

Full Length Research Paper

# Optimization of the fermentation conditions for ethanol production by new thermotolerant yeast strains of *Kluyveromyces* sp.

Mohamed Hashem<sup>1,2\*</sup>, Abdel Naser A. Zohri<sup>1</sup> and Maysa M. A. Ali<sup>1</sup>

<sup>1</sup>Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, 71516, Egypt.

<sup>2</sup>Biological Science Department, Faculty of Science, King Khalid University, P.O. Box 10255, Abha 61321, Saudi Arabia.

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This study introduces two new thermotolerant strains of *Kluyveromyces* sp. for the biofuel industry to reduce the overall cost of production. The fermentation conditions including temperature, pH, incubation period and sugar concentration were adjusted. The two yeast strains were identified by sequencing of ITS1 and ITS2 regions. Comparing the sequence results with the GenBank reference proved that the strain *Kluyveromyces* sp. ZMS1 had 100% of similarity with *Kluyveromyces maxianus*. Yeast strain *Kluyveromyces* sp. ZMS3 had only 97% of similarity with the reference species; consequently, it could be a new strain. At 35°C, *Kluyveromyces* sp. ZMS1 GU133329 and *Kluyveromyces* sp. ZMS3 GU133331 produced 9.55 (w/v) and 11.72% (w/v) of ethanol, respectively. The appropriate concentration of sugar that induced the maximum production of ethanol by these strains was 20 to 25%. The optimum pH range for both strains was 5.0-5.5. In fed-batch culture, the maximum ethanol production was 11.71 (w/v) and 11.62% (w/v) by *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329, respectively. This study concludes the two thermotolerant yeast strains are promising in the production of bioethanol, especially strain ZMS3 GU133331 that showed a good growth up to 45°C. It grew normally and carried out its fermentation process in 25% of sugar concentration. Application of these new strains will not only decrease the risk of contamination but also reduce in cooling costs that will lead to the reduction of the overall production cost.

**Key words:** Bioethanol, *Kluyveromyces* spp., thermotolerant, ITS, fed-batch.

## INTRODUCTION

Current industrial development and rapid pace of urbanization have called for an environmental sustainability and alternative energy. Recently, a new round of interest in bioenergy and biomass has been initiated with the recognition that the global crude oil reserves are finite, and its depletion is occurring much faster than previously predicted (Escobar et al., 2009). The depletion of fossil fuel reserves, the unstable panorama of the petrol prices and more recently, increasing environmental and political pressures have increased industrial focused

toward alternative fuel (Davis et al., 2005; Lim et al., 2013).

The emissions of greenhouse gases tend to elevate the temperature of the planet excessively. One of the most important greenhouse gases is CO<sub>2</sub>. Over the past century, the atmospheric concentration of CO<sub>2</sub> reached its highest levels (Conn, 2007). Since the pre-industrial times, the atmospheric concentrations of greenhouse gases have been increased a consequence, which has been just recognized, of human activities (IPCC, 2007).

\*Corresponding author. E-mail: drmhashem69@yahoo.com.

This rise is mainly caused by the unsustainable use of fossil fuels and the change in the use of the land. Interest in the use of bio-fuels worldwide has grown strongly in recent years due to the limited oil reserves, concerns about climate change from greenhouse gas emissions and the desire to promote domestic rural economies (Balat and Balat, 2009).

Bioethanol is an attractive alternative fuel because it is a renewable bio-based resource and it is oxygenated thereby provides the potential to reduce particulate emissions in compression-ignition engines (Hansen et al., 2005). It has a higher octane number, broader flammability limits, higher flame speeds and higher heats of vaporization than gasoline (Balat, 2007; Poonam and Anoop, 2011)

Ethanol is now produced from two major groups of bioresources: sugar substances and starchy materials. There is a competition between these two feedstocks for fuel ethanol production. While sugar substances were the feedstock for more than 60% of fuel ethanol production in the world at the beginning of the 2000s, its share decreased to 47% by 2006, when grains accounted for 53% of the production (Licht, 2006).

Ethanol fermentation at relatively high temperature is an important target for effective ethanol production in tropical countries where average day-time temperatures are usually high throughout the year. The advantages of rapid fermentation at high temperature are not only to decrease the risk of contamination but also to reduce the cooling costs (Eiadpum et al., 2012). To achieve high temperature fermentation it is necessary to use an efficient yeast strain that can tolerate high temperature (Limtong et al., 2007).

At present, industrial ethanol production employs a mesophilic strain of *Saccharomyces cerevisiae*. Although there have been numerous reports of potential applications of thermotolerant yeast strains in industrial ethanol production, only a few have been concerned with *S. cerevisiae* (Morimura et al., 1997; Sridhar et al., 2002); most of them focused on to *Kluyveromyces marxianus* (Zafar and Owais, 2006). Therefore, introduction of new thermotolerant strains into fermentation process has a good economic impact on the overall bioethanol production.

This study aimed at seeking the potentiality of new strains of *Kluyveromyces marxianus* to produce high concentration of ethanol at relative high temperature. Appropriate conditions of temperature, pH, sugar concentration and incubation time were investigated for production of the maximum amount of ethanol.

## MATERIALS AND METHODS

### Isolation and identification of the yeast strains

*Kluyveromyces* sp. GU133329 and *Kluyveromyces* sp. GU133331 were isolated from plum fruit and cantaloupe, respectively on yeast extract-malt extract agar (YMA) at 40°C using direct touch method

(Kurtzman and Fell, 1998). DNA of the yeast strains was extracted using Qiagen DNA extraction kit (Qiagen, Germany) as described by Ausubel et al. (1995). The primers of ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCG CTTATTGATATGC3') were used to amplify 18S rDNA. The PCR amplicons were sequenced in Genetic Engineering and Biotechnology Research Institute (GEBRI), city of scientific research and technology applications, Borg El-Arab, Egypt. DNA sequences were analyzed using the DNA Blast and the obtained nucleotide sequences were deposited in the GenBank under accession numbers; GU133329 and GU133331, respectively.

### Ethanol production by two strains of *Kluyveromyces* sp.

Inoculum of each yeast strain was prepared by transferring one loopfull of 48-h culture grown on slant of YMPGA into 250 ml Erlenmeyer flask containing 50 ml sterilized YMPG broth. After incubation on a shaker incubator (150 rpm) at 25°C for 36 h, the inoculum was transferred at the rate of 10% to the ethanol production medium. Production of ethanol was conducted at 25°C in 100 ml glass bottles containing 45 ml of a modified YMPG broth medium composed of yeast extract (0.3%), malt extract (0.3%), peptone (0.5%) and glucose (12.5%). The pH was adjusted to 4.5. Sugar was sterilized alone at 105°C for 15 min, while the other components were sterilized at 121°C for 15 min. The cultures were incubated on a rotary shaker (150 rpm) at 25°C for 5 h under aerobic condition and completed the fermentation period (72 h) under anaerobic condition.

### Quantitative estimation of ethanol

Ethanol content was estimated by bichromate method (Balasubramanian et al., 2011; Zohri and Eman Mostafa, 2000). Briefly, bio-ethanol content was estimated by bichromate method as the following; 5 ml of sample to be analyzed and 20 ml of 0.5 N NaOH was transferred to an evaporator and was distilled until approximately 10 ml was evaporated from the mixture. The evaporated gas was collected in the collection flask containing 30 ml of 0.2 N potassium bichromate solution and 10 ml of concentrated H<sub>2</sub>SO<sub>4</sub>. If the sample contains alcohol, the color of the solution in collecting flask turns from orange to dark green. The content of the collecting flask was quantitatively transferred into 250 ml Erlenmeyer flask with additional 100 ml distilled water, 10 ml of 0.2 N potassium iodide and 3 to 5 drops of 5% starch solution and then titrated against 0.1 N sodium thiosulfate solution.

Alcohol content (% volume) in the sample = (bichromate volume x 2-thiosulfate volume) x 0.0289.

This analytical method is very sensitive to alcohol concentrations up to 1.5% only. If the alcohol concentration in the sample is more than 1.5%, it must be diluted. Ethanol productivity and production efficiency were calculated according to Limtong et al. (2007).

### Determination of yeast growth

The optical density was measured as an indicator for yeast growth using a spectrophotometer (model LINICAL) at 600 nm as described by limiting et al. (2007).

### Optimization the fermentation conditions of ethanol production

#### Effect of incubation period

The selected two yeast strains were grown in YMPG broth medium

supplemented with 12.5% glucose for different incubation periods (24, 48, 72, 96, 120 and 144 h) at 25°C and 150 rpm. The pH was adjusted before inoculation at 4.5. Ethanol concentrations, optical density (O.D.) and pH were measured at the end of each incubation period.

#### Effect of sugar concentration

The two yeast strains were grown in YMPG broth medium supplemented individually with different sugar concentrations (12.5, 15, 17.5, 20, 22.5, 25, 30 and 35%) at 25 and 35°C and 150 rpm for 72 h. The pH was adjusted before inoculation at 4.5. Ethanol concentrations were measured at the end of the incubation period for each sugar concentration. Also, the percentage of ethanol yield related to the theoretical value was calculated.

#### Effect of temperature

YMPG broth medium supplemented with 25% glucose was selected for studying the effect of temperature on the ethanol production by the selected two yeast strains. The fermentation temperatures were 25, 30, 35 and 40°C. The pH was adjusted at 4.5. The fermentation process was achieved at 150 rpm. Ethanol concentrations and volumetric productivity of ethanol were measured at each degree of temperature after 0, 24, 48 and 72 and 96 h of incubation period.

#### Effect of pH value

The effects of different initial pH values (4.0, 5.0, 5.5 and 6.5) on ethanol production and their productivity in addition to the final pH values formed by the selected two yeast strains grown in YMPG broth medium with 25% sugar concentration at 35°C was determined. The fermentation process was achieved at 150 rpm for 144 h of fermentation. pH was adjusted by 1 N HCl or 0.1 N NaOH

#### Fed-batch strategy

Fed-batch fermentation strategy was applied for ethanol production by each of the selected two yeast strains. The initial glucose concentration was 25% and the initial pH was adjusted at 5.5. The fermentation was conducted at 30°C and 150 rpm. For feeding the fermentation process, a total of 8% glucose was added to the culture at the rate of 4% after 48 and 96 h of fermentation. The fermentation process was continued for 168 h. Ethanol concentrations were assessed every 24 h of incubation period.

#### Statistical analysis

All experiments were carried out in a completely randomized design. Each value is a mean of three readings and the standard error for each mean was calculated.

## RESULTS

### Identification of the two strains of *Kluyveromyces* sp.

The sequence of ITS1 rRNA and ITS2 rRNA of *Kluyveromyces* sp. ZMS1 and *Kluyveromyces* sp. ZMS3 was compared with the sequence of similar species in the GenBank database. Results of phylogeny showed that

yeast strain ZMS1 (GU133329) formed one group with *Kluyveromyces maxianus* DP3 HM363372 and had 100% of the similarity value (Figure 1). Whereas, yeast strain ZMS3 (GU133331) had only 97% of the similarity with the reference species. This result indicates that strain ZMS3 (GU133331) could be a new strain of *K. maxianus*. The two strains *Kluyveromyces* sp. ZMS1 GU133329 and *Kluyveromyces* sp. ZMS3 GU133331 have cluster value of 87% and inter-similarity as 96% indicating that they are somewhat different. At 42°C, both yeast strains grew well, however at 45°C only *Kluyveromyces* sp. ZMS3 (GU133331) showed good growth (data not shown). This could support our hypothesis that they are two different strains.

### Effect of incubation period on ethanol production

The results show that the ethanol concentration gradually increased by increasing the incubation period and reached its maximum after 60 and 72 h by *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329, respectively, and dramatically decreased with further extension of the incubation period (Figure 2). The ethanol concentration in case of the first strain was higher than the other one during the first 60 h of the incubation period. *Kluyveromyces* sp. ZMS3 GU133331 produced the maximum yield of ethanol (6.29% equal to 98.60% of the theoretical value) after 60 h. Also, this strain produced high levels of ethanol at 48 and 72 h of fermentation period. The other strain (*Kluyveromyces* sp. ZMS1 GU133329) produced the maximum ethanol level (6.21% equal to 97.41% of the theoretical value) after 72 h of incubation period.

The growth pattern of these strains obtained during the incubation period showed that the high optical density (O.D.) was recorded at the first 24 h for the two strains and slightly decreased by increasing the incubation period (Figure 3). At 600 nm, the O.D. was measured as 10.27 and 9.81 by *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329, respectively.

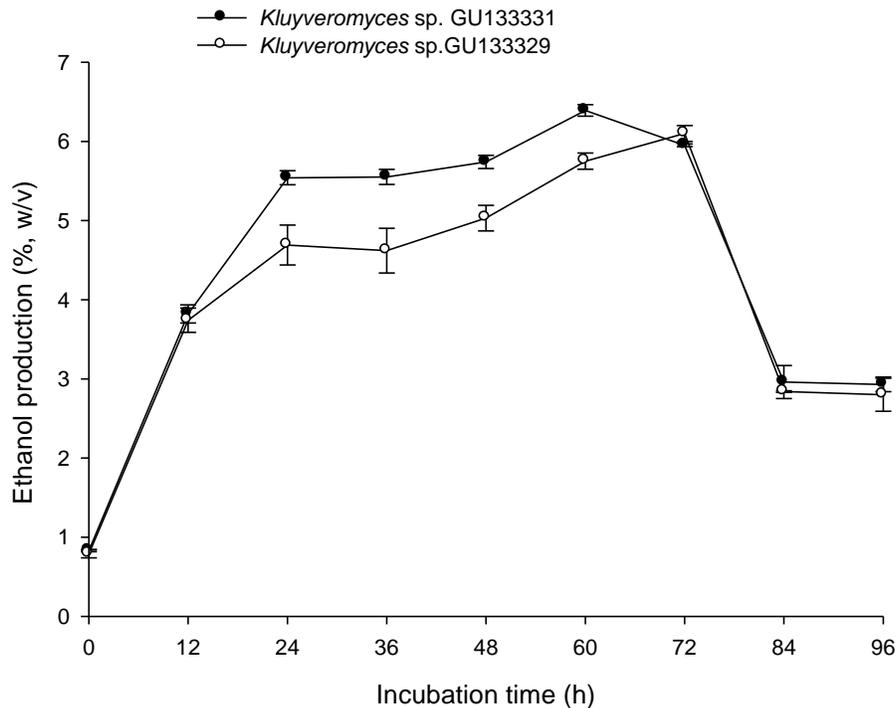
Final pH values of the fermentation medium after each of incubation periods were nearly similar for both yeast strains. During the first 12 h of fermentation, the pH greatly decreased from 4.5 to 3.8. However, during the next 48 h (24 -72 h) the change in pH was not considerable. After 84 to 96 h, pH increased to 4.2 (Figure 4).

### Effect of sugar concentration

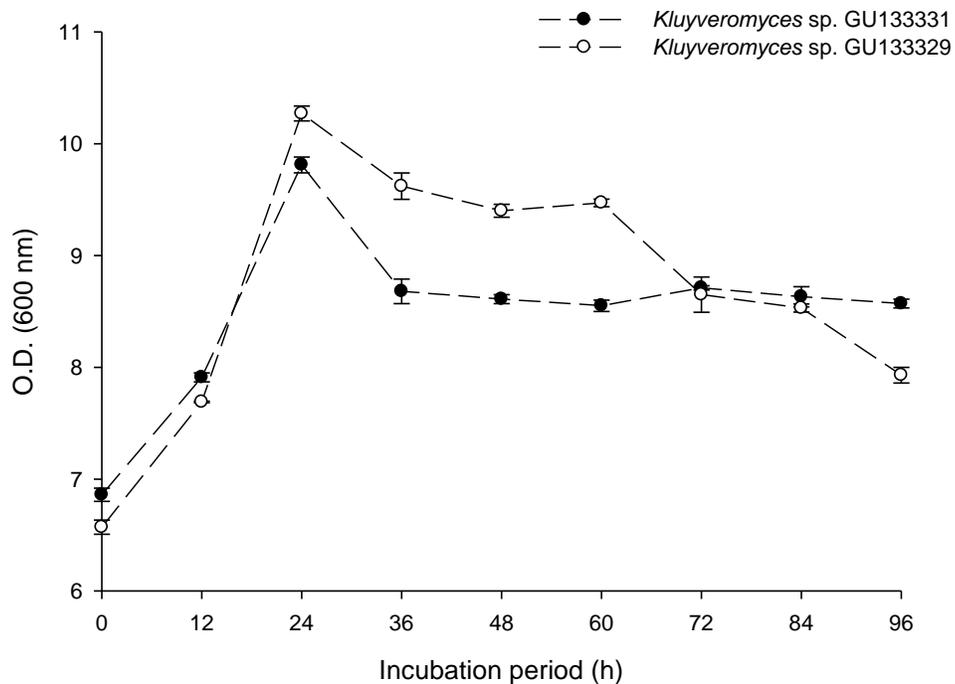
The two strains of *Kluyveromyces* sp. were grown in YMPG broth medium supplemented with different concentrations of glucose (12.5, 15.0, 17.5, 20.0, 22.5, 25.0, 30.0 and 35.0%) and were incubated at 25 and 35°C for 72 h in a shaking incubator (150 rpm). The obtained data revealed that at 25°C, both yeast strains



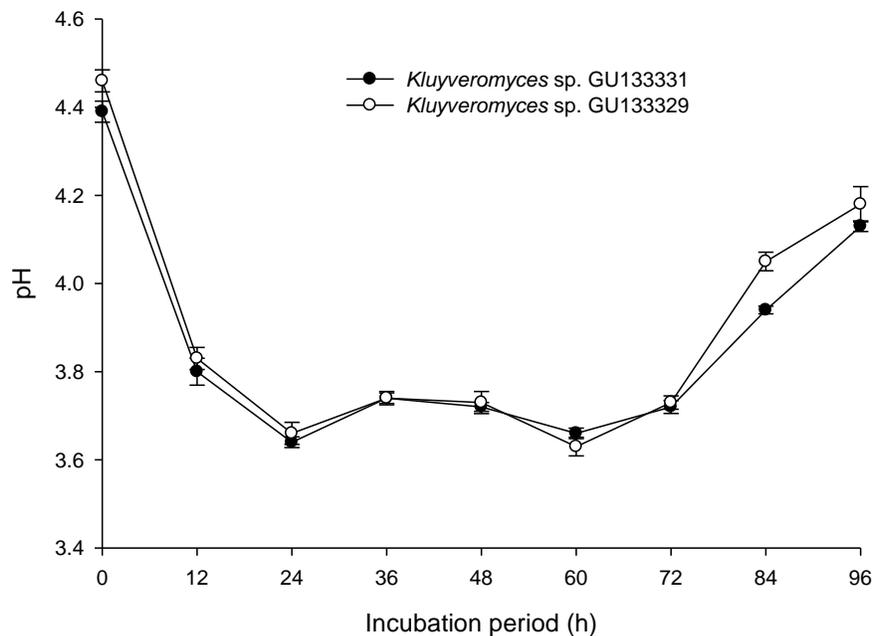
**Figure 1.** Phylogenetic relationship between *Kluyveromyces* sp. strains ZMS1 and ZMS3 and other ITS1 and ITS2 sequences of published strains in the GenBank. In the phylogenetic tree, *Kluyveromyces* sp. strains ZMS1, ZMS3 and *K. marixianus* were clustered together as one clade segments corresponding to an evolutionary distance of 0.01 shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree.



**Figure 2.** Ethanol production by the two strains of *Kluyveromyces* sp. grown on medium with 12.5% glucose for different incubation periods at 25°C.



**Figure 3.** Growth curve of the two strains of *Kluyveromyces* sp. grown on medium with 12.5% glucose for different incubation periods at 25°C.



**Figure 4.** Change in pH during ethanol production by the two strains of *Kluyveromyces* sp. grown on medium with 12.5% glucose at 25°C.

produced the maximum ethanol concentration when they were grown on 12.5% of sugar. The concentrations of ethanol formed by *Kluyveromyces* sp. ZMS3 GU133331,

*Kluyveromyces* sp. ZMS1 GU133329 were 5.97 and 6.26 equal to 93.61 and 98.25 of the theoretical value, respectively (Table 1). The increase in the sugar concen-

**Table 1.** Effect of sugar concentration on ethanol production by yeast strains grown on YM broth medium supplemented with the different concentrations of sugar and grown at both 25 and 35°C for 72 h at 150 rpm.

Sugar Conc. (%)	<i>Kluyveromyces</i> sp. ZMS3 GU133331				<i>Kluyveromyces</i> sp. ZMS1 GU133329			
	25°C		35°C		25°C		35°C	
	% (w/v)	% of theo.	%(w/v)	% of theo	% (w/v)	% of theo.	% (w/v)	% of theo
12.5	5.97 ± 0.01	93.61 ± 0.12	6.13 ± 0.01	96.16 ± 0.18	6.26 ± 0.03	98.25 ± 0.46	6.19 ± 0.03	97.15 ± 0.46
15	5.76 ± 0.06	75.25 ± 0.83	7.30 ± 0.03	95.38 ± 0.42	6.13 ± 0.02	80.13 ± 0.23	7.23 ± 0.02	94.55 ± 0.27
17.5	5.55 ± 0.01	62.15 ± 0.15	8.13 ± 0.01	91.13 ± 0.10	5.53 ± 0.01	61.90 ± 0.07	8.11 ± 0.06	90.87 ± 0.65
20	5.42 ± 0.01	53.17 ± 0.09	9.62 ± 0.01	94.28 ± 0.09	5.45 ± 0.02	53.46 ± 0.21	9.55 ± 0.02	93.63 ± 0.17
22.5	5.19 ± 0.04	45.26 ± 0.38	10.86 ± 0.02	94.64 ± 0.13	5.38 ± 0.01	46.88 ± 0.03	9.44 ± 0.02	82.24 ± 0.13
25	5.81 ± 0.03	45.54 ± 0.20	11.77 ± 0.01	92.29 ± 0.07	5.52 ± 0.01	43.29 ± 0.05	9.17 ± 0.04	71.95 ± 0.29
30	NG	-	11.46 ± 0.02	74.92 ± 0.14	NG	-	8.63 ± 0.02	56.38 ± 0.10
35	NG	-	10.36 ± 0.015	58.02 ± 0.081	NG	-	9.85 ± 0.018	55.20 ± 0.01

NG = No growth; Sugar conc. = sugar concentration; % (w/v) = ethanol levels as % weight/volume; % of theo = ethanol % of the theoretical value (ethanol production efficiency); ± standard deviation.

concentration resulted in a slight decrease in ethanol production by both strains. The two strains completely failed to grow in the fermentation medium containing high concentrations of sugar (30 and 35%) at 25°C. However, at 35°C, ethanol production by the two yeast strains increased by increasing the sugar concentrations up to 20 to 25%. *Kluyveromyces* sp. ZMS1 GU133329 produced the maximum level of ethanol as 9.55% (93.63% of the theoretical value) at 20% of sugar. *Kluyveromyces* sp. ZMS3 GU133331 produced 11.77% ethanol (92.29% of the theoretical value) as the maximum production in 25% of sugar.

### Effect of fermentation temperature

Effect of different fermentation temperatures (25, 30, 35 and 40°C) on ethanol production by the two yeast strains grown in medium containing 25% of sugar was evaluated. Figure 5 shows that yeast strain *Kluyveromyces* sp. ZMS3 GU133331 produced 11.72% (91.29% of the theoretical yield) at 35°C and 11.34% (88.94% of the theoretical yield) at and 30°C, respectively after 72 h. At 40°C, this strain produced only 9.21% (equal to 72.24% of the theoretical value) after 48 h. The optimum temperature for ethanol production by this strain was 35°C, however it is still effective in ethanol production up to 40°C and at this temperature production of ethanol was achieved at 48 h. At 96 h of incubation, the production dramatically decreased at all degrees of temperature.

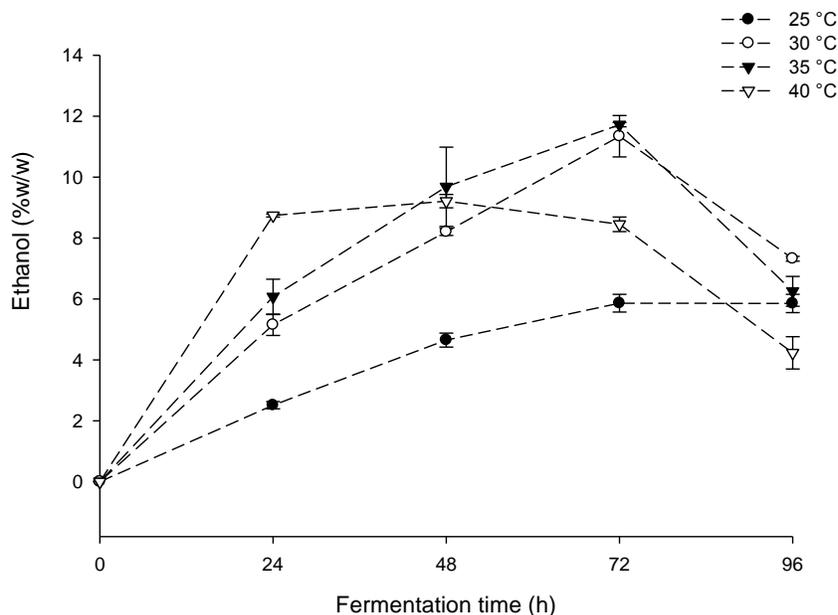
Figure 6 showed that the other strain *Kluyveromyces* sp. ZMS1 GU133329 nearly have the same pattern that was obtained by the previous one. The maximum production of ethanol was 10.06% (78.90 of the theoretical value) at 35°C after 48 h and 9.60% (75.29 of the theoretical value) at 30°C after 72 h. At 40°C, ethanol

concentration was 9.4% (73.73% of the theoretical value) after 48 h of fermentation period. During the first 24 h, ethanol production by this yeast strain increased by increasing the fermentation temperature to give 2.15, 5.70, 7.86 and 8.91% of ethanol at 25, 30, 35 and 40°C, respectively. The optimum fermentation temperature for ethanol production by this yeast strain was 35°C.

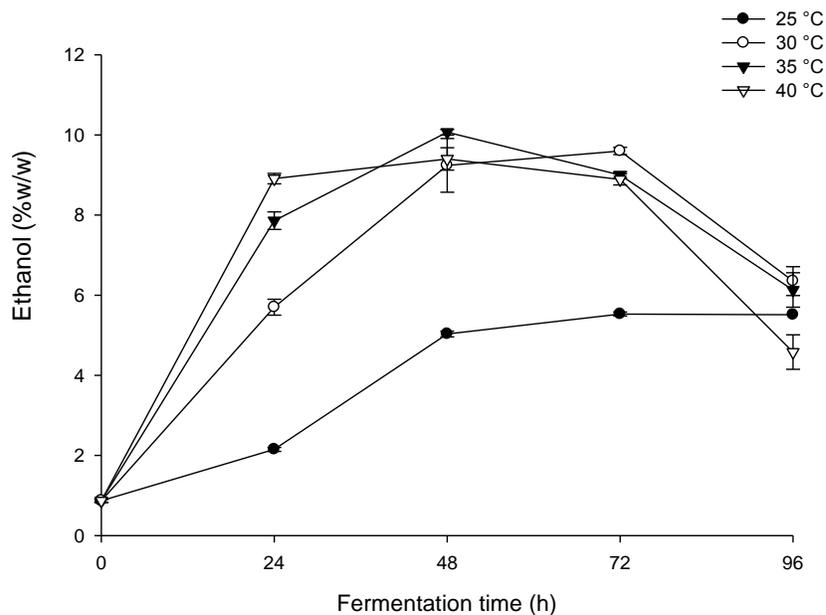
### Effect of hydrogen ion concentration (pH)

Figure 7 shows the effect of different initial pH values (4.0, 5.0, 5.5 and 6.5) on ethanol production by *Kluyveromyces* sp. ZMS3 GU133331 at 35°C after 144 h of incubation. These results clearly showed that the highest concentration of ethanol by this strain was produced after 72 h when the initial pH was 5.5. The ethanol concentration was 11.88% that represents 92.99% of the theoretical value. Very close result (11.82%) was recorded over the same period when the initial pH was 5.0. Other pH values (6.5 and 4.0) resulted in production of ethanol as 11.70 and 10.47%, respectively, but after longer incubation period (96 h). We noticed that the initial pH, 5.0-5.5 is the best range of pH for production of the maximum amount of ethanol in a short period of fermentation.

Figure 8 shows that the highest level of ethanol production by *Kluyveromyces* sp. ZMS1 GU133329 was recorded when the pH of the fermentation medium was adjusted at 6.5. This ethanol level was 11.60% equal to 90.8% of the theoretical value after 72 h of incubation period. In a fermentation medium with an initial pH of 5.5, the ethanol concentration reached 10.55% after 48 h of incubation period. Ethanol yield slightly decreased or was nearly constant with increasing the fermentation period using media with initial pH 5.5 or 6.5. It was observed that the initial pH 4.0 or 5.0 negatively affected the



**Figure 5.** Effect of temperature on ethanol production by *Kluyveromyces* sp. GU133331 grown on medium with 12.5% glucose.



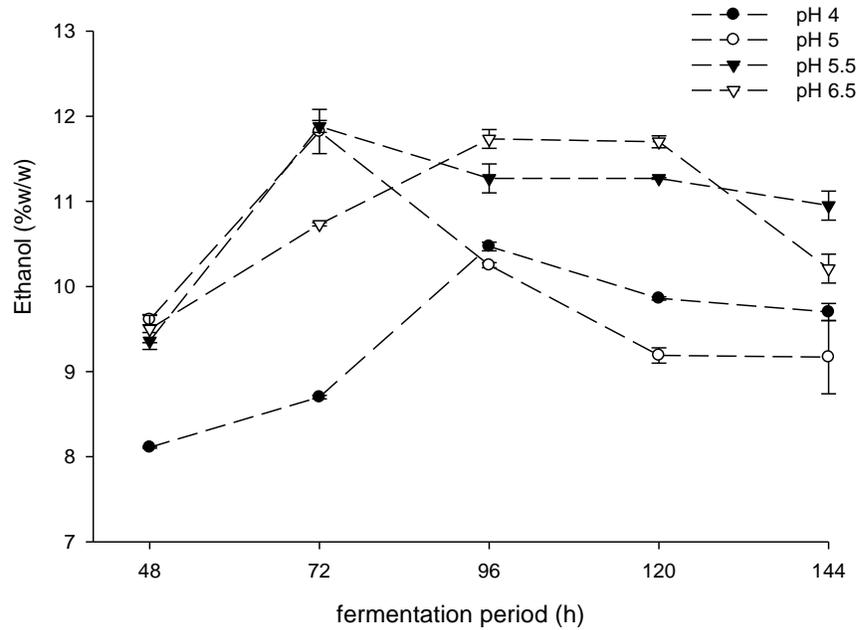
**Figure 6.** Effect of temperature on ethanol production by *Kluyveromyces* sp. GU133329 grown on medium with 12.5% glucose.

production of ethanol by this yeast strain as compared to pH 5.5 - 6.5.

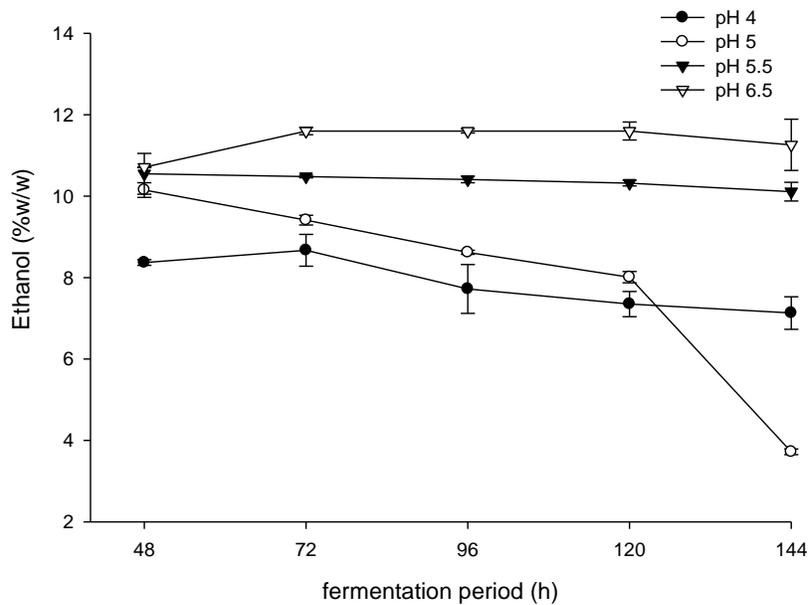
### Fed-batch fermentation

In fed-batch fermentation, the initial glucose concentration

of 25% was employed and the concentration of other nutrients and all other conditions were constant as in the batch fermentation. For feeding process, 8% glucose was added to the culture at the rate of 4% after 48 and 96 h of starting the fermentation. The fermentation process was continued until 168 h. The results of fed-batch fermentation are shown in Table 2. The ethanol production levels



**Figure 7.** Effect of pH on ethanol production by *Kluyveromyces* sp. GU133331 grown on medium with 12.5% glucose at 25°C.



**Figure 8.** Effect of pH on ethanol production by *Kluyveromyces* sp. GU133329 grown on medium with 12.5% glucose at 25°C.

levels were not increased by using this strategy and the results which recorded by fed-batch were completely similar to those recorded by batch technique. The maximum ethanol production levels, by *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329 were 11.71 and 11.62%, respectively. All

these ethanol production levels were recorded after 96 h of fermentation period. There was slight decrease in ethanol concentration by the two yeast strains after this period. The increase in glucose concentration after 48 and/or 96 h of fermentation did not increase the ethanol production. One or more factors like the non-ability of this yeast

**Table 2.** Ethanol concentration (%w/v)  $\pm$  standard deviation (based of three replicates) produced by two yeast strains during different fermentation period using fed-batch strategy.

Yeast strain	Fermentation time (h)			
	48	96	144	168
<i>K. marxianus</i> ZMS3 GU133331	9.55 $\pm$ 0.18	11.71 $\pm$ 0.23	11.62 $\pm$ 0.35	11.06 $\pm$ 0.10
<i>K. marxianus</i> ZMS1 GU133329	10.65 $\pm$ 0.20	11.62 $\pm$ 0.31	11.20 $\pm$ 0.04	11.11 $\pm$ 0.14

strains to tolerate the high ethanol concentration might have led to decrease in the ethanol level in the late incubation period despite the availability of glucose level in the fermentation medium.

## DISCUSSION

We selected *Kluyveromyces* sp. ZMS3 GU133331 and *Kluyveromyces* sp. ZMS1 GU133329 as very high ethanol producers and thermotolerant strains to study the effect of different fermentation conditions on their efficiency in ethanol production to introduce them as new strains to the industry. Identification of these strains by the sequence of the two non-coding regions ITS1 and ITS2 revealed that strain ZMS1 GU133329 had 100% similarity with *Kluyveromyces marxianus* DP3 HM363372 (in the GenBank). This result proves the identity of our strain as *Kluyveromyces marxianus*. But the other strain ZMS3 GU133331 had only 97% of similarity with the reference *Kluyveromyces marxianus*. So, strain ZMS3 GU133331 seemed to be a new strain of *K. marxianus*. The ITS regions length between the 18S and 5.8S rDNA (ITS1) and between the 5.8S and 28S rDNA (ITS2) in the eukaryotic genome are highly variable to provide sufficient information for the classification of eukaryotic microbes up to the species level (Sujaya et al., 2003; Ramos et al., 1998).

During ethanol production on the industrial scale, yeast may be confronted with a variety of environmental stresses that can cause the loss of yeast cell viability, reduced yeast growth and increased fermentation times, decreased fermentation rates (Graves et al., 2007). Fermentation time is a very important factor from an economic point of view in ethanol production. Our results revealed that the ethanol yield increased gradually by increasing the incubation time and reaching its maximum after 60-72 h and dramatically decreased with further extension of time. These findings are in agreement with those of Suryawati et al. (2008) and Faga et al. (2010), who reported that the appropriate time for different strains of *K. marxianus* to produce the highest amount of ethanol was 72 h. Also, the highest optical density (O.D.) was obtained at the first 24 h of fermentation period and slightly decreased by increasing the fermentation time. This finding is interpreted from the fact that yeast consumes all of the dissolved oxygen from the medium

usually within the first hours of fermentation. Under this initial aerobic condition, the Embden-Meyerhof-Parnas route (alternatively named EMP or glycolytic pathway) catabolizes the hexoses (glucose) to pyruvic acid. The yeast cell completely oxidizes the resultant pyruvate to CO<sub>2</sub> and water while producing energy for other metabolic processes. Only in the absence of oxygen, the pyruvate is converted into ethanol and CO<sub>2</sub> primarily by way of acetaldehyde (Munroe, 1994). Final pH values of the fermented mash after each fermentation periods were nearly similar (3.3 - 3.95). Those results indicated that the yeast strains had the ability to readjust the pH value in the fermentation medium. This result agrees with those recorded by Russell (2003) who reported that during any fermentation, H<sup>+</sup> ions are excreted by the yeast and this result in a decline in the pH of media. Also, he reported that in brewing or distilling processes with a pure yeast culture have an initial pH of 5.2-5.5, the final pH value decreased to about 3.8.

Our results showed that 12.5% of sugar was the best concentration for the two yeast strains at 25°C. The productivity decreased by increasing the sugar concentration and completely stopped when the concentration was 30%. We suggested that the increase in sugar concentration leads to increase of the viscosity in fermentation medium and this had a high inhibitory effect on yeast growth and their capability to produce ethanol. This suggestion is supported by Pratt-Marshall et al. (2003), who observed that fermentation of high gravity worts have a negative effect on the yeast performance due to the elevated osmotic pressure. Also, D'Amore et al., (1989) reported that as the initial wort gravity is increased, the rate of fermentation decreased and the amount of ethanol produced was lower than the theoretically expected value.

The obtained data clearly showed that ethanol production at 35°C increased by increasing the sugar concentrations up to 25 and 20% by *Kluyveromyces* sp. ZMS1 GU133329 and *Kluyveromyces* sp. ZMS3 GU133331, respectively. The productivity slightly decreased when the sugar concentration increased up to 30 or 35%. These results are nearly similar to those recorded by Al-Talibi et al. (1975), who observed that the alcohol produced by *S. cerevisiae* grown in Iraqi date juice increased with increasing sugar concentration in the juice from 10-25% and then decreased. Reddy and Reddy (2006) reported that the increase in the sugar con-

centration will decrease the sugar utilization, which results in reduction of the total ethanol production. This reduction could be due to several reasons including the production of other compounds like glycerol or acetic acid. Also, the intracellular ethanol, which may be increased by increasing ethanol production at high sugar concentration, exerts high toxicity on yeast and the nutrient may be deficient at the final stage of fermentation (Sols et al., 1971). All these factors lead to stopping the fermentation process and ethanol formation at the final stage of fermentation.

The maximum ethanol yield of *Kluyveromyces* sp. ZMS3 GU133331 was 11.77% (=2.29% of the theoretical value) at 35°C when it was grown in medium with 25% of sugar. While *Kluyveromyces* sp. ZMS1 GU133329 produced 9.55% ethanol (93.62% of the theoretical value) when it was cultivated in medium with 20% sugar at 35°C. These results are better than those recorded by Al-Talibi et al. (1975), who found that the highest yield of alcohol by *S. cerevisiae* grown on Iraqi date juice with 25% sugar content was 9.96% (equal to 77.96% of the theoretical value). Fleet and Heard (1993) reported that one of the most important factors affecting ethanol production is fermentation temperature that has a direct effect on the biochemical reactions of yeast. Torija et al. (2003) found that the alcohol fermentation increased as the temperature increased and recorded that the alcohol fermentation at 35 °C had no lag phase but they had a quick exponential phase and reached the maximal level earlier.

Increase in fermentation temperature was recorded and successfully employed to increase the fermentation rate (Dragone et al., 2004). Limtong et al. (2007) used a thermotolerant yeast strain named *K. marxianus* DMKU 3-1042 for fuel ethanol production from sugar cane juice supplemented with sucrose to 16-24% sugar and observed that increasing the sugar concentration resulted in an increase in the final ethanol concentration but only up to 22% total sugars at 37°C. They also reported that the sugar concentrations higher than 22% resulted in a decrease in ethanol production. The decrease in ethanol production at high sugar concentrations might be attributed to various factors including high osmotic pressure (Grubb and Mawson, 1993). Limtong et al. (2007) found that after 54 h of fermentation at 37°C using *K. marxianus* DMKU 3-1042 and fermentation medium with 22% sugar, the highest ethanol concentration reached 8.7%, represented 77.5% of the theoretical yield. Ethanol fermentation at high temperature is a key requirement for effective ethanol production in tropical countries where average day-time temperatures are usually high throughout most of the year. The advantages of rapid fermentation at high temperature are not only to decrease the risk of contamination but also to reduce cooling costs. The results of this experiment clearly showed that the two selected thermotolerant yeast strains were able to grow and produce high concentrations of

ethanol at 35 and 40°C. *Kluyveromyces* sp. ZMS3 GU133331 produced 11.72 and 9.21% as the maximum level of ethanol at 35 and 40°C after 72 and 48 h, respectively. Almost similar results were recorded by *Kluyveromyces* sp. ZMS3 GU133331 that produced 10.06 and 9.4% of ethanol at 35 and 40°C, respectively. There are numerous reports of potential applications of thermotolerant yeast strains especially those belonging to *Kluyveromyces marxianus* in industrial ethanol production (Zafar and Owais, 2006; Limtong et al., 2007). Ballesterose et al. (1991) examined the ability of *Saccharomyces*, *Candida* and *Kluyveromyces* to ferment glucose at temperatures above 40°C and reported that *K. marxianus* L.G. produced 3.76% ethanol at 42°C in glucose medium. Banat et al. (1992) tested a strain *K. marxianus* IMB3 and four other strains by enrichment medium at 45 and 50°C. They found that these strains produced high concentrations of ethanol from glucose at 45-50°C and from molasses at 37 and 40°C. Limtong et al. (2007) examined the production of fuel ethanol at high temperature by *K. marxianus* and their results are completely in agreement with ours. In tropical countries, industrial fuel ethanol fermentation is typically performed using mesophilic yeast under controlled conditions of temperature varying between 30 and 35°C. The fermentation cooling cost has an important impact on fuel ethanol production costs. It was demonstrated that if the fermentation temperature could be raised by 5°C, it would greatly reduce the fuel ethanol production costs (Abdel-Banat et al., 2010).

The effect of initial pH of the fermentation media on ethanol production showed that the highest ethanol concentration was obtained by *Kluyveromyces* sp. ZMS3 GU133331 in medium with initial pH 5.5, however pH 6.5 was more appropriate for *Kluyveromyces* sp. ZMS1 GU133329. In agreement with our results, Russell (2003) recorded that yeast prefers an acid pH and its optimum pH is 5.0-5.2 but brewing and distilling strains are capable of good growth at the pH range of approximately 3.5 to 6.0. Narendranath and Power (2005) found that the optimum pH for yeast growth and ethanol production by *S. cerevisiae* was pH 4.9. Limtong et al. (2007) examined the ethanol production by *K. marxianus* DMKU 3-1042 in sugar cane juice medium with 22% sugar at 37°C and found that the highest ethanol concentration (8.7%) and yield (77.5% of theoretical yield) were obtained when the fermentation was carried out at pH 5.0. They also found that the ethanol levels recorded at pH 5.0 were only slightly higher than those obtained from fermentation at pH 5.5, while the lowest ethanol values were obtained from fermentation at pH 4.0. The same authors in the same paper reported that the ethanol fermentation carried out at 40°C by the same yeast strain gave better results at pH 5.0 and 5.5 than those at pH 4.0 and 4.5.

In fed-batch culture, the initial glucose concentration was 25 and 8% glucose was added at the rate of 4% after 48 and 96 h of starting fermentation. Ethanol produc-

tion levels were not increased by using this strategy and the results which recorded by fed-batch were similar to those recorded by batch technique. Complete similar results were obtained by Rudolf et al. (2005). They compared between batch and fed-batch fermentation for ethanol production and reported that the final ethanol yields did not differ between batch and fed-batch techniques. Yoshida et al. (1973) introduced the term fed-batch cultures to describe batch cultures which are fed continuously or sequentially, with medium, without the removal of culture fluid. A fed-batch culture is established initially in batch mode and is then fed. The feed strategy used in our experiment was with a very concentrated solution of glucose for addition of 8% glucose at the rate of 4% after 48 and 96 h of starting fermentation. This strategy was described by Stanbury et al. (1995) as fixed volume strategy

In this study, the increase in glucose level after 48 and/or 96 h of fermentation could not affect the ethanol production by the two yeast strains under investigation. One or more factors, e.g. the non-ability of these strains to tolerate the high ethanol concentration might have led to decrease of the ethanol level in the late fermentation period despite the availability of glucose in the medium. Prescott and Dunn (1959) reported that higher level of alcohol produced in a fermentation process may inhibit the action of the yeast strain and some of the sugar is not utilized. Also, the intracellular ethanol may be increased by increasing ethanol production and exerts high toxicity on yeast in addition to the nutrient which may be deficient at the final stage of fermentation (Sols et al., 1971).

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