high temperatures on microbial growth have often been aggravated by ethanol concentrations greater than 3% [37]. Therefore, ethanol at 4% (v/v) and an incubation temperature of 35 °C served as selection pressures for the isolation of thermotolerant yeast strains in this work. As a result, sixty-two yeast isolates were obtained, and most of these yeasts were from soil samples. Their ability to grow and produce ethanol at high temperatures were tested by culturing the yeast strains in SSJ incubated at temperatures between 30 and 50 °C. The results showed that nine isolates of yeast, which were designated as DBKKU Y-53, DBKKU Y-55, DBKKU Y-58, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104,

DBKKU Y-105, DBKKU Y-106, and DBKKU Y-107, were able to grow and produce a relatively high level of ethanol at 45 °C. Of these, six isolates (*i.e.*, DBKKUY-102, DBKKUY-103, DBKKUY-104, DBKKUY-105, DBKKUY-106, DBKKUY-107) were capable of growth and ethanol production at 47 °C. Their ability to grow and produce ethanol at high temperatures were comparable to that of *K. marxianus* ATCC 8554, which is known as a thermotolerant yeast. Based on growth performance of the isolated yeast strains at high temperatures (above 40 °C), we speculated that these nine isolates of yeast were thermotolerant yeasts [38]. Therefore, all nine yeast isolates were selected for further study.

Successful isolation and selection of thermotolerant yeasts for ethanol production at high temperatures has been reported by several investigators. According to our literature review, SSJ has never been used directly as the substrate for yeast selection. Almost all previous research studies used glucose as a substrate, e.g., Ballesteros *et al.* [39] reported the screening and selection of thermotolerant yeast strains using glucose as the sole carbon source and found that *K. marxianus* L.G. was the most effective strain for ethanol production at high temperatures. This strain produced an ethanol concentration of approximately 3.76% (*w*/*v*) when the fermentation was carried out at 42 °C. Banat *et al.* [40] isolated and selected the thermotolerant yeast *K. marxianus* using an enrichment method with glucose as the carbon source and demonstrated that the selected *K. marxianus* strain produced an ethanol concentration of approximately 5.6%–6.0% (*w*/*v*) at 45–50 °C when using glucose as the substrate and ethanol concentration of approximately 7.5%–8.0% (*w*/*v*) and 6.5%–7.0% (*w*/*v*) at 37 °C and 40 °C, respectively, when using molasses as a substrate. Kiran Sree *et al.* [12] screened thermotolerant yeast *S. cerevisiae* VS3 using glucose as the sole carbon source, and they reported that the selected yeast strain produced an ethanol concentration of approximately 75 g· L^{-1} from a glucose concentration of 150 g· L^{-1} at 40 °C. Edgardo *et al.* [19] used glucose for the selection of thermotolerant yeast strains and obtained a good potential thermotolerant yeast *S. cerevisiae* that was able to produce approximately 75% of the theoretical ethanol yield at 40 °C. In addition to using glucose as a substrate, other carbon sources, such as sugarcane juice [13], xylose [41], sugarcane blackstrap molasses [42], and inulin [43] have also been used for screening and selecting thermotolerant yeasts.

3.2. Identification of the Selected Thermotolerant Yeasts

Based on the morphological and physiological characteristics, three isolated yeasts (*i.e.*, DBKKU Y-53, DBKKU Y-55, DBKKU Y-58) were found to be *Saccharomyces*, whereas six isolated yeasts (*i.e.*, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106, DBKKU Y-107) were revealed to be *Kluyveromyces*. To confirm this finding, molecular taxonomic analyses based on the nucleotide sequences of the D1/D2 domain of the 26S rDNA gene were carried out. As a result, the nucleotide sequences from the yeast strains DBKKU Y-53, DBKKU Y-55, DBKKU Y-58 and from *S. cerevisiae* (NL45 and NL51) were 99% identical, whereas strains DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106, DBKKU Y-107, and *K. marxianus* (FJ627963) were 99% identical. Phylogenetic analysis confirmed that strains DBKKU Y-53, DBKKU Y-55, and DBKKU Y-58 were clustered in the same group as *S. cerevisiae* whereas strains DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106, and DBKKU Y-107 were closely related to *K. marxianus* (Figure 1). Therefore, strains DBKKU Y-53, DBKKU Y-55, and DBKKU Y-58 were identified as *S. cerevisiae*, whereas the other strains were *K. marxianus*.

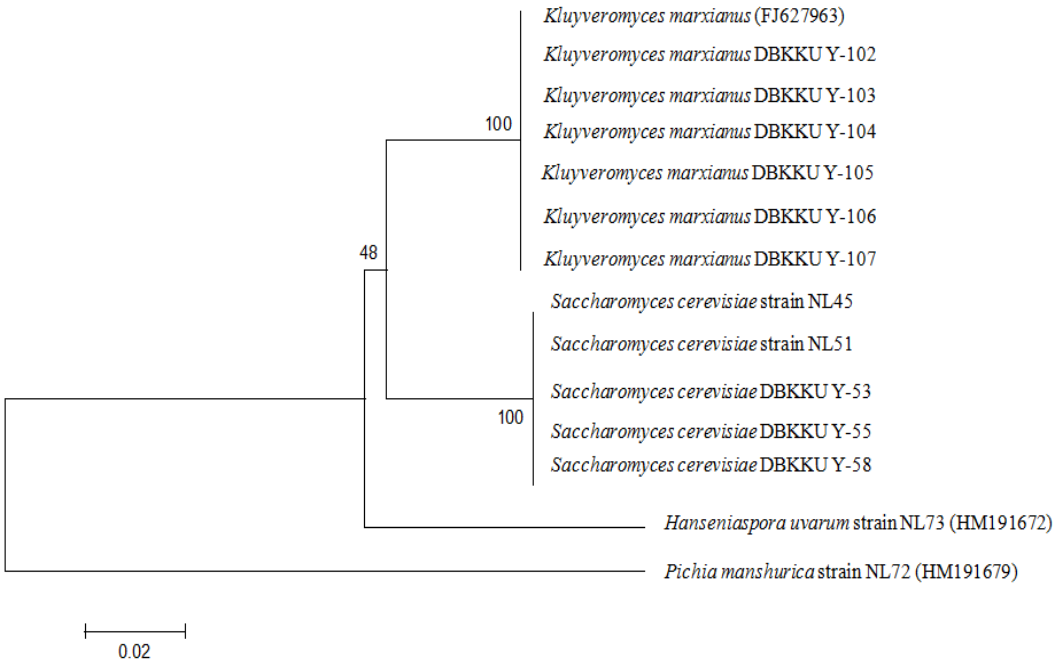

Figure 1. An unrooted phylogenetic tree showing the relationship between the isolated yeasts and the type strain *K. marxianus* and *S. cerevisiae* based on the D1/D2 domain of the 26s rDNA sequence analysis. The tree was built using the neighbor-joining method. Numbers given at the nodes indicate the percentage bootstrap values based on 1000 replications.

3.3. Comparative Study on the Ethanol Production by the Selected Thermotolerant Yeasts

A preliminary study of the ethanol production using SSJ by the selected thermotolerant yeasts revealed that only four strains, *i.e.*, DBKKU Y-53, DBKKU Y-102, DBKKU Y-103, and DBKKU Y-104, produced relatively high levels of ethanol at high temperatures after 24 h of fermentation. Therefore, these four strains were selected for further study of the ethanol production from SSJ at various incubation temperatures in a 500-mL flask scale. As shown in Table 2, strain DBKKU Y-53 produced the highest ethanol concentrations and volumetric ethanol productivities at 30 °C, 37 °C, 40 °C and 42 °C compared with the type strain, *K. marxianus* ATCC8554, and the other selected strains tested. At 45 °C, however, the ethanol concentration and volumetric ethanol productivity produced by this strain were lower than of the other strains tested. The ethanol concentrations and volumetric ethanol productivities produced by strains DBKKU Y-102, DBKKU Y-103, and DBKKU Y-104 were not significantly different compared with *K. marxianus* ATCC8554 at 30 °C, 37 °C, and 40 °C. At higher temperatures (42 °C and 45 °C), the ethanol concentrations and volumetric ethanol productivities from these strains were relatively greater than *K. marxianus* ATCC8554. It is evident from these results that increasing the fermentation temperature from 40 °C to 45 °C resulted in a reduction in the ethanol concentrations and productivities. Based on the maximum ethanol concentrations at 37 °C to 42 °C, which is a temperature commonly attained during fermentation in tropical regions, such as Thailand, *S. cerevisiae* DBKKU Y-53 was selected for further analysis.

Table 2. Ethanol production from sweet sorghum juice by isolated yeasts and the type strain, *K. marxianus* ATCC8554, at various temperatures.

Strain	30 °C		37 °C		40 °C		42 °C		45 °C	
	P	Q_p	***P***	Q_p	***P***	Q_p	***P***	Q_p	***P***	Q_p
ATCC 8554	19.55 ± 0.49^{a}	0.27 ± 0.01^{a}	25.06 ± 1.65^{a}	0.35 ± 0.02^{a}	25.83 ± 0.17^{a}	0.36 ± 0.00^{a}	12.45 ± 0.00^{a}	0.17 ± 0.00^{a}	12.45 ± 0.00^{b}	0.17 ± 0.00^{a}
DBKKU Y-53	63.67 ± 4.12^{b}	1.77 ± 0.11^{b}	61.99 ± 4.43^{b}	1.72 ± 0.12^{b}	58.20 ± 0.54^{d}	1.62 ± 0.02^{d}	38.77 ± 1.92^{c}	0.81 ± 0.04^{c}	$7.67b \pm 1.77^{a}$	0.16 ± 0.04^{a}
DBKKU Y-102	19.39 ± 0.63^{a}	0.27 ± 0.01^{a}	34.30 ± 8.99^{a}	0.48 ± 0.12^{a}	29.51 ± 0.34^{b}	0.41 ± 0.00^{b}	29.46 ± 1.10^{b}	0.41 ± 0.02^{b}	21.78 ± 1.27^{c}	0.30 ± 0.02^{b}
DBKKU Y-103	21.89 ± 0.80^{a}	0.30 ± 0.01^{a}	30.78 ± 0.07^{a}	0.43 ± 0.00^{a}	31.63 ± 0.05^{c}	0.44 ± 0.00^{c}	30.70 ± 1.09^{b}	0.43 ± 0.02^{b}	25.32 ± 0.82^{cd}	0.35 ± 0.00^{bc}
DBKKU Y-104	19.24 ± 1.00^{a}	0.27 ± 0.01^{a}	31.14 ± 1.94^{a}	0.43 ± 0.03^{a}	27.38 ± 1.23^{a}	0.38 ± 0.02^{a}	29.98 ± 1.34^{b}	0.42 ± 0.02^{b}	27.65 ± 2.87^{d}	0.38 ± 0.04^{c}

P: ethanol concentration produced ($g \cdot L^{-1}$); Q_p: volumetric ethanol productivity ($g \cdot L^{-1} \cdot h^{-1}$); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

3.4. Factors Influencing Ethanol Production When Using Thermotolerant Yeast S. cerevisiae DBKKU Y-53

There are several factors influencing the growth and ethanol production of yeast, such as the incubation temperature, the pH of the fermentation medium, the cell concentration, the sugar concentration, the nitrogen sources, and the aeration [13,44]. Therefore, the effects of some of the major factors on ethanol production using SSJ by thermotolerant yeast *S. cerevisiae* DBKKU Y-53 were investigated. In this work, the ethanol production efficiency of *S. cerevisiae* DBKKU Y-53 was compared with that of *S. cerevisiae* SC90, which is one of the industrial yeast strains widely used to produce ethanol on a commercial scale in Thailand.

The effect of temperature on ethanol production from SSJ using *S. cerevisiae* DBKKU Y-53 and *S. cerevisiae* SC90 was analyzed, and the results are summarized in Table 3. There was no significantly difference in ethanol concentration produced by both strains at 30 °C. However, at 37 °C and 40 °C, *S. cerevisiae* DBKKU Y-53 produced greater ethanol concentrations than *S. cerevisiae* SC09. The maximum ethanol concentrations produced by *S. cerevisiae* DBKKU Y-53 at 37 °C and 40 °C were 71.73 ± 2.62 g· L^{-1} and 58.14 ± 7.71 g· L^{-1}, respectively. When the incubation temperature was shifted from 40 °C to 42 °C and 45 °C, ethanol concentrations and volumetric ethanol productivities produced by both strains were remarkably decreased. This might be due to the negative effect of high temperature on growth and metabolic processes in yeast cells. It has been reported that high temperature causes a modification of plasma membrane fluidity and a reduction in the effectiveness of the plasma membrane as a semipermeable barrier allowing leakage of essential cofactors and coenzymes required for the activity of enzymes involved in glucose metabolism and ethanol production [44]. Roukas [45] reported that high temperatures caused denaturation of cellular proteins, which resulted in the reduction of cell growth and fermentation activity. Moreover, the reduction in yeast growth at high temperatures was also due to the accumulation of intracellular ethanol, which modifies the cell membrane structure of yeast cell [46]. Our results were in good agreement with Banat *et al.* [40], who observed a reduction in ethanol concentration produced by the thermotolerant yeast *K. marxianus* at 40 °C. Kiran Sree *et al.* [12] reported the ethanol production from glucose (150 g· L^{-1}) using thermotolerant yeast *S. cerevisiae* VS3 and observed a reduction in ethanol concentration when the incubation temperature shifted from 30 °C to 40 °C. Limtong *et al.* [13] demonstrated that the ethanol concentration produced by the newly isolated thermotolerant yeast *K. marxianus* DMKU3-1042 decreased significantly when the incubation temperature was increased from 37 °C to 40 °C. Tofighi *et al.* [47] reported the reduction in cell mass productivity and ethanol fermentation ability of the thermotolerant yeast *S. cerevisiae* when the incubation temperature increased to greater than 40 °C. Recently, Charoensopharat *et al.* [43] found that the ethanol concentration produced by the newly isolated thermotolerant yeast *K. marxianus* decreased dramatically when ethanol fermentation was carried out at temperatures greater than 40 °C.

The pH of the medium is an important factor influencing ethanol yield. Generally, the optimum pH for yeast growth and ethanol production is in the range of 4.0 to 6.0 depending on growth conditions, such as the temperature, the presence of oxygen, the yeast species, and the type of raw material. For instance, the optimum pH for ethanol production from sugarcane juice at high temperatures using the thermotolerant yeast *K. marxianus* DMKU 3-1042 was 5.0 [13]. During ethanol fermentation, the pH of the fermentation medium is almost in the range of 4.0 to 5.5. This pH level typically prevents bacterial contamination during the fermentation process [48]. In this study, the effect of pH on ethanol production by the thermotolerant yeast *S. cerevisiae* DBKKU Y-53 and a reference strain was investigated, and the results are summarized in Tables 4 and 5. The optimum pH for ethanol production from SSJ by both strains at 37 °C and 40 °C was 5.5, which is in agreement with reports by Ercan *et al.* [49] and Sign and Bishnoi [50]. At a pH less than or greater than 5.5, ethanol concentrations and volumetric ethanol productivities tended to decrease. This might be related to the activity of enzymes involved in the ethanol production pathway. It has been reported that enzymes may be inactivated at a pH level that is less than or greater than the optimum value causing a reduction in cell growth and ethanol fermentation ability [48]. The initial pH of the SSJ was in the range of 5.2–5.5. Therefore, a pH of 5.5, which gave the highest ethanol concentration, was selected for further study.

Table 3. Kinetic parameters of ethanol production from sweet sorghum juice at various temperatures by *S. cerevisiae* DBKKUY-53 and *S. cerevisiae* SC90.

Temperature (°C)	*S. cerevisiae* DBKKU Y-53			*S. cerevisiae* SC90		
	P	Q_p	E_y	P	Q_p	E_y
30	82.77 ± 1.99 [a]	2.30 ± 0.06 [a]	88.40 ± 0.59	83.30 ± 2.26 [a]	1.74 ± 0.05 [a]	96.47 ± 2.30
37	71.73 ± 2.62 [b]	1.49 ± 0.05 [b]	87.76 ± 3.22	63.22 ± 2.42 [b]	1.32 ± 0.05 [b]	98.30 ± 0.46
40	58.14 ± 7.71 [c]	1.61 ± 0.20 [b]	88.25 ± 2.73	53.68 ± 1.17 [c]	1.12 ± 0.02 [c]	94.04 ± 0.70
42	32.30 ± 0.49 [d]	0.67 ± 0.01 [c]	88.20 ± 6.37	32.54 ± 1.56 [d]	0.68 ± 0.03 [d]	96.59 ± 0.97
45	9.08 ± 4.02 [e]	0.19 ± 0.08 [d]	62.64 ± 5.60	17.69 ± 2.54 [e]	0.37 ± 0.05 [e]	98.07 ± 0.58

P: ethanol concentration (g· L^{-1}); Q_p: volumetric ethanol productivity (g· L^{-1}· h^{-1}); E_y: percentage of ethanol production efficiency (%); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

Table 4. Kinetic parameters of ethanol production from sweet sorghum juice under various pHs, cell concentrations, and sugar concentrations by *S. cerevisiae* DBKKUY-53 at 37 °C and 40 °C.

Conditions	37 °C			40 °C		
	P	Q_p	E_y	P	Q_p	E_y
			pH			
4.0	68.21 ± 3.74 [a]	1.42 ± 0.08 [a]	87.47 ± 3.61	48.79 ± 2.09 [a]	1.02 ± 0.04 [a]	86.48 ± 0.94
4.5	67.80 ± 1.46 [a]	1.41 ± 0.03 [a]	91.27 ± 3.80	49.40 ± 0.13 [a]	1.03 ± 0.00 [a]	89.89 ± 4.43
5.0	71.73 ± 2.62 [a]	1.49 ± 0.05 [a]	87.76 ± 3.22	59.17 ± 5.33 [b]	1.61 ± 0.20 [c]	88.25 ± 2.73
5.5	74.23 ± 1.76 [a]	1.55 ± 0.08 [a]	92.06 ± 5.70	62.78 ± 0.67 [b]	1.31 ± 0.01 [b]	88.43 ± 0.53
6.0	72.14 ± 1.23 [ab]	1.50 ± 0.03 [a]	91.81 ± 0.66	61.98 ± 0.43 [b]	1.72 ± 0.01 [c]	92.35 ± 0.66
			Cell concentration (cells· mL^{-1})			
1×10^6	74.23 ± 3.62 [a]	1.55 ± 0.08 [a]	92.06 ± 5.70	62.78± 0.67 [a]	1.31 ± 0.01 [a]	88.43 ± 0.53
1×10^7	84.06 ± 1.11 [b]	1.75 ± 0.02 [b]	87.72 ± 0.53	77.14 ± 1.58 [b]	2.14 ± 0.04 [b]	87.78 ± 1.37
1×10^8	88.17 ± 0.17 [b]	2.94 ± 0.01 [c]	84.73± 0.28	83.35 ± 2.37 [c]	3.47 ± 0.10 [c]	89.07 ± 0.04
			Sugar concentration (g· L^{-1})			
200	88.17 ± 0.17 [a]	2.94 ± 0.01 [a]	90.42 ± 0.28	83.35 ± 2.37 [a]	3.47 ± 0.10 [a]	89.07 ± 0.04
250	99.56 ± 0.88 [c]	4.15 ± 0.04 [c]	98.44 ± 0.53	88.97 ± 1.23 [b]	3.71 ± 0.05 [b]	96.53 ± 2.55
300	92.69 ± 0.25 [b]	3.86 ± 0.01 [b]	98.89 ± 0.08	83.25 ± 0.43 [a]	3.47 ± 0.02 [a]	97.40 ± 1.11

P: ethanol concentration (g· L^{-1}); Q_p: volumetric ethanol productivity (g· L^{-1}· h^{-1}); E_y: percentage of ethanol production efficiency (%); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

The initial cell concentration affects not only the ethanol yield but also the substrate consumption rate and ethanol fermentation rate. Generally, high initial cell concentrations reduce the lag phase of growth and increase the sugar consumption and ethanol fermentation rate, leading to a high ethanol yield and productivity. In this study, the effect of initial cell concentrations (1×10^6, 1×10^7, 1×10^8 cells· mL^{-1}) on ethanol fermentation from SSJ containing 220 g· L^{-1} of total sugars was investigated, and the results are summarized in Tables 4 and 5. As a result, increasing the cell concentration resulted in an increase in the ethanol concentration and the volumetric ethanol productivity. The maximum ethanol concentrations and volumetric ethanol productivities produced by *S. cerevisiae* DBKKU Y-53 and SC09 at 37 °C and 40 °C were achieved at an initial cell concentration of 1×10^8 cells· mL^{-1}, which is in good agreement with Charoensopharat *et al.* [43] and Laopaiboon *et al.* [51].

High sugar concentrations (more than 20% w/v) are not often used in industrial ethanol production because they may reduce the yeast cell viability, the substrate conversion rate, and the ethanol yield [52,53]. However, ethanol production with high sugar concentrations have also been reported, and the fermentation efficiencies vary depending on the yeast species and fermentation conditions. For example, Laopaiboon *et al.* [54] reported a maximum ethanol concentration of

120.68 g· L^{-1} and volumetric ethanol productivity of 2.01 g· L^{-1}· h^{-1} when *S. cerevisiae* NP01 was used to produce ethanol from SSJ under a very high gravity fermentation. Charoensopharat *et al.* [43] reported a maximum ethanol concentration of 104.83 g· L^{-1} and a volumetric ethanol productivity of 4.37 g· L^{-1}· h^{-1} when *K. marxianus* DBKKU Y-102 was used to produce ethanol from Jerusalem artichoke tubers at 37 °C during consolidated bioprocessing. To verify the effect of sugar concentration on ethanol production efficiency of the thermotolerant yeast *S. cerevisiae* DBKKU Y-53, SSJ containing various sugar concentrations (200, 250, 300 g· L^{-1}) was tested. As shown in Tables 4 and 5 increasing in the sugar concentration from 200 g· L^{-1} to 250 g· L^{-1} resulted in an increase in the ethanol concentration. However, at a sugar concentration of 300 g· L^{-1} the ethanol concentration was remarkably decreased, and a large amount of sugar remained in the fermentation broth at both 37 °C and 40 °C. High sugar concentrations have been reported to cause negative effects on cell viability and morphology due to an increase in the osmotic pressure, which leads to a reduction in the cell biomass and ethanol yield [53,55]. The maximum ethanol concentrations produced by *S. cerevisiae* DBKKU Y-53 and SC90 were achieved at a sugar concentration of 250 g· L^{-1}. Therefore, this sugar concentration was used for subsequent experiments.

Table 5. Kinetic parameters of ethanol production from sweet sorghum juice under various pHs, cell concentrations, and sugar concentrations by *S. cerevisiae* SC90 at 37 °C and 40 °C.

Conditions	37 °C			40 °C		
	P	Q_p	E_y	P	Q_p	E_y
			pH			
4.0	72.90 ± 2.31 ^{a}	1.22 ± 0.04 ^{bc}	96.21 ± 2.71	55.08 ± 2.67 ^{ab}	0.92 ± 0.04 ^{ab}	97.58 ± 0.28
4.5	70.70 ± 2.55 ^{a}	1.18 ± 0.04 ^{c}	97.15 ± 0.52	53.01 ± 1.35 ^{a}	0.88 ± 0.02 ^{a}	97.55 ± 1.77
5.0	63.22 ± 2.42 ^{b}	1.32 ± 0.05 ^{b}	98.30 ± 0.46	53.68 ± 1.17 ^{ab}	1.12 ± 0.02 ^{c}	94.04 ± 0.70
5.5	75.19 ± 2.40 ^{a}	1.25 ± 0.04 ^{bc}	95.08 ± 2.92	57.62 ± 0.64 ^{b}	0.96 ± 0.01 ^{b}	98.26 ± 1.90
6.0	72.41 ± 1.75 ^{a}	1.51 ± 0.04 ^{a}	75.99 ± 0.05	57.09 ± 0.42 ^{b}	0.96 ± 0.01 ^{b}	80.29 ± 1.89
			Cell concentration (cells· mL^{-1})			
1×10^6	75.19 ± 2.40 ^{a}	1.25 ± 0.04 ^{a}	95.08 ± 2.92	62.78± 0.67 ^{a}	1.31 ± 0.01 ^{a}	88.43 ± 0.53
1×10^7	75.16 ± 0.06 ^{a}	1.57 ± 0.00 ^{b}	90.50 ± 1.06	77.14 ± 1.58 ^{b}	2.14 ± 0.04 ^{b}	87.78 ± 1.37
1×10^8	76.41 ± 1.99 ^{a}	2.55 ± 0.07 ^{c}	79.98 ± 4.31	83.35 ± 2.37 ^{c}	3.47 ± 0.10 ^{c}	89.07 ± 0.04
			Sugar concentration (g· L^{-1})			
200	76.41 ± 1.99 ^{a}	2.55 ± 0.07 ^{c}	79.98 ± 4.31	70.91 ± 0.96 ^{a}	2.96 ± 0.04 ^{a}	88.45 ± 1.81
250	83.05 ± 0.02 ^{b}	1.73 ± 0.00 ^{a}	93.79 ± 2.20	73.00 ± 0.28 ^{a}	2.03 ± 0.01 ^{b}	94.34 ± 0.21
300	78.98 ± 0.78 ^{a}	2.19 ± 0.02 ^{b}	94.79 ± 0.65	71.66 ± 0.96 ^{a}	1.99 ± 0.03 ^{b}	90.93 ± 1.15

P: ethanol concentration (g· L^{-1}); Q_p: volumetric ethanol productivity (g· L^{-1}· h^{-1}); E_y: percentage of ethanol production efficiency (%); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

Approximately 10% of yeast's dry weight is nitrogen [44]. Therefore, nitrogen is one of the important constituents for cell growth and synthesis of structural and functional proteins involved in metabolic processes. Various types of nitrogen sources have been used to supplement ethanol fermentation medium both in organic (yeast extract, corn steep liquor) and inorganic forms (ammonium sulfate, ammonium nitrate, urea, diammonium phosphate). In this study, the effects on ethanol production of using yeast extract, urea, and ammonium sulfate at different concentrations from SSJ by *S. cerevisiae* DBKKU Y-53 and SC90 were determined, and the results are summarized in Tables 6 and 7. Supplementation of urea in the SSJ was shown to significantly enhance the ethanol production by both strains of yeast at 37 °C and 40 °C, which is in good agreement with reports from Yue *et al.* [56]. There was no significant difference in ethanol production at 37 °C when comparing yeast extract supplementation and supplementation-free fermentations. However, at 40 °C, supplementation of yeast extract tended to result in a lower ethanol concentration compared with the control condition. Yeast extract has been reported to be a good organic nitrogen source for ethanol production using *S. cerevisiae*. However, its availability to be utilized by yeast cells is depended on the fermentation

conditions. For example, Laopaiboon *et al.* [54] reported that supplementation of yeast extract in SSJ improved the ethanol production by *S. cerevisiae* NP01 for very high gravity fermentation. With respect to ammonium sulfate, supplementation of this nitrogen compound resulted in a reduction of the ethanol concentration in all conditions tested compared with the control without nitrogen supplementation. These results clearly indicate that ammonium sulfate was not a good nitrogen source for ethanol production at high temperatures using *S. cerevisiae* DBKKU Y-53 and SC90 when SSJ was used as a substrate. One possibility is that this nitrogen compound was not taken up by the yeast cells during high temperature fermentation. Ter Schure *et al.* [57] and Magasanik and Kaiser [58] reported that the uptake of nitrogen by yeast cells is regulated by the mechanism known as nitrogen catabolite repression (NCR). NCR enables yeast cells to select the best nitrogen sources by repressing the transcription of genes involved in the utilization of the poorer nitrogen [59]. However, the effectiveness of NCR mechanism is influenced by many factors including fermentation temperature and the presence of ethanol. Normally, high temperatures and high ethanol concentrations cause the modification of the plasma membrane. Therefore, the nitrogen sources sensing system, which is mainly located in the plasma membrane of yeast cells, may be affected by these stress conditions, leading to the impairment of ammonium sulfate uptake [60,61]. In this study, urea proved to be the best nitrogen source for ethanol production from SSJ during high temperature fermentation using *S. cerevisiae* DBKKU Y-53 and SC90; therefore, it was chosen for further analysis.

Table 6. Kinetic parameters of ethanol production from sweet sorghum juice with various nitrogen sources by *S. cerevisiae* DBKKUY-53 at 37 °C and 40 °C.

Conditions	37 °C			40 °C		
	P	Q_p	E_y	P	Q_p	E_y
Control	99.56 ± 0.88^{bcd}	4.15 ± 0.04^{a}	98.44 ± 0.53	88.97 ± 1.23^{a}	3.71 ± 0.05^{a}	95.56 ± 1.83
Yeast extract						
6	101.30 ± 0.84^{bc}	3.38 ± 0.03^{b}	93.43 ± 1.70	79.19 ± 5.53^{bcd}	3.30 ± 0.23^{bc}	91.78 ± 3.06
9	100.35 ± 0.43^{bcd}	3.35 ± 0.01^{b}	92.39 ± 2.90	76.81 ± 8.84^{cde}	3.20 ± 0.37^{bcd}	94.98 ± 1.66
12	97.05 ± 0.98^{d}	3.23 ± 0.03^{c}	88.20 ± 2.84	83.76 ± 0.26^{abc}	3.49 ± 0.01^{ab}	96.01 ± 1.27
Urea						
0.25	97.97 ± 3.29^{cd}	2.72 ± 0.09^{d}	86.47 ± 1.70	91.18 ± 2.70^{a}	3.04 ± 0.09^{cde}	95.42 ± 3.93
0.50	102.72 ± 1.94^{ab}	2.14 ± 0.04^{e}	96.01 ± 1.16	88.27 ± 0.36^{a}	2.94 ± 0.01^{de}	95.30 ± 3.66
0.75	105.17 ± 1.37^{a}	2.19 ± 0.03^{e}	94.31 ± 4.16	88.66 ± 0.59^{a}	2.96 ± 0.02^{de}	87.82 ± 0.26
1.00	99.90 ± 1.41^{bcd}	2.78 ± 0.04^{d}	85.65 ± 7.36	86.41 ± 2.43^{ab}	2.88 ± 0.08^{e}	83.82 ± 2.15
$(NH_4)_2\ SO_4$						
0.25	97.38 ± 0.23^{d}	3.25 ± 0.01^{c}	94.37 ± 2.64	67.25 ± 1.60^{f}	1.40 ± 0.02^{g}	94.51 ± 2.69
0.50	96.77 ± 2.41^{d}	3.23 ± 0.08^{c}	90.37 ± 5.24	69.90 ± 1.02^{ef}	1.94 ± 0.01^{f}	94.50 ± 1.04
0.75	97.70 ± 0.06^{cd}	2.71 ± 0.00^{d}	86.76 ± 6.82	70.29 ± 0.59^{ef}	1.95 ± 0.01^{f}	96.18 ± 1.51
1.00	99.81 ± 1.44^{bcd}	2.77 ± 0.04^{d}	87.76 ± 6.82	74.27 ± 1.14^{def}	2.06 ± 0.02^{f}	96.25 ± 0.48

P: ethanol concentration ($g \cdot L^{-1}$); Q_p: volumetric ethanol productivity ($g \cdot L^{-1} \cdot h^{-1}$); E_y: percentage of ethanol production efficiency (%); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

Table 7. Kinetic parameters of ethanol production from sweet sorghum juice with various nitrogen sources by *S. cerevisiae* SC90 at 37 °C and 40 °C.

Conditions	37 °C			40 °C		
	P	Q_p	E_y	P	Q_p	E_y
Control	83.05 ± 0.02^{c}	1.73 ± 0.00^{d}	93.79 ± 2.20	73.00 ± 0.28^{a}	2.03 ± 0.01^{c}	94.34 ± 0.21
Yeast extract						
6.0	83.53 ± 0.55^{c}	2.78 ± 0.02^{b}	97.83 ± 0.52	67.17 ± 2.78^{b}	2.24 ± 0.09^{b}	92.54 ± 4.06
9.0	88.23 ± 1.71^{b}	2.94 ± 0.06^{a}	94.07 ± 0.58	67.47 ± 1.81^{b}	2.25 ± 0.06^{b}	97.98 ± 0.50
12.0	87.38 ± 0.96^{b}	2.91 ± 0.03^{a}	94.68 ± 2.30	69.25 ± 1.16^{ab}	2.31 ± 0.04^{b}	98.18 ± 1.50

Table 7. *Cont.*

Conditions	37 °C			40 °C		
	P	***Q_p***	***E_y***	***P***	***Q_p***	***E_y***
			Urea			
0.25	92.48 ± 0.87 ^{a}	2.57 ± 0.02 ^{c}	97.48 ± 0.95	72.87 ± 0.72 ^{a}	3.04 ± 0.03 ^{a}	87.17 ± 1.23
0.50	92.68 ± 0.29 ^{a}	2.57 ± 0.01 ^{c}	98.03 ± 0.08	72.20 ± 2.15 ^{ab}	3.01 ± 0.09 ^{a}	91.91 ± 0.60
0.75	86.76 ± 3.35 ^{b}	2.89 ± 0.11 ^{a}	97.86 ± 0.11	69.90 ± 1.31 ^{ab}	2.91 ± 0.05 ^{a}	94.36 ± 0.37
1.00	87.39 ± 0.39 ^{b}	2.91 ± 0.01 ^{a}	98.36 ± 0.84	73.78 ± 1.17 ^{a}	2.05 ± 0.03 ^{c}	91.65 ± 2.09
			$(NH_4)_2$ SO_4			
0.25	57.57 ± 1.67 ^{f}	1.20 ± 0.02 ^{g}	91.90 ± 0.53	54.03 ± 0.56 ^{d}	1.13 ± 0.01 ^{d}	92.66 ± 2.92
0.50	71.85 ± 0.36 ^{d}	1.00 ± 0.00 ^{h}	91.77 ± 0.42	57.82 ± 0.93 ^{cd}	1.20 ± 0.02 ^{d}	92.23 ± 4.00
0.75	66.54 ± 2.11 ^{e}	1.39 ± 0.04 ^{f}	97.53 ± 0.01	56.65 ± 5.91 ^{cd}	1.18 ± 0.12 ^{d}	86.90 ± 5.20
1.00	72.10 ± 0.78 ^{d}	1.50 ± 0.02 ^{e}	94.84 ± 1.20	59.81 ± 1.34 ^{c}	1.25 ± 0.03 ^{d}	97.68 ± 1.26

P: ethanol concentration (g· L^{-1}); Q_p: volumetric ethanol productivity (g· L^{-1}· h^{-1}); E_y: percentage of ethanol production efficiency (%); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

3.5. Ethanol Production in 2-L Bioreactor

The time profiles of ethanol production from SSJ using *S. cerevisiae* DBKKU Y-53 and SC90 at 37 °C and 40 °C in a 2-L bioreactor are illustrated in Figure 2. During the first 12 h after fermentation, ethanol concentrations produced by both strains sharply increased and reached their maximum values at 24 h for *S. cerevisiae* SC90 and 48 h for *S. cerevisiae* DBKKU Y-53. Table 8 summarizes the kinetic parameters of ethanol production from the SSJ at high temperatures using *S. cerevisiae* DBKKU Y-53 and SC90. It can be seen from this finding that the newly isolated thermotolerant yeast *S. cerevisiae* DBKKU Y-53 resulted in a greater ethanol concentration as well as sugar utilization capability compared with SC90.

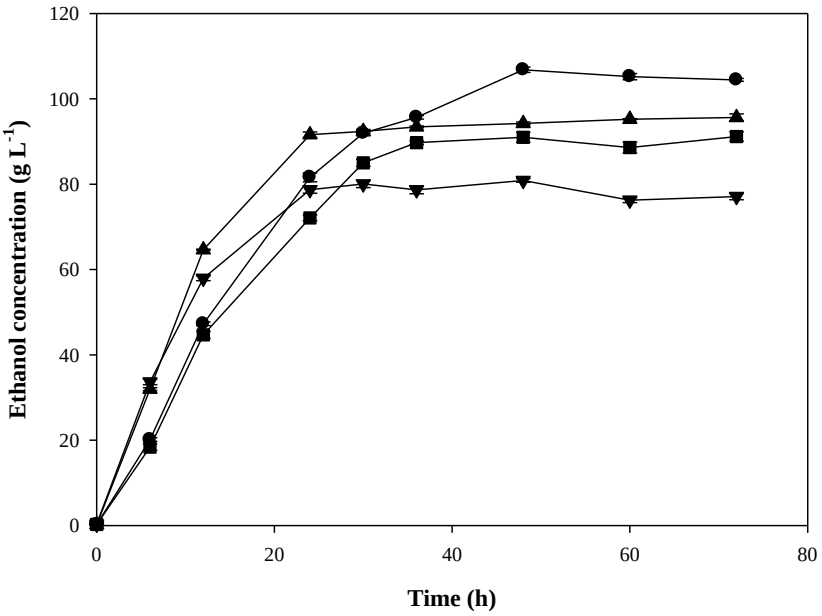

Figure 2. Ethanol production from sweet sorghum juice by *S. cerevisiae* DBKKUY-53 and *S. cerevisiae* SC90 in a 2-L bioreactor at high temperatures. *S. cerevisiae* DBKKUY-53 at 37 °C (●); *S. cerevisiae* DBKKUY-53 at 40 °C (■); *S. cerevisiae* SC90 at 37 °C (▲); *S. cerevisiae* SC90 at 40 °C (▾).

Table 8. Kinetic parameters of ethanol production from sweet sorghum juice by *S. cerevisiae* DBKKU Y-53 and *S. cerevisiae* SC90 in a 2-L bioreactor at 37 °C and 40 °C.

Strains	T (°C)	Parameters (mean ± SD)					
		P	Q_p	$Y_{p/s}$	E_y	t	Sugar Utilized (%)
DBKKU Y-53	37	106.82 ± 0.01 [a]	2.23 ± 0.01 [a]	0.45 ± 0.02	88.42 ± 0.11	48	91.84 ± 0.20
	40	85.01 ± 0.03 [a]	2.83 ± 0.02 [a]	0.42 ± 0.01	82.06 ± 0.09	30	79.35 ± 0.05
SC90	37	91.59 ± 0.01 [b]	3.82 ± 0.02 [b]	0.47 ± 0.01	91.52 ± 0.02	24	82.68 ± 0.02
	40	78.69 ± 0.02 [b]	3.28 ± 0.03 [b]	0.46 ± 0.02	89.08 ± 0.01	24	68.19 ± 0.02

T: incubation temperature; P: ethanol concentration (g·L^{-1}); Q_p: volumetric ethanol productivity (g·L^{-1}·h^{-1}); $Y_{p/s}$: ethanol yield (g·g^{-1}); E_y: percentage of ethanol production efficiency (%); t: fermentation time (h); Mean values ± SD with different letters in the same column are significant different at $p < 0.05$ based on DMRT analysis.

A comparative analysis of the ethanol production from SSJ using the thermotolerant yeast *S. cerevisiae* DBKKU Y-53 with values reported in the literatures using different raw materials and yeast strains was performed, and the results are summarized in Table 9. The ethanol concentration and volumetric ethanol productivity produced by *S. cerevisiae* DBKKU Y-53 were greater than values reported in other works, suggesting that the newly isolated thermotolerant yeast *S. cerevisiae* DBKKU Y-53 was a good candidate for ethanol production from SSJ at high temperatures. Although several isolates of the thermotolerant yeast *K. marxianus* have been reported to be capable of growth and ethanol production at temperatures greater than 45 °C, almost all of these isolates had relatively lower ethanol yields and were less tolerant to ethanol than *S. cerevisiae*. Furthermore, these isolates also required oxygen during ethanol fermentation resulting in an increase in the operating cost. Therefore, the thermotolerant yeast *S. cerevisiae* is more promising for ethanol production at high temperatures on a commercial scale compared with *K. marxianus*.

Table 9. Comparison of ethanol production by *S. cerevisiae* DBKKU Y-53 and other yeast strains reported in the literatures using different raw materials.

Strains	S_0 (g·L^{-1})	C-Source	T (°C)	P (g·L^{-1})	Q_p (g·L^{-1}·h^{-1})	References
S. cerevisiae UV-VS3 100	250	Glucose	30 42	98.0 62.0	2.04 1.29	Sridhar *et al.* [62]
S. cerevisiae VS3	150	Glucose	35 40 42	75.0 60.0 58.0	1.56 1.25 1.21	Kiran Sree *et al.* [12]
S. cerevisiae F111	180	Sugarcane molasses	43	84.0	2.33	Abdel-fattah *et al.* [63]
K. marxianus WR12	180	Sugarcane molasses	43	80.6	2.88	
Pichia kudriavzevii	170	Sugarcane juice	40	71.9	4.00	Dhaliwal *et al.* [64]
K. marxianus DMKU 3-1042	220	Sugarcane juice	37 40	87.0 67.8	1.45 1.13	Limtong *et al.* [13]
Issatchenkia orientalis IPE 100	150	Glucose	37 42	64.3 65.5	1.07 1.37	Kwon *et al.* [65]
S. cerevisiae C3751	100	Glucose	37 41	37.3 38.0	1.55 1.58	Auesukaree *et al.* [20]
S. cerevisiae DBKKU Y-53	250	SSJ	37 40	106.82 85.01	2.23 2.83	This study

S_0: initial sugar concentration; T: incubation temperature; P: ethanol concentration; Q_p: volumetric ethanol productivity.

This work will contribute a significant amount of information on ethanol production at an industrial scale. However, the ethanol production cost at a large-scale should be concerned. There

are many techniques which can be employed to reduce the production cost; for example, cell recycling, various fermentation approaches, such as very high gravity fermentation, consolidated bioprocessing, and continuous ethanol fermentation using stirred tank bioreactor coupling with plug flow bioreactor [17,18,43,54].

3.6. Real-Time RT-PCR Analysis of Gene Expression

Stressful conditions during ethanol fermentation, such as high temperatures, and high concentrations of ethanol or sugar, have been reported to trigger the expression of several stress-responsive genes including those encoding HSPs, enzymes involved in protein degradation and in the glycolysis pathway, and other proteins involved in the synthesis of compatible solutes and reserve carbohydrates [22,23,25,66,67]. In the present study, the expression levels of *hsp26*, *hsp70*, *hsp90*, *hsp104*, *cdc*, *tps*, *nth*, *gsy*, and *rsp* in the thermotolerant yeast *S. cerevisiae* DBKKU Y-53 and SC90 were determined using real-time RT-PCR. As shown in Figure 3, the expression of all genes was activated in both strains of yeast during ethanol fermentation at 40 °C. Although the expression levels of *tps*, *nth*, *gsy*, and *rsp* in *S. cerevisiae* DBKKU Y-53 and SC90 were not dramatically different, the genes encoding HSPs (*hsp26*, *hsp70*, *hsp90*, *hsp104*) in DBKKU Y-53 were much greater than in SC90. This finding suggests that high growth and ethanol fermentation capabilities of DBKKU Y-53 at a high temperature might be related to increased expression levels of HSP genes. Conversely, the expression level of *cdc* encoding pyruvate kinase, which is involved in ATP production, in SC90 was greater than that in DBKKU Y-53, suggesting that SC90 required more ATP for growth and ethanol production at high temperature. The results in this study were in good agreement with Auesukaree *et al.* [20], who observed high expression levels of genes encoding the small HSP, HSP70, HSP90, and HSP100 family and those genes encoding trehalose-6-phosphate synthase, neutral trehalase, and glycogen synthase in *S. cerevisiae* after heat shock and long-term heat exposure at 37 °C. Piper *et al.* [68] reported that several HSPs are constitutively expressed at appropriate temperatures and play a crucial role in folding and assembling proteins. Many of the HSPs, such as HSP70, HSP90, and HSP104, play an important role as molecular chaperones. These molecular chaperones depend on the energy of ATP hydrolysis for function [69]. Therefore, increasing the expression level of *cdc* during ethanol fermentation at a high temperature may provide sufficient energy not only for growth and ethanol fermentation activity but may also function as molecular chaperones to protect the structural integrity of the proteins in yeast cells. In addition to the HSPs, the accumulation of trehalose has also been reported to be associated with heat stress protection and thermotolerance [70]. Trehalose can stabilize the protein structure, reduce the aggregation of denatured proteins, and cooperate with HSPs to promote protein refolding [71–73].

In *S. cerevisiae*, ubiquitin ligase plays a key regulatory role in many cellular processes, such as trafficking, sorting, modifying gene expression, DNA repair, RNA transport as well as the degradation of a large number of proteins in multiple cellular compartments [74]. This protein is also involved in the pathways responsible for the regulation of chromatin function and ultimately controls gene expression under limited nutrient conditions [75]. Most recently, Shahsavarani *et al.* [76] also demonstrated that overexpression of *RSP5* encoding ubiquitin ligase improved the ability of *S. cerevisiae* to tolerate high temperatures. An increase in the ubiquitin ligase, which was observed in this study by the high expression level of *rsp* during ethanol fermentation at high temperature, might regulate the transcription of some genes and induce the heat stress response through the ubiquitination process. Therefore, the ability of *S. cerevisiae* DBKKU Y-53 and SC90 to grow and produce a relatively high level of ethanol at a high temperature might be explained by this mechanism.

In the thermotolerant yeast *K. marxianus*, the molecular mechanisms conferring thermotolerance are complicated and are controlled by multiple genes not only for HSPs biosynthesis but also for those genes encoding the proteins involved in DNA replication and repair, RNA processing, ribosome biogenesis, and carbohydrate metabolism process [21]. The expression of *hsp* genes and those functioning to prevent protein denaturation may be insufficient to allow growth and efficient ethanol

fermentation of *S. cerevisiae* at high temperatures. To gain a better understanding and provide useful information for fundamental and applied research for innovative applications, further studies are required to clarify the precise mechanism conferring thermotolerance in *S. cerevisiae*.

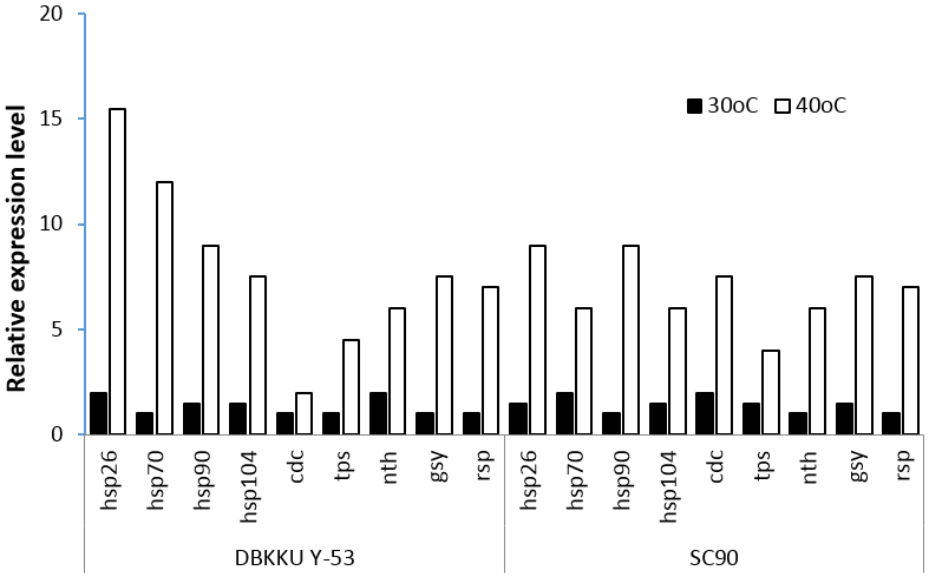

Figure 3. Expression levels of genes encoding HSP26 (*hsp26*), HSP70 (*hsp70*), HSP90 (*hsp90*), HSP104 (*hsp104*), pyruvate kinase (*cdc*), trehalose-6-phosphate synthase (*tps*), neutral trehalase (*nth*), glycogen synthase (*gsy*), and ubiquitin synthase (*rsp*) in the exponential-growth phase of the thermotolerant yeast *S. cerevisiae* DBKKU Y-53 and a reference strain *S. cerevisiae* SC90 during ethanol fermentation at 40 °C. Values presented are the means and relative expression levels of each gene as described in the Materials and Methods section.

4. Conclusions

Utilization of high-potential thermotolerant ethanol-producing yeast is a promising approach to reduce the energy used in cooling systems and to also reduce the operating cost of ethanol production at high temperatures. In this study, the newly isolated thermotolerant ethanol-producing yeast designated as *S. cerevisiae* DBKKU Y-53 exhibited high growth and ethanol production efficiencies at high temperatures (37 °C and 40 °C) compared with the other isolated strains and the industrial ethanol producer *S. cerevisiae* SC90. The optimum conditions for ethanol production by this thermotolerant yeast using SSJ as a raw material were the following: a pH of 5.5, a sugar concentration of 250 g· L^{-1}, a cell concentration of 1.0×10^8 cells· mL^{-1}. The SSJ without the addition of an exogenous nitrogen source can be used directly as substrate for ethanol production at high temperatures by the thermotolerant yeast *S. cerevisiae* DBKKU Y-53. During ethanol fermentation at 40 °C, genes encoding HSP26, HSP70, HSP90, HSP104, pyruvate kinase, trehalose-6-phosphate synthase, neutral trehalase, glycogen synthase, and ubiquitin ligase were highly expressed in *S. cerevisiae* DBKKU Y-53 and SC90 compared with the expression levels at 30 °C. This finding suggests that the growth and ethanol fermentation activity of yeast at high temperatures were not only correlated with the expression of genes involved in heat-stress response but were also correlated with genes involved in ATP production, trehalose and glycogen metabolism, and the protein degradation process.

Acknowledgments: This research was financially supported by the Energy Policy and Planning Office, Ministry of Energy, Thailand and the Research Fund for Supporting Lecturer to Admit High Potential Student to Study and Research on His Expert Program Year 2009, the Graduate School, Khon Kaen University, Thailand. Apart of this work was also supported by the Center for Alternative Energy Research and Development, the Fermentation Research Center for Value Added Agricultural Products (FerVAAP), The New Core to Core Program (CCP) A. Advanced Research Networks on "Establishment of an International Research Core for New Bio-research

Fields with Microbes from Tropical Areas World Class Research Hub of Tropical Microbial Resources and Their Utilization", and a grant from Khon Kaen University.

Author Contributions: Sunan Nuanpeng performed the experiments; Sudarat Thanonkeo analyzed the data; Mamoru Yamada contributed reagents and analysis tools; Pornthap Thanonkeo conceived and designed the experiments, analyzed the data and wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Farrell, A.E.; Plevin, R.J.; Turner, B.T.; Jones, A.D.; O'Hare, M.; Kammen, D.M. Ethanol can contribute to energy and environmental goals. *Sciences* **2006**, *311*, 506–508. [CrossRef] [PubMed]
2. Hill, J.; Nelson, E.; Tilman, D.; Polasky, S.; Tiffany, D. Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 11206–11210. [CrossRef] [PubMed]
3. Yan, J.; Lin, T. Biofuels in Asia. *Appl. Energy* **2009**, *86*, S1–S10. [CrossRef]
4. Zhou, A.; Thomson, E. The development of biofuels in Asia. *Appl. Energy* **2009**, *86*, S11–S20. [CrossRef]
5. Salassi, M.E. *The Economic Feasibility of Ethanol Production from Sugar Crops*; LSU AgCenter: Baton Rouge, LA, USA, 2007.
6. Daniel, P.H.; Lueschen, W.E.; Kanne, B.K.; Hoverstad, T.R. A comparison of sweet sorghum cultivars and maize for ethanol production. *J. Prod. Agric.* **1991**, *4*, 377–381.
7. Bvochora, J.M.; Read, J.S.; Zvauya, R. Application of very high gravity technology to the cofermentation of sweet stem sorghum juice and sorghum grain. *Ind. Crops Prod.* **2000**, *11*, 11–17. [CrossRef]
8. Woods, J. Integrating Sweet Sorghum and Sugarcane for Bioenergy: Modeling the Potential for Electricity and Ethanol Production in SE Zimbabwe. Ph.D. Thesis, University of London, London, UK, 2000.
9. Chan-u-tit, P.; Laopaiboon, L.; Jaisil, P.; Laopaiboon, P. High level ethanol production by nitrogen and osmoprotectant supplementation under very high gravity fermentation conditions. *Energies* **2013**, *6*, 884–899. [CrossRef]
10. Yu, M.; Li, J.; Chang, S.; Du, R.; Li, S.; Zhang, L.; Fan, G.; Yan, Z.; Cui, T.; Cong, G.; *et al*. Optimization of ethanol production from NaOH-pretreated solid state fermented sweet sorghum bagasse. *Energies* **2014**, *7*, 4054–4067. [CrossRef]
11. Gnansounou, E.; Dauriat, A.; Wyman, C.E. Refining sweet sorghum to ethanol and sugar: Economic trade-offs in the context of North China. *Bioresour. Technol.* **2005**, *96*, 985–1002. [CrossRef] [PubMed]
12. Kiran Sree, N.; Sridhar, M.; Suresh, K.; Banat, I.M.; Venkateswar, R.L. Isolation of thermotolerant, osmotolerant, flocculating *Saccharomyces cerevisiae* for ethanol production. *Bioresour. Technol.* **2000**, *72*, 43–46. [CrossRef]
13. Limtong, S.; Sringiew, C.; Yongmanitchai, W. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour. Technol.* **2007**, *98*, 3367–3374. [CrossRef] [PubMed]
14. Kourkoutas, Y.; Bekatorou, A.; Banat, I.M.; Marchant, R.; Koutinas, A.A. Immobilization technologies and support materials suitable in alcohol beverages production: A review. *Food Microbiol.* **2004**, *21*, 377–397. [CrossRef]
15. Zafar, S.; Owais, M. Ethanol production from crude whey by *Kluyveromyces marxianus*. *Biochem. Eng. J.* **2006**, *27*, 295–298. [CrossRef]
16. Yuan, W.J.; Zhao, X.Q.; Ge, X.M.; Bai, F.W. Ethanol fermentation with *Kluyveromyces marxianus* from Jerusalem artichoke grown in salina and irrigated with a mixture of seawater and freshwater. *J. Appl. Microbiol.* **2008**, *105*, 2076–2083. [CrossRef] [PubMed]
17. Yuan, W.J.; Chang, B.L.; Ren, J.G.; Liu, J.P.; Bai, F.W.; Li, Y.Y. Consolidated bioprocessing strategy for ethanol production from Jerusalem artichoke tubers by *Kluyveromyces. marxianus* under high gravity conditions. *J. Appl. Microbiol.* **2011**, *112*, 38–44. [CrossRef] [PubMed]
18. Hu, N.; Yuan, B.; Sun, J.; Wang, S.A.; Li, F.L. Thermotolerant *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* strains representing potentials for bioethanol production from Jerusalem artichoke by consolidated bioprocessing. *Appl. Microbiol. Biotechnol.* **2012**, *95*, 1359–1368. [CrossRef] [PubMed]

19. Edgardo, A.; Carolina, P.; Manuel, R.; Juanita, F.; Baeza, J. Selection of thermotolerant yeast strains *Saccharomyces cerevisiae* for bioethanol production. *Enzyme Microb. Technol.* **2008**, *43*, 120–123. [CrossRef]
20. Auesukaree, C.; Koedrith, P.; Saenpayavai, P.; Asvarak, T.; Benjaphokee, S.; Sugiyama, M.; Kaneko, Y.; Harashima, S.; Boonchird, C. Characterization and gene expression profiles of thermotolerant *Saccharomyces cerevisiae* isolates from Thai fruits. *J. Biosci. Bioeng.* **2012**, *114*, 144–149. [CrossRef] [PubMed]
21. Lertwattanasakul, N.; Kosaka, T.; Hosoyama, A.; Suzuki, Y.; Rodrussamee, N.; Matsutani, M.; Murata, M.; Fujimoto, N.; Suprayogi; Tsuchikane, K.; *et al*. Genetic basis of the highly efficient yeast *Kluyveromyces marxianus*: Complete genome sequence and transcriptome analyses. *Biotechnol. Biofuels* **2015**, *8*. [CrossRef] [PubMed]
22. Walter, S.; Buchner, J. Molecular chaperones-cellular machines for protein folding. *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 1098–1113. [CrossRef]
23. Borges, J.C.; Ramos, C.H. Protein folding assisted by chaperones. *Protein Pept. Lett.* **2005**, *12*, 257–261. [CrossRef] [PubMed]
24. Purvis, J.E.; Yomano, L.P.; Ingram, L.O. Enhanced trehalose production improved growth of *Escherichia coli* under osmotic stress. *Appl. Environ. Microbiol.* **2005**, *71*, 3761–3769. [CrossRef] [PubMed]
25. Li, L.L.; Ye, Y.R.; Pan, L.; Zhu, Y.; Zheng, S.P.; Lin, Y. The induction of trehalose and glycerol in *Saccharomyces cerevisiae* in response to various stresses. *Biochem. Biophy. Res. Commun.* **2009**, *387*, 778–783. [CrossRef] [PubMed]
26. Parrou, J.L.; Teste, M.A.; Francois, J. Effects of various types of stress on the metabolism of reserve carbohydrates in *Saccharomyces cerevisiae*: Genetic evidence for a stress-induced recycling of glycogen and trehalose. *Microbiology* **1997**, *143*, 1891–1900. [CrossRef] [PubMed]
27. Unnikrishnan, I.; Miller, S.; Meinke, M.; LaPorte, D.C. Multiple positive and negative elements involved in the regulation of expression of *GSY1* in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **2003**, *278*, 26450–26457. [CrossRef] [PubMed]
28. Kurtzman, C.; Robnett, C.J. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek* **1998**, *73*, 331–371. [CrossRef] [PubMed]
29. Harju, S.; Fedosyuk, H.; Peterson, K.R. Rapid isolation of yeast genomic DNA: Bust n' Grab. *BMC Biotechnol.* **2004**, *4*. [CrossRef] [PubMed]
30. O'Donnell, K. *Fusarium* and its near relatives. In *The Fungal Holomorph: Mitotic, Meiotic and Pleomorphic Speciation in Fungal Systematics*; Reynolds, D.R., Taylor, J.W., Eds.; CAB International: Wallingford, UK, 1993; pp. 225–233.
31. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. Mega 5: Molecular evolutionary genetics analysis using maximum likelihood evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
32. Saitou, N.; Nei, M. The neighbor-joining method. A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4*, 406–425. [PubMed]
33. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
34. Zoecklein, B.W.; Fugelsang, K.C.; Gump, B.H.; Nury, F.S. *Wine Analysis and Production*; Chapman & Hall: New York, NY, USA, 1995.
35. Dubois, M.; Gilles, K.A.; Hamilton, J.R.; Robers, P.K.; Smith, F. Colourimetric method for determination of sugar and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]
36. Banat, I.M.; Nigam, P.; Singh, D.; Marchant, R.; McHale, A.P. Review: Ethanol production at elevated temperatures and alcohol concentrations: Part I—Yeasts in general. *World J. Microbiol. Biotechnol.* **1998**, *14*, 809–821. [CrossRef]
37. Van Uden, N. Cardinal temperatures of yeast. In *Handbook of Microbiology*; Laskin, A.I., Lechevalier, H.H.A., Eds.; CRC Press: Boca Raton, FL, USA, 1984; Volume VI, pp. 1–8.
38. Lee, C.; Yamakawa, T.; Kodama, T. Rapid growth of a thermotolerant yeast on palm oil. *World J. Microbiol. Biotechnol.* **1993**, *9*, 187–190. [CrossRef] [PubMed]
39. Ballesteros, I.; Ballesteros, M.; Cabanas, A.; Carrasco, J.; Martin, C.; Negro, M.; Saez, F.; Saez, R. Selection of thermotolerant yeasts for simultaneous saccharification and fermentation (SSF) of cellulose to ethanol. *Appl. Biochem. Biotechnol.* **1991**, *28–29*, 307–315. [CrossRef] [PubMed]

40. Banat, I.M.; Nigam, P.; Marchant, R. Isolation of thermotolerant, fermentative yeasts growing at 52 °C and producing ethanol at 45 °C and 50 °C. *World J. Microbiol. Biotechnol.* **1992**, *8*, 259–263. [CrossRef] [PubMed]
41. Tanimura, A.; Nakamura, T.; Watanabe, I.; Ogawa, J.; Shima, J. Isolation of a novel strain of *Candida shehatae* for ethanol production at elevated temperature. *SpringerPlus* **2012**, *1*, 27. [CrossRef] [PubMed]
42. Kaewkrajay, C.; Dethoup, T.; Limtong, S. Ethanol production from cassava using a newly isolated thermotolerant yeast strain. *ScienceAsia* **2014**, *40*, 268–277. [CrossRef]
43. Charoensopharat, K.; Thanonkeo, P.; Thanonkeo, S.; Yamada, M. Ethanol production from Jerusalem artichoke tubers at high temperature by newly isolated thermotolerant inulin-utilizing yeast *Kluyveromyces marxianus* using consolidated bioprocessing. *Antonie Van Leeuwenhoek* **2015**, *108*, 173–190. [CrossRef] [PubMed]
44. Walker, G.M. The roles of magnesium in biotechnology. *Crit. Rev. Biotechnol.* **1994**, *14*, 311–354. [CrossRef] [PubMed]
45. Roukas, T. Solid-state fermentation of carob pods for ethanol production. *Appl. Microbiol. Biotechnol.* **1994**, *41*, 296–301. [CrossRef]
46. Torija, M.J.; Beltran, G.; Novo, M.; Poblet, M.; Guillamon, J.M.; Mas, A.; Rozes, N. Effects of fermentation temperature and *Saccharomyces* species on the cell fatty acid composition and presence of volatile compounds in wine. *Int. J. Food Microbiol.* **2003**, *85*, 127–136. [CrossRef]
47. Tofighi, A.; Assadi, M.M.; Asadirad, M.H.A.; Karizi, S.Z. Bio-ethanol production by a novel autochthonous thermo-tolerant yeast isolated from wastewater. *J. Environ. Health Sci. Eng.* **2014**, *12*, 107. [CrossRef] [PubMed]
48. Narendranath, N.V.; Power, R. Relationship between pH and medium dissolved solids in terms of growth and metabolism of *Lactobacilli* and *Saccharomyces cerevisiae* during ethanol production. *Appl. Environ. Microbiol.* **2005**, *71*, 2239–2243. [CrossRef] [PubMed]
49. Ercan, Y.; Irfan, T.; Mustafa, K. Optimization of ethanol production from carob pod extract using immobilized *Saccharomyces cerevisiae* cells in a stirred tank bioreactor. *Bioresour. Technol.* **2013**, *135*, 365–371. [CrossRef] [PubMed]
50. Singh, A.; Bishnoi, N.R. Ethanol production from pretreated wheat straw hydrolyzate by *Saccharomyces cerevisiae* via sequential statistical optimization. *Ind. Crops Prod.* **2013**, *41*, 221–226. [CrossRef]
51. Laopaiboon, L.; Thanonkeo, P.; Jaisil, P.; Laopaiboon, P. Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*. *World J. Microbiol. Biotechnol.* **2007**, *23*, 1497–1501. [CrossRef]
52. Ingledew, W.M. Alcohol production by *Saccharomyces cerevisiae*: A yeast primer. In *The Alcohol Textbook*, 3rd ed.; Jacques, K.A., Lyons, T.P., Kelsall, D.R., Eds.; Nottingham University Press: Nottingham, UK, 1999; pp. 49–87.
53. Ozmichi, S.; Kargi, F. Ethanol fermentation of cheese whey powder solution by repeated fed-batch operation. *Enzyme Microb. Technol.* **2007**, *41*, 169–174.
54. Laopaiboon, L.; Nuanpeng, S.; Srinophakun, P.; Klanrit, P.; Laopaiboon, P. Ethanol production from sweet sorghum juice using very high gravity technology: Effects of carbon and nitrogen supplementations. *Bioresour. Technol.* **2009**, *100*, 4176–4182. [CrossRef] [PubMed]
55. Bai, F.W.; Chen, L.J.; Zhang, Z.; Anderson, W.A.; Moo-Young, M. Continuous ethanol production and evaluation of yeast cell lysis and viability loss under very high gravity medium conditions. *J. Biotechnol.* **2004**, *110*, 287–293. [CrossRef] [PubMed]
56. Yue, G.; Yu, J.; Zhang, X.; Tan, T. The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. *Biomass Bioenergy* **2012**, *39*, 48–52. [CrossRef]
57. Ter Schure, E.G.; van Riel, A.A.W.; Verrips, C.T. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **2000**, *24*, 67–83. [CrossRef] [PubMed]
58. Magasanik, B.; Kaiser, C.A. Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* **2002**, *290*, 1–18. [CrossRef]
59. Magasanik, B. Regulation of nitrogen utilization. In *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene expression*; John, E.W., Pringle, J.R., Broach, J.R., Eds.; Cold Spring Harbor: New York, NY, USA, 1992; pp. 283–317.
60. Beltran, G.; Esteve-Zarzoso, B.; Rozès, N.; Mas, A.; Guillamón, J.M. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *J. Agric. Food Chem.* **2005**, *53*, 996–1002. [CrossRef] [PubMed]

61. Beltran, G.; Rozès, N.; Mas, A.; Guillamón, J.M. Effect of low-temperature fermentation on yeast nitrogen metabolism. *World J. Microbiol. Biotechnol.* **2007**, *23*, 809–815. [CrossRef]
62. Sridhar, M.; Kiran Sree, N.; Venkateswar, R.L. Effect of UV radiation on thermotolerance, ethanol tolerance and osmotolerance of *Saccharomyces cerevisiae* VS1 and VS3 strains. *Bioresour. Technol.* **2002**, *83*, 199–202. [CrossRef]
63. Abdel-fattah, W.R.; Fadil, M.; Nigam, P.; Banat, I.M. Isolation of thermotolerant ethanologenic yeasts and use of selected strains in industrial scale fermentation in an Egyptian distillery. *Biotechnol. Bioeng.* **2000**, *68*, 531–535. [CrossRef]
64. Dhaliwal, S.S.; Oberoi, H.S.; Sandhu, S.K.; Nanda, D.; Kumar, D.; Uppal, S.K. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. *Bioresour. Technol.* **2011**, *102*, 5968–5975. [CrossRef] [PubMed]
65. Kwon, Y.J.; Ma, A.Z.; Li, Q.; Wang, F.; Zhuang, G.Q.; Liu, C.Z. Effect of lignocellulosic inhibitory compounds on growth and ethanol fermentation of newly-isolated thermotolerant *Issatchenkia. orientalis*. *Bioresour. Technol.* **2011**, *102*, 8099–8104. [CrossRef] [PubMed]
66. Ni, H.T.; LaPorte, D.C. Response of a yeast glycogen synthase gene to stress. *Mol. Microbiol.* **1995**, *16*, 1197–1205. [CrossRef] [PubMed]
67. Verghese, J.; Abrams, J.; Wang, Y.; Morano, K.A. Biology of the heat shock responses: Building yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiol. Mol. Biol. Rev.* **2012**, *76*, 115–158. [CrossRef] [PubMed]
68. Piper, P.W. Molecular events associated with acquisition of heat tolerance by the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **1993**, *11*, 339–356. [CrossRef] [PubMed]
69. Lindquist, S. Heat-shock proteins and stress tolerance in microorganisms. *Curr. Opin. Genet. Dev.* **1992**, *2*, 748–755. [CrossRef]
70. Singer, M.A.; Lindquist, S. Thermotolerance in *Saccharomyces cerevisiae*: The Yin and Yang of trehalose. *Trends Biotechnol.* **1998**, *16*, 460–468. [CrossRef]
71. Hottiger, T.; De Virgilio, C.; Hall, M.N.; Boller, T.; Wiemken, A. The role of trehalose synthesis for the acquisition of thermotolerance in yeast II. Physiological concentrations of trehalose increase the thermal stability of proteins *in vitro*. *Eur. J. Biochem.* **1994**, *219*, 187–193. [CrossRef] [PubMed]
72. Zancan, P.; Sola-Penna, M. Trehalose and glycerol stabilize and renature yeast inorganic pyrophosphate inactivated by very high temperatures. *Arch. Biochem. Biophys.* **2005**, *444*, 52–60. [CrossRef] [PubMed]
73. Lindquist, S.; Kim, G. Heat-shock protein 104 expression is sufficient for thermotolerance in yeast. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5301–5306. [CrossRef] [PubMed]
74. Krsmanović, T.; Kőlling, R. The HECT E3 ubiquitin ligase Rsp5 is important for ubiquitin homeostasis in yeast. *FEBS Lett.* **2004**, *577*, 215–219. [CrossRef] [PubMed]
75. Cardona, F.; Aranda, A.; Del Olmo, L. Ubiquitin ligase Rsp5 is involved in the gene expression changes during nutrient limitation in *Saccharomyces cerevisiae*. *Yeast* **2009**, *26*, 1–15. [CrossRef] [PubMed]
76. Shahsavarani, H.; Sugiyama, M.; Kaneko, Y.; Chuenchit, B.; Harashima, S. Superior thermotolerance of *Saccharomyces cerevisiae* for efficient bioethanol fermentation can be achieved by overexpression of *RSP5* ubiquitin ligase. *Biotechnol. Adv.* **2012**, *30*, 1289–1300. [CrossRef] [PubMed]