CHAPTER III
ISOLATION AND SELECTION OF THERMOTOLERANT YEAST CAPABLE OF PRODUCING ETHANOL FROM JERUSALEM ARTICHOKE JUICE

3.1 Introduction

Ethanol production is usually accomplished by microbial conversion of carbohydrates present in agricultural products such as corn, sugarcane, wheat or rice straw and cassava root. Fermentative production of ethanol from renewable biomass is an attractive source of energy because plant biomass is the only sustainable source of organic fuels, chemicals, and materials available to humanity. Jerusalem artichoke (*Helianthus tuberosus* L.) is one of the most interesting materials among those agricultural products. It belongs to the sunflower family, which can grow in a wide variety of climates. This plant contains nearly 20% of carbohydrates, 70–90% of which is inulin. Inulin consists of linear chains of β (2→1) linked D-fructose units. Each chain is terminated by a D-glucose residue linked to fructose by an α (1→2) bond (Ge and Zhang, 2005), which has potential for ethanol fermentation, fructose syrup production, single cell oil and inulooligosaccharide (IOS) production (Chi et al., 2009; Zhao et al., 2010). Ethanol and temperature tolerance for a yeast strain at high temperatures and high ethanol concentration are important characteristics of microorganisms of interest to industrial ethanol production. Thermotolerant yeasts have been investigated for many potential applications (Dhaliwal et al., 2011; de Souza et al., 2012; Hashem et al., 2013). Fakruddin et al. (2013) isolated and characterized the strain P (*Saccharomyces Unisporus*), strain C (*Saccharomyces cerevisiae*), strain T (*Saccharomyces cerevisiae*) and strain DB2 (*Candida piceae*) from agro industrial waste. Most of the strains were thermotolerant, pH tolerant, ethanol tolerant as well as osmotolerant. These strains could be potential for ethanol production from sugarcane molasses. Ethanol fermentation process at high temperature with thermotolerant yeasts have several advantages such as reduce cost for cooling system, reduce risk of contamination, and increase the speed of catalytic

reactions related to fermentation. In this work, isolation and characterization of thermotolerant yeasts capable of producing ethanol from Jerusalem artichoke (*Helianthus tuberosus* L.) at high temperature were investigated.

3.2 Materials and methods

3.2.1 Isolation of the yeast strains

Various sources of samples such as sugarcane juice, rotten fruits and soil and plant materials from Jerusalem artichoke plantations were collected from different locations in Thailand including Khon Kaen, Udon Thani, Lop Buri, Nong Khai, and subjected to the isolation and screening of thermotolerant yeasts by using enrichment culture technique (Limtong et al., 2007). Isolation was carried out at 35^{o}C in 250-ml Erlenmeyer flask containing 50 ml yeast extract malt extract (YM) broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) supplemented with 4% (v/v) ethanol on a rotary shaker at 150 rpm for 2 days. After incubation, the enriched cultures were then streaked on YM agar plates and incubated at 35^{o}C until the yeast colonies appeared. Pure cultures were maintained on YM medium and stored at 4^{o}C with subculturing every 2 months.

3.2.2 Screening for the thermotolerant yeasts

Thermotolerant yeast strains were selected based on their growth performances at temperatures ranged from 30-50°C by streak plate and drop plate technique. The isolated yeast strains were inoculated for 24 h at 30°C on a rotary shaker with shaking speed at 200 rpm. Enriched cultures were then streaked on YM agar plate. The drop plate technique was performed on YM agar, the aliquots of 10-fold culture dilutions of cells were dropped onto YM agar plate. Successful cultures were collected and screened further for their ethanol production efficiency at elevated temperature.

3.2.3 Screening for inulin-utilizing yeasts

The inulin medium (1.4% KH_2PO_4, 1% inulin, 0.6% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, 0.1% trisodium citrate, 0.02% $MgSO_4.7H_2O$) was used to screen for the inulin utilizing yeasts as described by Castro et al. (1995). The screening method was carried out in test tube containing inulin medium and the Durham fermentation tube was placed into each tube. The CO_2 accumulation was then observed, and the strains

that filled the Durham tube with gas were selected. The drop plate technique was performed on inulin agar medium, the aliquots of 10-fold culture dilutions of cells were dropped onto inulin agar plate. The cultures were kept at 30°C for 24-48 h.

3.2.4 Ethanol production from Jerusalem artichoke juice by the isolated yeast strains

Ethanol fermentations ability of the isolated yeasts in Erlenmeyer flask was compared using Jerusalem artichoke juice without acid or enzymatic pre-treatment as a raw material. Batch fermentation was performed in 500-ml Erlenmeyer flask under static and shaking condition at 30, 37, 40 and 45^{o}C. Inoculum was prepared by transferring one loopful of 24 h pure culture to inoculate 125-ml Erlenmeyer flask containing 50 ml YM medium and incubated at 30^{o}C on a rotary shaker with shaking speed at 200 rpm for 15-18 h. The culture was transferred to the screening medium to give an initial cell concentration of 1×10^{6} cells/ml. During fermentation, samples were withdrawn at certain time intervals for further analysis.

3.2.5 Analytical methods

Total sugars were assayed by the Phenol-sulfuric acid method (Dubois et al., 1956). The yeast cell numbers and total soluble solids of the fermentation broth were determined by direct counting method using haemacytometer and hand-held refractometer, respectively (Zoecklien et al., 1995). The pH was measured by pH meter. Ethanol concentration in the culture medium was measured by gas chromatography (GC) (Shimadzu GC-14B, Japan) using polyethylene glycol (PEG-20M) packed column with a flame ionization detector. N_2 was used as a carrier gas and isopropanol was used as an internal standard (Laopaiboon et al., 2009). The ethanol yield (*Y*ps) was calculated as the actual ethanol produced and expressed as g ethanol per g sugar utilized (g/g). The volumetric ethanol productivity (*Qp*, g/l.h) was calculated by the following equations:

$$Qp = P/t$$

Where *P* is the ethanol concentration (g/l) and *t* is the fermentation time (h) giving the highest ethanol concentration.

3.2.6 Identification of the selected yeast strains

Identification of the yeast strains was carried out using morphological and D1/D2 domain of 26S rDNA gene sequencing analysis (Kurtzman and Robnett,

1998). Yeasts genomic DNA was prepared as described by Harju et al. (2004) with slight modifications. Cells were grown at 30^{o}C for 24 h in 1.5 ml of YM broth on a rotary shaker with shaking speed at 200 rpm and cells were collected by centrifugation at 5,000 rpm for 5 min at 4^{o}C. The cell pellets were washed once with distilled water and then resuspended in 200 µl of lysis buffer [2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA (pH 8.0)]. The tubes were placed in a -80^{o}C freezer until they were completely frozen, then boiled for 1 min to thaw quickly. The process was repeated, and the tubes were vortexed vigorously for 30 s and extracted with 200 µl phenol/chloroform/isoamyl. The tubes were vortexed for 2 min and then centrifuged at 10,000 rpm for 3 min. The upper aqueous layer was transferred to a tube containing 400 µl of ice-cold absolute ethanol. Precipitating the samples at room temperature for 5 min and then centrifuged at 10,000 rpm for 5 min. The DNA pellets were washed with 500 µl of 70% ethanol followed by drying the DNA pellets for 5 min at 60°C. The DNA was resuspended in 20 µl TE buffer [10 mM Tris, 1 mM EDTA (pH 8.0)]. The D1/D2 domain of 26S rDNA was amplified by PCR with forward primer NL-1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer NL-4 (5′-GGT CCG TGT TTC AAG ACG G-3′) (O'Donnell, 1993). After PCR amplification with specific primers, the PCR product was separated on 1.0% agarose gel by agarose gel electrophoresis and purified using NucleoSpin® Extract II (Machery-nagel, Germany) according to the manufacture's instruction. PCR products of 26S rDNA were sequenced by the First BASE Laboratories Sdn Bhd (Seri Kembangan, Selangor Darul Ehsan, Malaysia) and homology analysis was performed using BLAST program by comparing pairwise of the sequences.

3.3 Results and discussion

3.3.1 Isolation and selection of thermotolerant yeasts

Based on the enrichment culture technique, a total of fifty isolates of yeast were obtained from various sources. These isolated of yeast grew well on YM agar medium at 30-40^{o}C. Among them, six isolates were able to grow at temperature higher than 40^{o}C as well as in the medium containing inulin as carbon source, suggesting that these isolated of yeasts are inulin-utilizing yeast. All these six isolates

(designated as DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107) exhibited the ability to grow in YM medium containing 4% (v/v) ethanol and at temperature up to 45^{o}C (Figure 3.1 and 3.2).

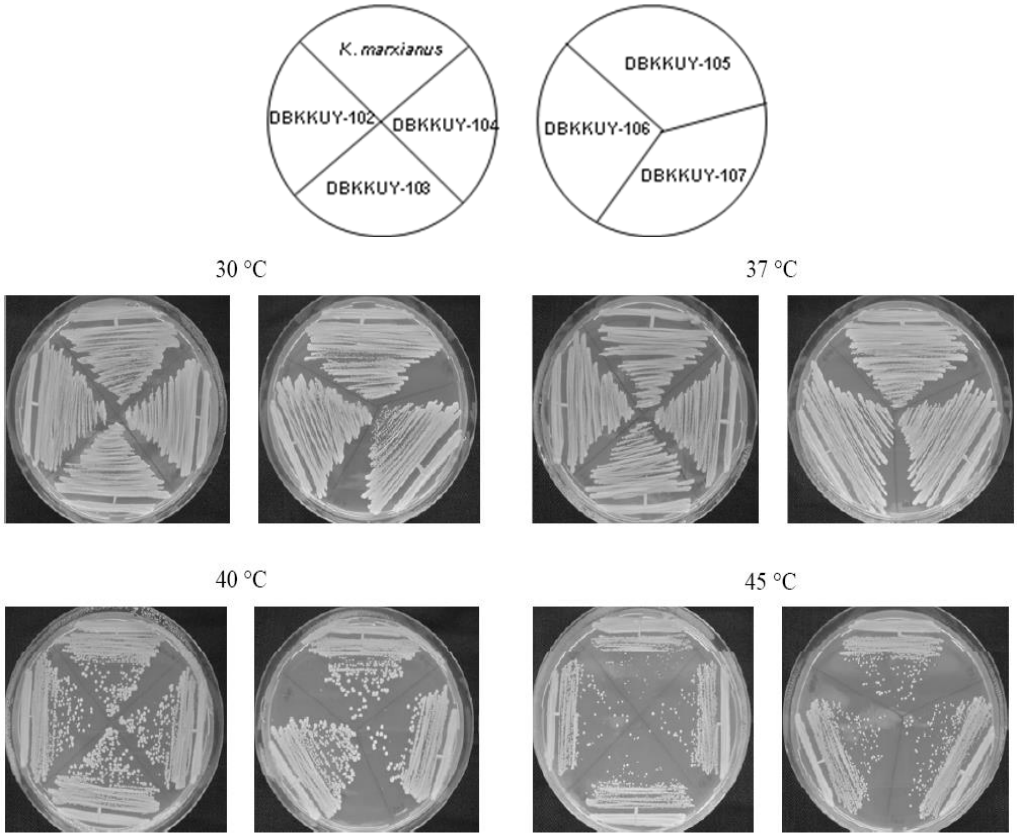

Figure 3.1 Growth properties of the type strain, *K. marxianus,* and newly isolated yeast, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107 on YM agar plates and incubated at the indicated temperatures for 24 h

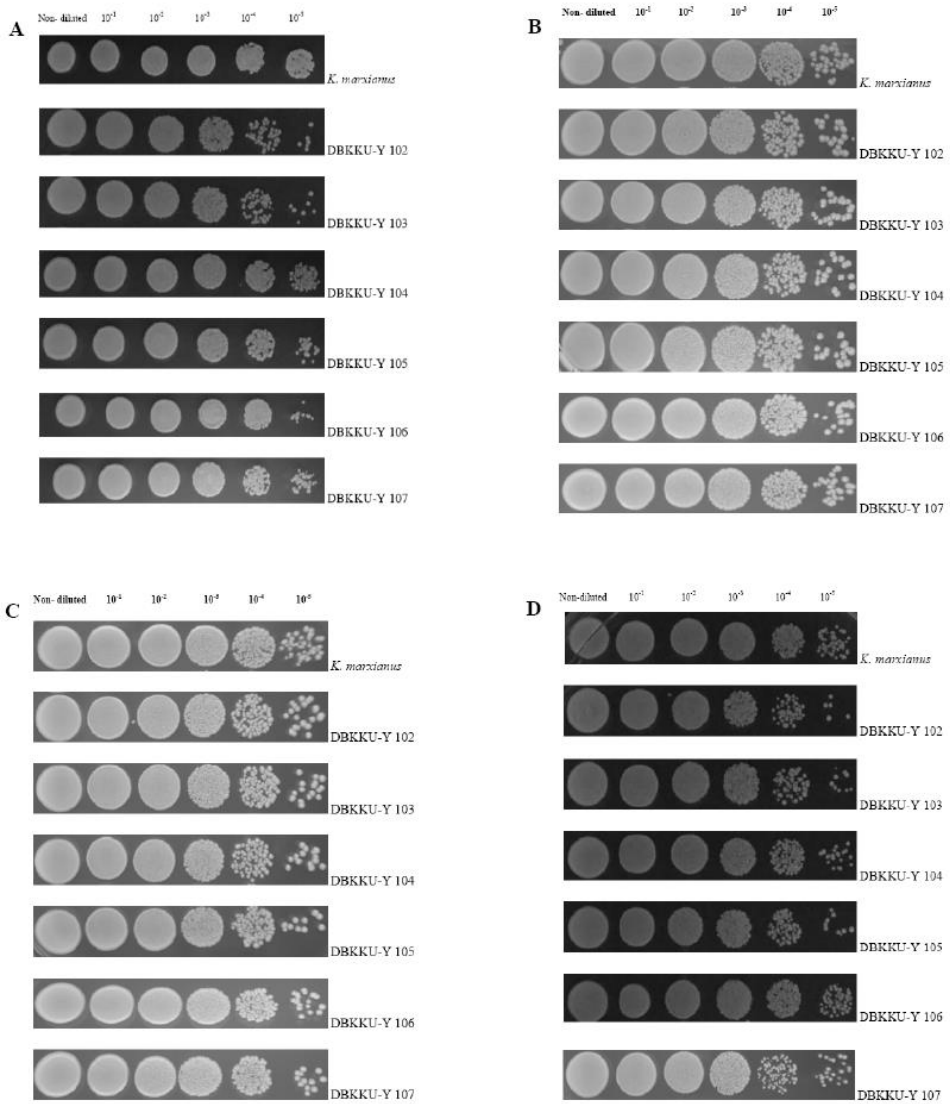

Figure 3.2 Exponentially growing yeast cultures of *K. marxianus*, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107 were spotted in 10-fold serial dilutions onto YM agar plates and grown at 30 (A), 37 (B), 40 (C) and 45°C (D) for 24 h

All these isolates also produced CO_2 after cultivated in inulin medium, same as that observed in the type strain, *K. marxianus* (data not shown). As shown in Figure 3.3A and 3B, all yeast strains can utilize inulin as carbon and energy sources for growth, and high growth level was observed after 48 h of incubation on inulin agar plate. These results indicated that the isolated yeast strains can produce the enzymes inulinase, which can be hydrolyze inulin into fructose and glucose that essential for

growth of microorganisms. The most of the inulinase producing microorganisms such as *K. marxianus*, *Cryptococcus aureus*, and *K. fragilis* are common inulinase producer (Kalil et al. 2005; Pandey et al. 1999). Based on their abilities to grow at high temperature (Figure 3.1 and 3.2), we speculated that all these six isolated are thermotolerant yeast, as the description of thermotolerant microorganism given by McCracken and Gong (1982), the type strains were classified as thermotolerant yeast since their maximum temperatures ranging from 37-45°C.

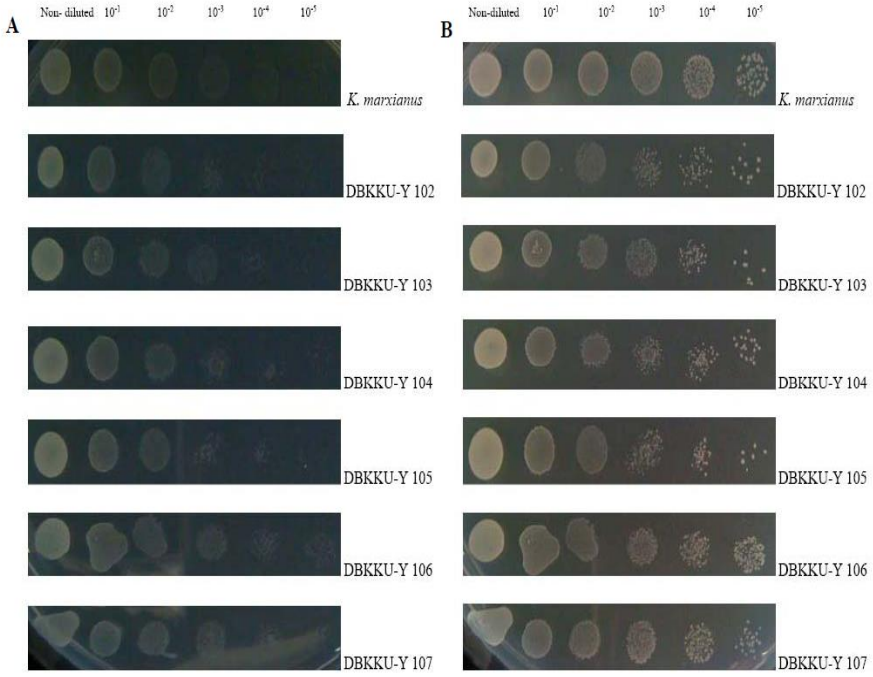

Figure 3.3 Exponentially growing yeast cultures of *K. marxianus*, DBKKU Y-102, DBKKU Y-103, DBKKU Y-104, DBKKU Y-105, DBKKU Y-106 and DBKKU Y-107 were spotted in 10-fold serial dilutions onto inulin agar plates and grown at 30°C for 24 (A) and 48 h (B)

The morphological characteristics of the isolated yeast colony grown on YM and inulin medium were white to cream in color, circular in shape, convex profile with matte surface. The yeast cells were round-oval shape with budding, when observed under microscope (Figure 3.4). There have been several studies on the

isolation of thermotolerant yeasts capable of producing ethanol at high temperature. For example, Limtong et al. (2007) isolated *K. marxianus* DMKU 3-1042 by enrichment technique in a sugar cane juice medium supplemented with 4% (v/v) ethanol at 35°C. This strain produced high concentrations of ethanol at both 40 and 45°C. Koedrith et al. (2008) isolated a thermotolerant yeast strain from banana leaves and characterized it as *Saccharomyces cerevisiae* TR2 that grew efficiently in a complete medium up to 40-41°C. Kwon et al. (2011) isolated thermotolerant ethanologenic yeast strain, *Issatchenkia orientalis* IPE 100, by enrichment in a slightly modified M9 medium at 42°C that possessed a high efficiency of ethanol fermentation at 42°C.

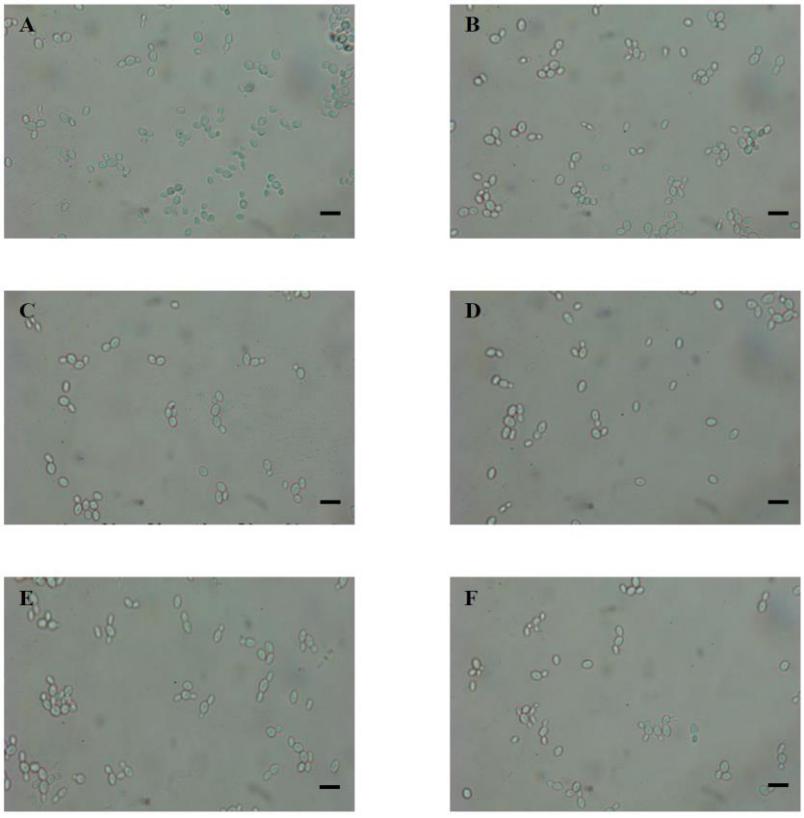

Figure 3.4 Light microscopy image showing the cell morphology of DBKKU Y-102 (A), DBKKU Y-103 (B), DBKKU Y-104 (C), DBKKU Y-105 (D), DBKKU Y-106 (E) and DBKKU Y-107 (F) when grown on a YM medium for 24 h at 30°C under 400x magnification and bar (-) = 10 µm

3.3.2 Ethanol production from Jerusalem artichoke by the selected yeast strains

A comparative study on ethanol production from Jerusalem artichoke at various temperatures between static and shaking conditions was carried out and the results showed that the maximum ethanol concentrations under shaking condition were higher than static condition at all temperatures tested. Ethanol fermentation carried out at 30, 37 and 40°C by shaking conditions provided the similar results, however the highest ethanol concentration (62.38±0.18 g/l) and yield (88.79%) of theoretical yield were achieved from strain DBKKU Y-102 in shaking condition at 40°C (Table 3.1).

Table 3.1 Ethanol production from Jerusalem artichoke by the isolated yeasts at various temperatures under static and shaking conditions

Strain	Ethanol concentration (g/l)							
	30 °C		37 °C		40 °C		45 °C	
	Static	Shaking	Static	Shaking	Static	Shaking	Static	Shaking
DBKKU Y-102	50.09± 0.25[a] (108 h)	59.94± 0.18[a] (36 h)	30.20± 0.49[a] (96 h)	61.35± 0.16[a] (36 h)	27.52± 0.11[a] (84 h)	62.38± 0.18[a] (36 h)	15.92± 0.08[a] (72 h)	39.44± 0.21[a] (36 h)
DBKKU Y-103	49.38± 0.04[b] (108 h)	57.35± 0.36[b] (36 h)	28.42± 0.41[b] (96 h)	58.51± 0.28[b,c] (48 h)	26.53± 0.35[b] (96 h)	60.64± 0.16[b,c] (36 h)	15.53± 0.13[b] (84 h)	39.44± 0.25[a] (36 h)
DBKKU Y-104	49.38± 0.05[b] (108 h)	59.01± 0.35[c] (48 h)	30.31± 0.47[a] (96 h)	58.22± 0.35[b,c] (48 h)	22.03± 0.05[c] (96 h)	61.01± 0.21[c] (36 h)	17.27± 0.08[c] (84 h)	35.66± 0.23[b] (36 h)
DBKKU Y-105	46.54± 0.13[c] (108 h)	56.23± 0.25[d] (36 h)	28.88± 0.47[b,c] (96 h)	58.14± 0.21[b,c] (48 h)	25.95± 0.27[d] (96 h)	60.53± 0.24[b] (36 h)	15.36± 0.08[d] (84 h)	33.71± 0.25[c] (36 h)
DBKKU Y-106	48.04± 0.37[d] (120 h)	59.26± 0.44[c] (48 h)	29.90± 0.33[a] (96 h)	58.75± 0.51[c] (48 h)	26.29± 0.22[b,d] (96 h)	59.62± 0.10[d] (36 h)	15.10± 0.01[d] (84 h)	36.15± 0.21[b] (36 h)
DBKKU Y-107	49.00± 0.46[b] (120 h)	56.06± 0.46[d] (48 h)	26.68± 0.04[c] (96 h)	57.85± 0.24[b] (36 h)	25.22± 0.06[e] (84 h)	58.41± 0.10[e] (36 h)	15.15± 0.23[d] (84 h)	40.36± 0.27[d] (36 h)

[a-e] Means followed by the same letter within a same column are not significantly different using Duncan's multiple range test at the level of 0.05.

The results were expressed as mean ±SD.

Shaking condition had a clear influence on ethanol production of the strain DBKKU Y-102. *K. marxinus* did not show a high growth rate under anaerobic conditions, which was a common characteristic of *S. cerevisiae,* but it could undergo

fermentation in anaerobic condition (Walker 1998; Banat et al. 1998). Diaz et al. (1996) mentioned that in static conditions as there is no physical mixing procedure but in shaking conditions a physical mixing is provided by a proper shaking of the vessel which ultimately results in a homogenous supply of nutrients to the cells and as a result a higher fermentation rate.

Study of ethanol fermentation by thermotolerant yeast at high temperature has been reported by several investigators. For example, Kumar et al. (2009) reported the isolation of *Kluyveromyces* sp. IIPE453 from soil sample collected from dumping sites of crushed sugarcane bagasse in a sugar mill that could ferment 20% glucose within 48 h at 50°C and yield a maximum ethanol concentration of 8.2% (w/v). Watanabe et al. (2010) screened and selected *S. cerevisiae* NFRI3225 for simultaneous saccharification and fermentation (SSF) of very high gravity (VHG), and produced 13.7% (w/v) of ethanol from potato mash at 37°C for 24 h. Mehdikhani et al. (2011) reported the screening of *Saccharomyces cerevisiae* (PTCC^{5269} M3 and Areni M7) by gamma irradiation treatments and selected for their ability to grow and ferment molasses in a temperature range of 35-45°C. Ethanol yield of 23.50% (v/v) and 22.60% (v/v) at 72 h was obtained. Yuangsaard et al. (2012) isolated *Pichia kudriavzevii* DMKU 3-ET15 from traditional fermented pork sausage by an enrichment technique in a yeast extract peptone dextrose (YPD) broth. The strain produced a final ethanol concentration of 7.35% (w/v) after 33 h, a productivity of 2.23 g/l.h and a yield of 79.9% of the theoretical yield from cassava starch hydrolysate at 40°C in a 2.5L jar fermenter with an agitation speed of 300 rpm and an aeration rate of 0.1 vvm throughout the fermentation.

3.3.3 Identification of the selected yeast strains

On the basis of sequence analysis of morphological and D1/D2 domain of 26S rDNA, all of the sequences of six isolated yeasts were 99% identical to the sequence of *K. marxianus*. The phylogenetic analysis confirmed that all of the sequences are closely related to *K. marxianus*. Therefore, the selected yeast strains were identified as *K. marxianus*.

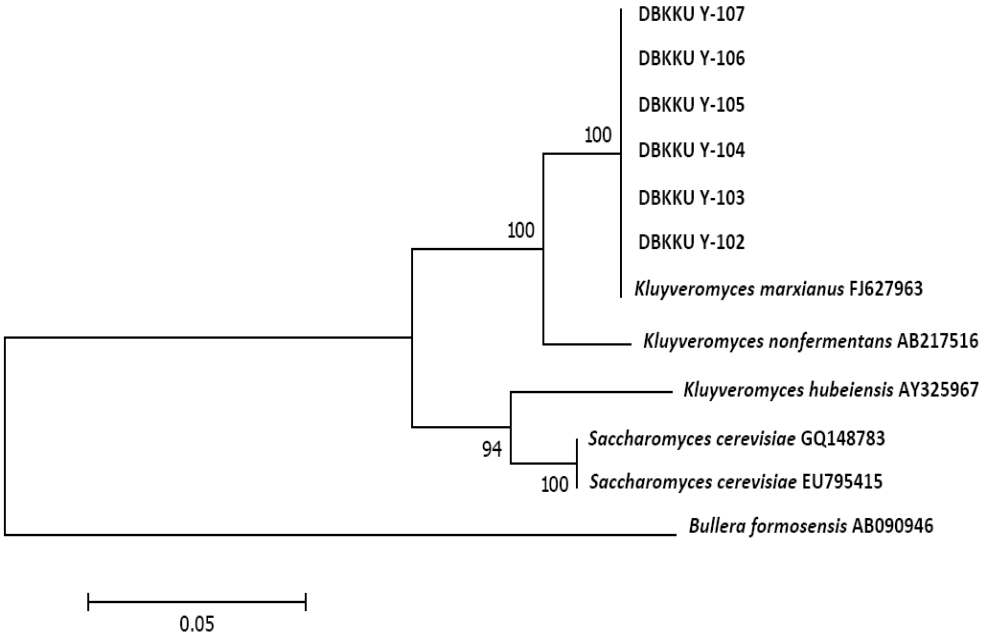

Figure 3.5 Phylogenetic tree of the D1/D2 domain of 26S rDNA from neighbor-joining depicting relationships among type strains of selected species of the yeast

3.4 Conclusion

As the results showed in this study, we obtained thermotolerant inulin-utilizing strain *K. marxianus* DBKKU Y-102, which can be used as effective strain in ethanol production using Jerusalem artichoke juice without acid or enzymatic pre-treatment as a raw material. The highest ethanol concentration (62.38±0.18 g/l) and yield (88.79%) of theoretical yield were obtained from this strain in shaking condition at 40°C. These results suggested that the newly thermotolerant yeast, DBKKU Y-102, had high potential for ethanol production from Jerusalem artichoke at high temperature.

3.5 Acknowledgements

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