INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
16–0873/2017/19–4–825–833
DOI: 10.17957/IJAB/15.0368
http://www.fspublishers.org

Full Length Article

Production of Bioethanol from Spoilage Date Fruits by New Osmotolerant Yeasts

Mohamed Hashem[1, 2,3*], Abd El-Latif Hesham[4], Sulaiman A. Alrumman[1] and Saad A. Alamri[1]
[1]*Biology Department, Faculty of Science, King Khalid University, Abha, Saudi Arabia*
[2]*Research Center for Advanced Materials Science (RCAMS)*
[3]*Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt*
[4]*Genetics Department, Faculty of Agriculture, Assiut University, Assiut, Egypt*
[*]For correspondence: drmhashem69@yahoo.com

Abstract

This research aimed at the production of bioethanol from a cheap and renewable resource (spoilage dates) by nonconventional yeasts to reduce total cost of the production. Chemical, physical or biological pretreatment of the spoilage date juice (SDJ) did not affect the availability of fermentable sugars significantly. The isolated osmotolerant yeast strains: *Pichia kudriavzevii* KKUY-0034, *Hanseniaspora opuntiae* KKUY-0152 and *H. uvarum* KKUY-0078, which were genetically identified based on sequences of D1/D2 domain 26S rRNA gene and phylogenetic analysis, were tested for their fermentability of the SDJ. The fermentation conditions were adjusted to induce the maximum production of ethanol. Results showed that the highest quantities of ethanol were obtained when the yeasts were grown on 20% of date juice at 30°C and when the pH was adjusted at 4-6 for 60 h. Addition of either Zn or Mg (0.4 g/L) and $NH_4H_2PO_4$ (4 g/L) had a good impact on the ethanol productivity by the three species, however, *H. uvarum* KKUY-0078 was the leader that produced 60 g/L of ethanol. In 7-L fermentor, when the optimum conditions were kept constant, ethanol production reached to 80 g/L after 60 h. The study concludes that SDJ is a promising and costless substrate for production of the bioenergy and using the osomtolerant yeasts is an economic strategy. The partial 26S rRNA gene sequences of *P. kudriavzevii* KKUY-0034, *H. uvarum* KKUY-0078 and *H. opuntiae* KKUY-0152 were deposited in the DDBJ, EMBL, and GenBank database under the accession Nos. JQ690250, JQ690236 and KC110834, respectively.

Keywords: Biofuel; Spoilage dates; Osmotolerant yeasts; Optimization; 26S rRNA sequences

Introduction

The growing demand for energy, increase in oil price and the environmental problems as a result of the use of fossil fuels has become a great challenge facing the world. Therefore, we are compelled to move towards sustainable alternative sources for energy (Wang *et al.,* 2013). Biofuel as a cheap and clean energy is a good alternative to fossil fuels (Braide *et al.*, 2016; Dhaliwal *et al.,* 2011). Bioethanol is an attractive renewable bio-based resource; however, the ability to produce high concentrations of ethanol rapidly is a key factor to maintain a high yield of ethanol during the fermentation process (Grahovac *et al.,* 2012; Hossain *et al.,* 2017).

To enhance the productivity and cost effectiveness of ethanol production, low-cost substrates should be used (Wang *et al.,* 2013). A wide range of feedstock, such as starch-based (wheat and corn), sugar-based (sugarcane juice and molasses and sugar beet), and cellulosic (wood and bagasse) resources can be used in ethanol production (Sarkar *et al.,* 2012; Khan and Dwivedi, 2013). Sugar-based feedstock can be considered as an ideal substrate for biofuel production because it contains readily available fermentable sugars (Balasubramanian *et al.,* 2011). Exhaustion of these crops in energy production will lead to the increase in their price all over the world and shortage in the human food resources. Therefore, using of renewable of agro-wastes and crop residue comprises an additional resource that alleviated the negative effects of consuming the energy crops (Hossain *et al.,* 2017). Spoilage dates, which contain a large amount of fermentable sugars, are a good alternative for the production of ethanol on a large scale, especially in countries, which are known for growing date palms. Date palm (*Phoenix dactylifera* L.) is one of the most important fruit trees growing in Arabian countries. Saudi Arabia is famous for a huge number of date palm trees and produces about 13% of the world production of dates. However, a considerable part of the date yield is subjected to spoilage and rotting because of pest attacking, handling, inappropriate transporting, and lack of cold stores (El-Juhany, 2010). We found many markets and farmers suffering from an accumulation of rotten and spoilage dates in their stores and they are facing problem to get rid of

To cite this paper: M. Hashem, A. El-Latif Hesham, S.A. Alrumman and S.A. Alamri, 2017. Production of bioethanol from spoilage date fruits by new osmotolerant yeasts. *Int. J. Agric. Biol.*, 19: 825–833

wasted dates. Recycling of this huge quantity of the spoilage date fruits and giving farmers a way to profit from it will help to minimize the risk.

Microorganisms of primary interest to industrial operations in the fermentation of ethanol include *Saccharomyces uvarum, S. cerevisiae, Kluyueromyces* sp., and *Schizosaccharomyces pombe.* The microflora of traditional and industrial fermentation processes may constitute a good source of microbial isolates with industrially relevant characteristics (Hossain and Jalil, 2015). Specifically, stress-tolerant yeast variants may be found in alcoholic fermentation processes, in which the yeast is subjected to several stresses, including osmotic and ethanol stresses (Basso *et al.,* 2008). Such stress-tolerant isolates may be good candidates for better fermentation process than the conventional microorganisms. Also, we assumed that introduction of osmotolerant yeasts, which could ferment more concentration of sugars, will reduce the workspace in fermentation tank that leads to the reduction of the capital cost of the fermentation process. Therefore, the main objective of this work was the recycling of the spoilage dates fruits into bioethanol by new isolated osmotolerant yeasts to minimize the capital cost of biofuel production process that makes it a competitive alternative in future for the classical oil production.

Materials and Methods

Substrate Preparation

Two date cultivars (Arihy and Nabt Ali) that showed a high degree of spoilage were selected in this study. Spoiled date fruits without stones of the two cultivars were mixed (1:1; w:w) and used to prepare spoilage date juice (SDJ) (Hashem *et al.,* 2014). Physical and chemical characteristics of the spoilage date including pH, moisture content, total solids, sugars, protein, lipids, fibers, ash and metal contents were estimated following the procedures of the AOAC (2000). Reducing sugar (glucose and fructose) concentration was detected by using 3, 5-dinitrosalicylic acid (DNS) reagent as described by Miller (1959).

Yeast Strains Isolation and Genetic Identification

Hanseniaspora uvarum KKUY-0078, *H. opuntiae* KKUY-0152 and *Pichia kudriavzevii* KKUY-0034 were selected among 150 isolates based on their efficiency to ferment the SDJ (data not shown). These isolates were recovered from spoilage dates on malt extract yeast extract agar (YMA) by the dilution plate method. They were genetically identified by sequencing the D1/D2 domain of the 26S rDNA region and phylogenetic analysis. The extraction of total yeast genomic DNA was performed according to the procedures described by Hesham (2014). The DNA was amplified using primers described by Kurtzman and Robnett (1998). These were NL1 (5'–GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5' GGTCCGTGTTTCAAG ACGG –3'). PCR reaction was performed in a final volume of 50 µL containing GoTaq green master mix (Promega, Madison, WI, USA), 1 µL of each primer at a concentration of 0.5 mM, and 1 µL template DNA. The PCR conditions were as described by Kurtzman and Robnett (1998). The amplified DNA was purified using the GFXTM – PCR DNA and gel band purification kit (Amersham, Biosciences), and the purified PCR was sequenced at the Macrogen Company (Seoul, Korea). The DNA sequence was analyzed using the DNA Blast at http://www.ncbi.nlm.nih.gov/BLAST/. The isolates identification was confirmed by the construction of phylogenetic trees using MEGA version 4.0 program.

Primary Ethanol Production by the Selected Yeast Strains

The procedures for the primary screening for ethanol production by the selected yeast strains have been done as described by Hashem *et al.* (2014).

Quantitative Estimation of Ethanol and Glucose Concentrations

Concentrations of ethanol and glucose in the samples were determined enzymatically using estimation kits (K620-100 for ethanol and K606-100 for glucose) according the procedures provided by BioVision company, USA (Hashem *et al.,* 2014).

Effect of Temperature

The SDJ (20%) was used to study temperature effect on the ethanol production by the selected yeasts. The fermentation temperatures were adjusted at 25, 30 and 35°C. The pH was adjusted at 4.5. The fermentation process was achieved at 150 rpm. Ethanol levels were estimated gravimetrically at each degree of temperature after 72 h of incubation.

Effect of pH Value

The effects of different initial pH levels (4, 5, 6, 7 and 8) on ethanol production by the selected yeasts grown on 20% of the date juice at 30°C was studied. The fermentation process was achieved at 150 rpm for 72 h of fermentation. pH was adjusted by 1N HCl or 0.1N NaOH.

Effect of Fermentation Period

The selected yeasts were grown on 20% of the date juice for different fermentation periods (24, 48, 60, 72 and 96 h) at 30°C and 150 rpm. The pH was adjusted before inoculation at 4. Ethanol concentrations were measured at the end of each fermentation period.

Effect of Sugar Concentration

The three yeasts were grown on different concentrations of

SDJ (10, 15, 20 and 25%) and incubated at 30°C and 150 rpm. for 72 h. The pH was adjusted before inoculation at 4. Ethanol concentration was measured at the end of fermentation period.

Effect of Metal Addition

Different metals; Zn (0.5 g/L), Mn (0.3 g/L), Co (0.2 g/L) and Mg (0.5 g/L) were added singly to the growing medium of the yeasts (20% of SDJ). The fermentation process was achieved at 150 rpm, 30°C and pH 4 for 72 h. Ethanol concentration was measured at the end of fermentation period. Different concentrations of Mn and Mg (0, 0.1, 0.2, 0.3, 0.4 and 0.5 g/L) as the most effective metals were tested in another set of the experiment.

Effect of Nitrogen Sources

Different available nitrogen sources including; yeast extract, malt extract, tryptone, ammonium nitrate and ammonium dihydrogen phosphate (5 g/L) were added singly to the growing medium of yeasts (20% of SDJ). The fermentation process was achieved at 150 rpm, 30°C and pH 4 for 72 h. Ethanol concentration was measured at the end of fermentation period. Different concentrations of ammonium dihydrogen phosphate (0, 2, 4, 6, 8, 10 g/L) as the most effective nitrogen source were tested in another set of experiment.

Pilot Test

Fermentation was carried out in a BioFlo/CelliGen 115 fermentor provided by New Brunswick Co., USA, with all necessary controls. The reactor was of 7-L capacity and the working volume was 3 L. The fermenter is equipped with an agitator, pH, and temperature control systems. The fermenter was cleaned and steam sterilized at 121°C for 20 min. Then the sterilized medium [20% of SDJ, ammonium dihydrogen phosphate (4 g/L), Mg (0.4 g/L) and Zn (0.4 g/L)] containing the inoculum (150 mL of 10^8 cell/mL) was transferred to the fermenter. The seed culture was grown at 25°C for 24 h in 250 mL flask containing 100 mL of YPD medium. The temperature of fermentation was maintained at 30 ± 1°C and pH was regulated at 4.5. The agitator speed was maintained constant throughout the experiment at 200 rpm. The reactor was maintained under anaerobic conditions. Samples were taken during the course of 72 h fermentation to monitor the ethanol concentrations.

Statistical Analysis

All experiments were repeated twice. The replicates were arranged in a completely randomized design and the data were analyzed using one-way analysis of variance (ANOVA) and the significance of differences among the treatments was determined according to Least Significant Difference (LSD) at $P < 0.05$ (Gomez and Gomez, 1984).

Results

Fig. 1 shows that boiling of the SDJ for 30 min at 100 °C, adding 5% H_2SO_4 or adding cellulose, as pretreatment, increased the concentration of the reducing sugar up to 470 g/kg fresh dates. However, the difference among these treatments was not significant ($P < 0.05$). The hot H_2SO_4 and cold water had a negative impact on the released sugars. Analysis of SDJ shows that the fruit mixture of the two selected cultivars contains 62.5% of the dry weight as sugars (Table 1). Glucose account for 28.5% of the dry weight of the date, however the total reducing sugars were 462 g/kg. Protein content was 1.8% and the lipid content was 0.52%. Manganese was estimated as 1.82 mg/kg and other metals were detected in low concentrations. Cadmium was detected as 0.8 ppm, followed by nickel (1.84 ppm).

The selected yeasts: KKUY-0078, KKUY-0034 and KKUY-0152 produced 43.70, 41.48 and 39.07 g/L of ethanol from the SDJ (20%, w:v) (Fig. 2), respectively. They consumed 83.1%, 80.88% and 78.47% of the available sugars (92.4 g/L), respectively. Based on the theoretical expected yield (51% of glucose), the productivity of ethanol by these yeasts was 92.74%, 88.03% and 82.92%, respectively.

The sequence analysis of the large subunit of 26S rRNA gene of KKUY-0034, KKUY-0078 and KKUY-0152 isolates showed high identity with those of *P. kudriavzevii*, *H. uvarum* and *H. opuntiae*, respectively i.e. had 99% or 100% of similarity. Analysis of the phylogenetic tree of the three selected isolates with the related species (Fig. 3) confirmed their taxonomic positions. Therefore, the isolates KKUY-0034, KKUY-0078 and KKUY-0152 were identified as *P. kudriavzevii*, *H. uvarum* and *H. opuntiae* respectively. The sequence analyses of the identified yeasts were deposited in the DDBJ, EMBL and GenBank database under the accession Nos. JQ690250, JQ690236 and KC110834, respectively.

The results obtined from the optimization studies showed that 30°C was the most appropriate temperature for all the three yeasst, at which the maximum quantities of ethanol were produced. While the production started to decrease when the tempreature reached to 35°C except the *H. uvarum* KKUY-0078, which strongly decreased at this tempreature (Fig. 4A). *P. kudriavzevii* KKUY-0034 produced the highest quantity of ethanol (58.6 g/L) at 30°C folowed by *H. uvarum* KKUY-0078 (57.75 g/L). These data indicate that these yeast strains are mesophylic ethanol-producers. Fig. 4B shows that the three yeast strains produced the maximum quantities of ethanol at pH 4 and 5, however the production decreased at pH 6–8. This could be due to that the enzyme system of ethanol production has slightly acidic affinity. To test the appropriate concentration of the SDJ for ethanol production, juice concentration range from 10 to 25% was used. Results show that all yeasts produced their maximum concentration of ethanol when they were grown in 20% of

Table 1: Chemical and physical properties of spoilage date fruit juice

Constituents	Concentration
Moisture	28.4%
Total sugars	625 g/kg
Disaccharides	125 g/kg
Monosaccharides (reducing sugars)	462 g/kg
Glucose	285 g/kg
Total Protein	18 g/kg
Lipids	5.2 g/kg
Fibers	56.4 g/kg
Ash	5.75 g/kg
Iron (Fe)	5.08 ppm
Cupper (Cu)	4.16 ppm
Cobalt (Co)	3.2 ppm
Nickel (Ni)	1.84 ppm
Zinc (Zn)	5.4 ppm
Lead (Pb)	2.12 ppm
Cadmium (Cd)	0.8 ppm
Manganese (Mg)	1.82 mg/ kg

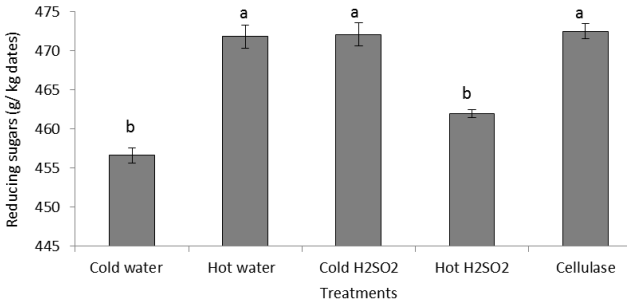

Fig. 1: Effect of different pretreatments on reducing sugars availability from spoilage date fruits. Columns followed by the same letter are not significant at LSD ($P < 0.05$) and bars represent the standard error

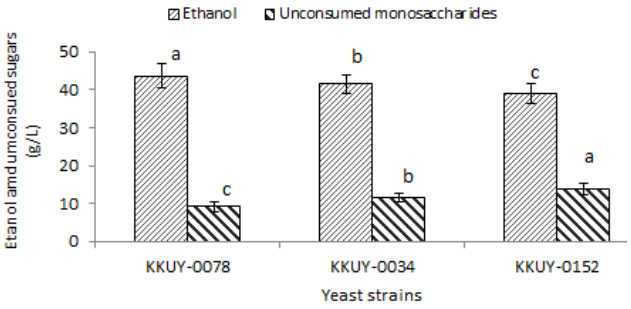

Fig. 2: Production of ethanol by three yeast strains and their consumption of sugar. KKUY-0078 (=*H. uvarum*), KKUY-0034 (=*Pichia kudriavzevii*) and KKUY-0152 (=*H. opuntiae*). Columns with the same pattern having the same letters are not significant at LSD ($P < 0.05$)

date juice (Fig. 4C). *H. uvarum* KKUY-0078 produced its maximum ethanol concentration as 61.2 g/L that is equal to 92% of the theoretical yield. The results indicate the high efficiency of the three yeasts to assimilate and ferment the date juice into ethanol. However, in 25% of SDJ, ethanol

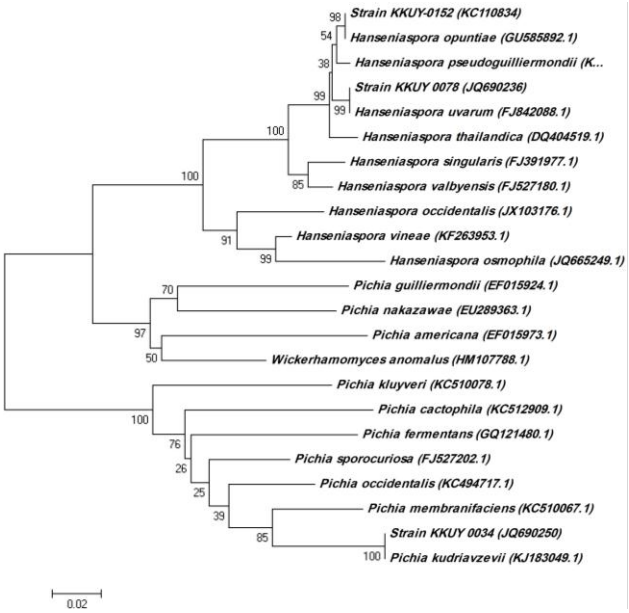

Fig. 3: Phylogenetic relationship between isolated yeast strains (KKUY-0152, KKUY-0078 and KKUY-0034) and other 26S rRNA gene sequences of published strains. Accession numbers for sequences are as shown in the phylogenetic tree

concentrations were lower than those obtained in 20%. The maximum yield of ethanol by the three yeast strains was achieved at 60 h (Fig. 5). The quantity of ethanol produced by the three yeasts had approximate values (57.2–63.5 g/L). Then, the production began to decrease by further extension of time.

The influence of metal addition on ethanol concentration is depicted in Fig. 6. The results approved that both Zn (0.5 g/L) and Mg (0.5 g/L) significantly enhanced the production of ethanol by the three yeasts. Both Mn and Co showed some toxicity on the yeasts activity that involved in decreasing the ethanol production (Fig. 6A). When different concentrations of Zn were added, 0.4 g/L was found the most appropriate concentration for the three yeasts; however, its stimulatory effect on *H. opuntiae* KKUY-0152 was noticeable. It produced 67.89 g/L of ethanol because of addition of Zn (0.4 g/L). The productivity of the other two yeasts was enhanced but still lower than *H. opuntiae* KKUY-0152 (Fig. 6B). The higher doses of this metal decreased the ethanol productivity by all yeasts. Addition of Mg in different concentrations was tested and the data were represented in Fig. 6C. Low doses of this metal (up to 0.4 g/L) had a stimulative effect of the ethanol production by the yeasts. The higher doses showed negative effect on the productivity. Addition of this metal as 0.4 g/L increased the ethanol production by *H. opuntiae* KKUY-0152 to 67.0 g/L; however, *H. uvarum* KKYU-0078 and *P. kudriavzevii* KKUY-0034 produced 58.04 and 56.69 g/L, respectively.

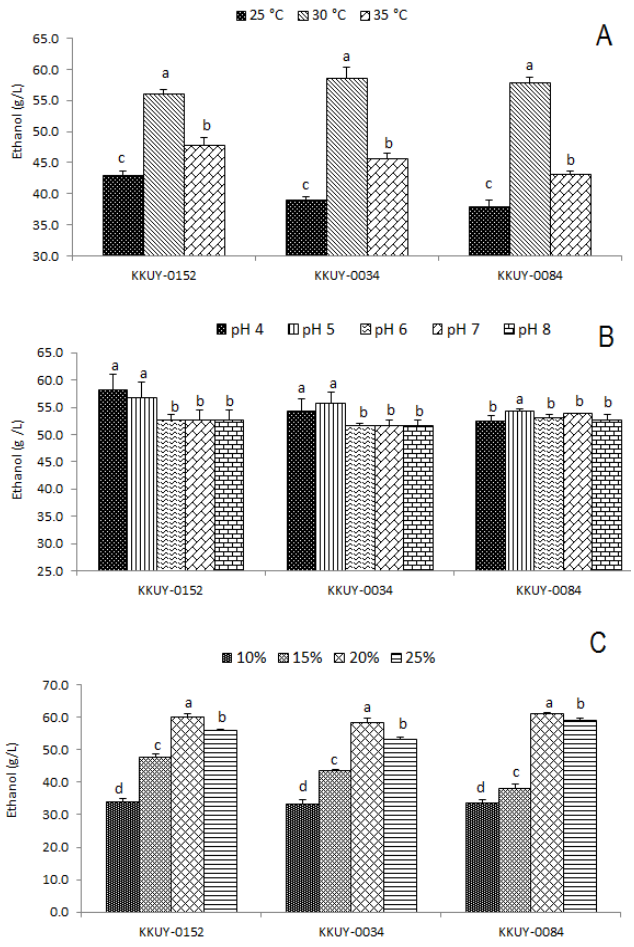

Fig. 4: Effect of temperature (A), pH (B) and date juice concentration (C) on ethanol production. KKUY-0152 (=*H. opuntiae*), KKUY-0034 (=*P. kudriavzevii*) and KKUY-0078 (=*H. uvarum*). Columns with the same pattern having the same letters are not significant at LSD ($P < 0.05$). Bar above each column represents the standard errors

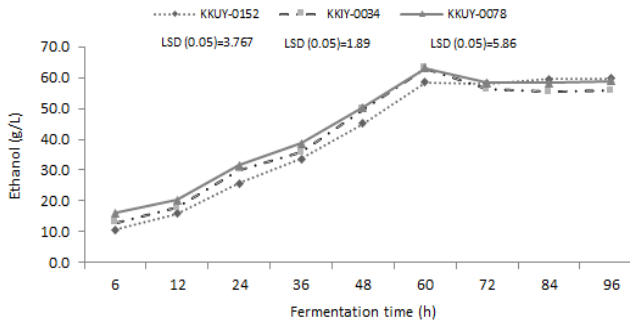

Fig. 5: Effect of fermentation time on ethanol production. KKUY-0078 (=*H. uvarum)*, KKUY-0152 (=*H. opuntiae*) and KKUY-0034 (=*P. kudriavzevii*). Bar at each mark represents the standard errors

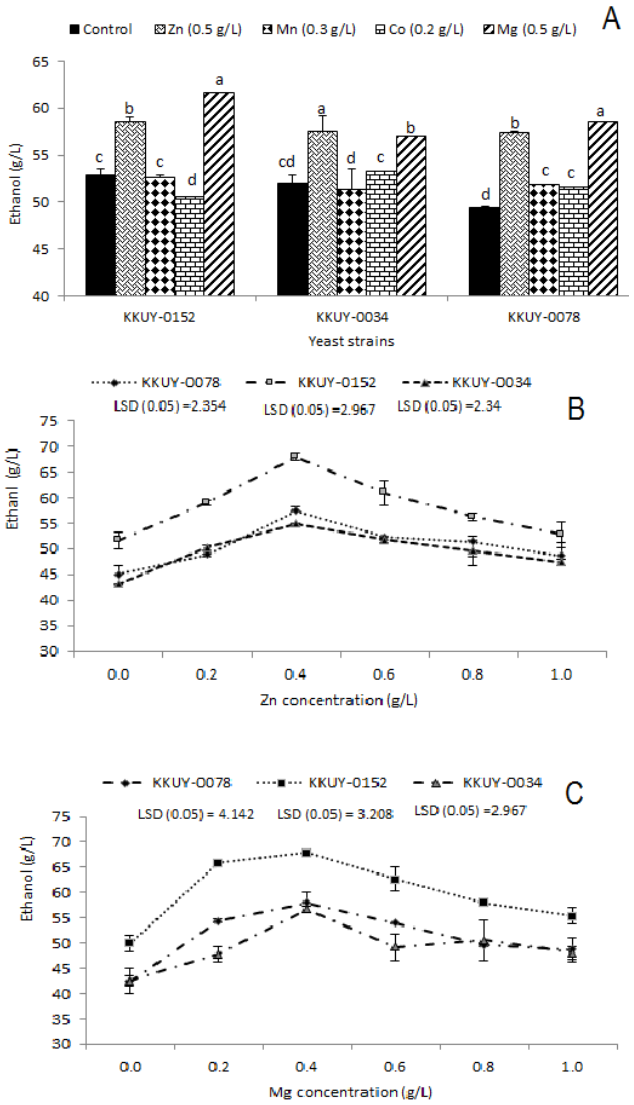

Fig. 6: Effect of addition of different metals (Zn, Mn, Co and Mg) on ethanol production (A), effect of Zn concentration on ethanol production (B) and effect of Mg concentration on ethanol production (C) by KKUY-0152 (=*Hanseniaspora opuntiae*), KKUY-0034 (=*Pichia kudriavzevii*) and KKUY-0078 (=*Hanseniaspora uvarum*). Columns with the same letter opposite to each yeast strain are not significant at LSD ($P < 0.05$). Bar above each column represents the standard errors

The effect of different nitrogen sources (5 g/L) on ethanol production by the yeasts was tested and the results showed that all nitrogen sources had a significant positive effect on the productivity (Fig. 7A). The highest increase in ethanol productivity was due to the addition of ammonium dihydrogen phosphate. The maximum ethanol concentration was 62.75, 60.20 and 59.36 by *H. opuntiae* KKUY-0152, *P. kudriavzevii* KKUY-0034 and *H. uvarum* KKUY-0078, respectively. When ammonium dihydrogen

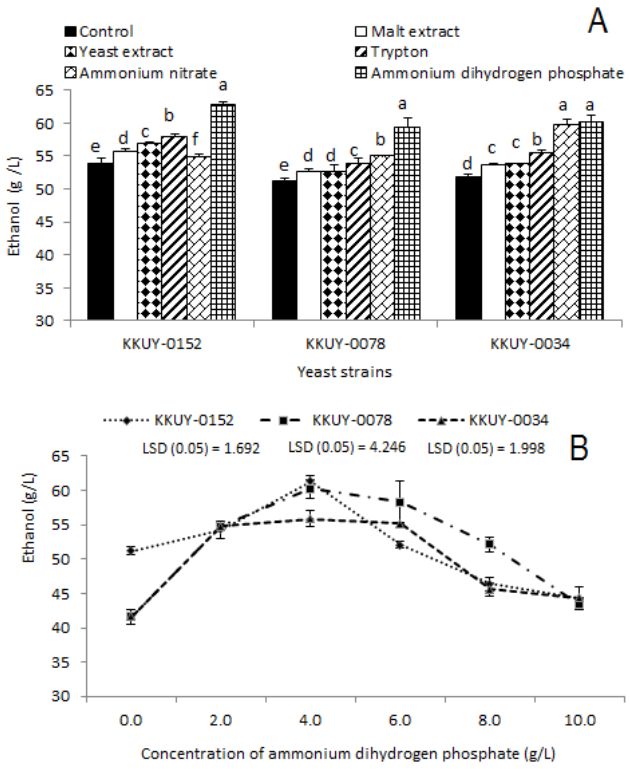

Fig. 7: Effect of different nitrogen sources (g/L) on ethanol production (A) and effect of ammonium dihydrogen phosphate consecration on ethanol production (B) by KKUY-0152 (=*Hanseniaspora opuntiae*), KKUY-0034 (=*Pichia kudriavzevii*) and KKUY-0078 (=*Hanseniaspora uvarum*). Columns with the same letter opposite to each yeast strain are not significant at LSD ($P < 0.05$). Bar above each column represents the standard errors

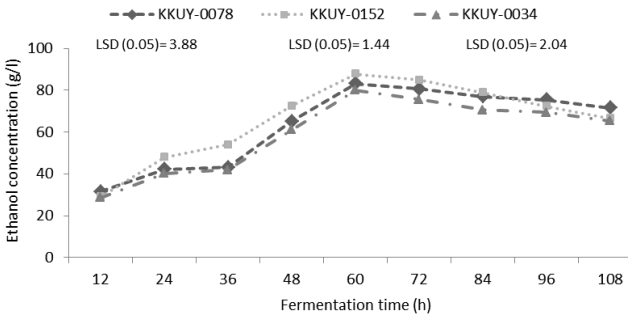

Fig. 8: Production of ethanol in 7-L fermentor by KKUY-0152 (=*Hanseniaspora opuntiae*), KKUY-0034 (=*Pichia kudriavzevii*) and KKUY-0078 (=*Hanseniaspora uvarum*). Bars represent the standard error

phosphate was added in concentration gradient, 4 g/L was the best dose for all yeasts (Fig. 7B).

Fig. 8 shows the ethanol productivity of the yeast strains in 7 L fermentror. As it was aproved from the laboratory, *H. opuntiae* KKUY-0152 and *H. uvarum* KKUY-0078 are the highest ethanol producers; however, the first species was a superior one. The ethanol production increased gradually until 60 h to record the maximum value as 87.74 and 83.24 g/L by *H. opuntiae* KKUY-0152 and *H. uvarum* KKUY-0078, respectively. Then the concentration begain to decline deliberately to the end of the fermentation time. It was noticed that the ethanol production in the fermentor is considerably higher than that was obtained in the shaked conical flasks (60 g/L). This could be due to the large enviroenment and avialbility of the nutrients in addition to shaking procedures that adapt the fermentation process to increase the ethanol productivity.

Discussion

Results of the present study showed that there was no significant difference among hot water, cold acid or cellulase enzyme as pretreatments of SDJ. We assume that the most degradable sugar contained in the spoilage tissues was degraded by action of the endogenous microflora. So, there was no need for further pretreatment. This is very interesting from the economic point of view, because using only a hot water is enough to produce all fermentable sugar from the spoilage date fruits, and this will reduce the cost of the pretreatments. Chemical analysis showed the richness of SDJ in sugars and many minerals that make them an appropriate medium for growth of yeasts without any additions (Hossain *et al.*, 2017). Also, SDJ is a good source of fermentation because it contains high amount of the fermentable sugars.

The sequence analysis of the large subunit 26S rRNA gene confirmed the identity of the tested strains as *P. kudriavzevii* KKUY-0034, *H. uvarum* KKUY-0078 and *H. opuntiae* KKUY-0152. This technique is now accepted as a standard, rapid and accurate method for yeast identification compared with the classical method (Kurtzman and Robnett, 1998). Because, 600 bp length of D1/D2 domain of the 26S rDNA contains satisfactory variation to define individuals at the species level (De Lanos *et al.*, 2004).

P. kudriavzevii KKUY-0034, *H. uvarum* KKUY-0078 and *H. opuntiae* KKUY-0152 were able to ferment SDJ efficiently and produced considerable amount of ethanol. Their transformation rate was 92.74%, 88.03% and 82.92%, respectively, based on the expected theoretical production that is equal to 51% of glucose (Govindaswamy and Vane 2007). In agreement with our results, Al-Talibi *et al.* (1975) reported that *S. cerevisiae* yielded 9.96% ethanol by fermentation of 20% pure sugar solution (represented 97.65% of the theoretical value). Mehaia and Cherayan (1991) obtained 48.27% ethanol by *S. cerevisiae* (= 94.64% of the theoretical value) and 47.0% (= 92.16% of the theoretical yield) of the total concentration of the sugar when used in the batch fermentation the sugars date juice 9.8 and 13.83%, respectively. Zohri and Mostafa (2000)

have shown that the quantities of ethanol produced by *S. cerevisiae* were 96.54, 99.17 and 78%, while those formed by *S. bayamus* were 98.65, 99.17 and 78.58% of the theoretical values, respectively, when 13.5, 18 and 22.5% sugar date juice.

Results showed that 30°C is the most appropriate temperature for the three yeasts. This indicates that these yeasts are mesophylic ethanol-producers. *H. uvarum* was more frequently reported as a principal species in many technological interest (Kachalkin *et al.*, 2015). For our relevance, this is first report to use non-saccharomyces yeasts in production of ethanol from spoilage date palm fruits in Saudi Arabia. It seems that such yeast species will carry out much fermentation instead of *S. cerevisiae* in the future. Temperature is one of the most important factors affecting ethanol production is fermentation, which has a direct effect on the biochemical reactions of yeast (Albertin *et al.*, 2014, 2016). Also, temperature is known to affect yeast metabolism and, as a result, the formation of some metabolites such as ethanol, glycerol, acetic acid (Lafon-Lafourcade, 1983). The enzyme activities are expected low at low temperature (Torija *et al.*, 2003). In this study, the maximum production of ethanol was produced at pH 4 and 5. This could be due to that the enzyme system of ethanol production has slightly acidic affinity. pH has a significant impact on the fermentation, because it effects on the growth of yeasts, the fermentation rates and the formation of by-products. So, maintenance of pH constant during fermentation is very important for the processes (Albertin *et al.*, 2016). In accordance with our results, Pramanik (2003) reported that the maximum ethanol concentration produced by *S. cerevisiae* was achieved at pH 4.25–5.0. 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. He also, reported that during any fermentation, H^+ ions are excreted by the yeast and these results in a pH decline in the 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 ~3.8. 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) reported that *K. marxianus* DMKU 3-1042 produced the highest ethanol concentration (8.7%) and yield (77.5% of theoretical yield) in sugar cane juice medium with 22% sugar at pH 5.0.

To enhance the ethanol production, it is important to use yeast strains that have ability to tolerate and utilize high concentrations of sugars (Converti *et al.*, 1985; Shiyuan *et al.*, 1987). So, we used concentration of SDJ up to 25% with an intention to get high yields of ethanol in reasonable time. Results showed that all yeasts producee the maximum concentration of ethanol when they were grown in 20% of date juice, however, 25% decrease the productivity. We assume that high concentration of sugar has an inhibitory effect on ethanol production, that may be caused due to the yeast cells plasmolysis (Pramanik, 2003). In agreement with this hypothesis, Pratt-Marshall *et al.* (2003) mentioned that increase in sugar concentrations lead to increase the viscosity in fermentation medium and this had a highly inhibiting effect on yeast growth and their capability to ethanol production. Reddy and Reddy (2006) reported that the increasing in the sugar concentration will decrease in sugar utilization, which results in reduction of the total ethanol production. This reduction is due to several reasons including the production of other compounds than ethanol 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 of these factors lead to stopping the fermentation process and ethanol formation at the final stage of fermentation.

The maximum yield of ethanol by the three yeasts was achieved at 60 h. Fermentation period is a very important factor from the economics of ethanol on the industrial scale. We think that the similarity in fermentation time by the three yeasts is because their taxonomically intimacy and their similarity in physiology and the required cultural conditions. Our findings are in agreement with those of Limtong *et al.* (2007), who used four isolates of *Kluyveromyces marxianus* for ethanol production from sugar cane juice and found that the maximum ethanol concentrations were formed by the four yeast isolates after 72 h of starting the fermentation at 30°C.

Our results approved that both Zn (0.5 g/L) and Mg (0.5 g/L) significantly enhanced the production of ethanol by the three yeasts. While, both Mn and Co showed some toxicity on the yeasts activity that involved in decreasing the ethanol production. Zinc is a basic element for all living organisms, which affects both cell growth and metabolism. Zinc improved the production of ethanol by promoting the accumulation of trehalose and ergosterol in the yeast cells that improved alcohol tolerance (Shobayashi *et al.*, 2005; Tosun and Ergun, 2007; Zhao *et al.*, 2009). Addition of Mg in low doses (up to 0.4 g/L) induced the ethanol production by the three yeasts; however, the higher doses showed suppressive effect on the productivity. Magnesium is necessary for the growth of yeast, metabolism and fermentation (Walker, 2000). Thanonkeo *et al.* (2007) found that addition of magnesium (10–20 mM) dramatically increased the ethanol production by *Zymomonas mobilis*. The decrease in ethanol productivity as a result of increasing the concentration of magnesium could be due to its toxicity in high concentration or due to its cleating with other growth necessary cofactor like calcium (Okon and Nwabueze, 2010).

Addition of different nitrogenous compounds showed a significant increase in ethanol production by the tested yeasts. Ammonium dihydrogen phosphate showed the highest increase in the productivity among all sources. Our results are supported by those of Grahovac *et al.* (2012),

who found that the addition of a nitrogen source such as ammonium sulphate significantly increased the ethanol yield. In similar findings, Yue *et al.* (2012) reported that the addition of nitrogen to the fermentation medium greatly improved the production of ethanol. They found that with urea as the nitrogen source, the maximum ethanol concentration reached 135 g/L after 60 h by *S. cerevisiae*.

The ethanol productivity in 7 L bioreactor showed a similar trend as in the case of laboratory. The ethanol concentration increased gradually until 60 h to reach its maxiumum, and then begain to decline. It is worth to mention that the production in the fermentor was hgiher than in the shaked conical flasks by 38.7–46.2%. This could be due to the large enviroenment and avialbility of the nutrients in addition to shaking procedures that adapt the fermentation process to increase the ethanol productivity.

Conclusion

In conclusion, this work introduces a very cheap and sustainable raw material "spoilage dates" for the biofuel production sector. This material contains a large amount of monosaccharide sugars that are available for fermentation into ethanol. We suppose the usage of spoilage dates in bioethanol production will decrease the overall production cost, since it significantly decreases the raw material cost and the pretreatment expense. Using of non-conventional and osmotolerant yeasts that could ferment such materials efficiently represent an additional achievement of this study. We recommend application of such cheap material and fermentative osmotolerant yeasts to produce bioenergy on large and commercial scale.

Acknowledgement

The authors would like to express their gratitude to King Khalid University, Saudi Arabia for providing administrative and technical support.

References

Albertin, W., C. Miot-Sertier, M. Bely, P. Marullo, J. Coulon, V. Moine, B. Colonna-Ceccaldi and I. Masneuf-Pomarede, 2014. Oenological prefermentation practices strongly impact yeast population dynamics and alcoholic fermentation kinetics in Chardonnay grape must. *Int. J. Food Microbiol.*, 178: 87–9.

Albertin, W. Setati, M.E. Miot-Sertier, C. Mostert, T.T. Colonna-Ceccaldi, B. Coulon, J. Girard, P. Moine, V. Pillet, M. Salin, F. Pillet, F. Salin, M. Bely, B. Divol and I. Masneuf-Pomarede, 2016. *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. *Front Microbiol*., 6: 1569

Al-Talibi, A.A., N.D. Benjamin and A.R. Abboud, 1975. Relationship between ethyl alcohol produced by *Saccharomyces cerevisiae* from Iraqi dates, pure sugar solution and the amount of inoculums. *Nahrung*, 19: 335–340

AOAC, 2000. *The Association of Official Analytical Chemists*. Wiscsin: George Banta Co. Inc

Balasubramanian, K., V. Ambikapathy and A. Panneerselvam, 2011. Studies on ethanol production from spoiled fruits by batch fermentations. *J. Microbiol. Biotechnol. Res.*, 1: 158–163

Basso, L.C., H.V. de Amorim, A.J. de Oliveira and M.L. Lopes, 2008. Yeast selection for fuel ethanol production in Brazil. *FEMS Yeast Res.*, 8: 1155–1163

Braide, W., I.A. Kanu, U.S. Oranusi and S.A. Adeleye, 2016. Production of bioethanol from agricultural waste. *J. Fundam. Appl. Sci*., 8: 372–386

Converti, A.P., P.A. Lodi, F. Parisi and M.D. Borghi, 1985. A kinetic study of saccharomyces strains: Performance at high sugar concentrations. *Biotechnol. Bioeng.*, 27: 1108

De Lanos, R., A. Querol, A.M. Planes and M.T. Fernández-Espinar, 2004. Molecular characterization of clinical *Saccharomyces cerevisiae* isolates and their association with non-clinical strains. *Syst. Appl. Microbiol*., 27: 427–435

Dhaliwal, S.S., H.S. Oberoi, S.K. Sandhu, D. Nanda, D. Kumar and S.K. Uppal, 2011. Enhanced ethanol production from sugarcane juice by galactose adaptation of a newly isolated thermotolerant strain of *Pichia kudriavzevii*. *Biorsour. Technol.*, 102: 5968–5975

El-Juhany, L.I., 2010. Degradation of date palm trees and date production in Arab countries: causes and potential rehabilitation. *Aust. J. Basic Appl. Sci*., 4: 3998–4010

Gomez, K.A. and A.A. Gomez, 1984. *Statistical Procedures for Agricultural Research*, 2nd edition, pp: 13–175. John Wiley and sons, Inc. London, UK

Govindaswamy, S. and L.M. Vane, 2007. Kinetics of growth and ethanol production on different carbon substrates using genetically engineered xylose-fermenting yeast. *Biorsour. Technol*., 98: 677–685

Grahovac, J., J. Dodic, A. Jokic, S. Dodic and S. Popov, 2012. Optimization of ethanol production from thick juice: A response surface methodology approach. *Fuel*, 93: 221–228

Hashem, M., A. Hesham, S.A. Alrumman and S.A. Alamri, 2014. Indigenous yeasts of the rotten date fruits and their potentiality in bioethanol and single-cell protein production. *Int. J. Agric. Biol.*, 16: 752–758

Hesham, A., 2014. New safety and rapid method for extraction of genomic DNA from bacteria and yeast strains suitable for PCR amplifications. *J. Pure Appl. Microbiol.*, 8: 383–388

Hossain, N., J.H. Zaini and T.M.I. Mahlia, 2017. A review of bioethanol production from plant-based waste biomass by yeast fermentation. *Int. J. Technol.*, 1: 5–18

Hossain, N. and R. Jalil, 2015. Analyses of Bio-energy Properties from Malaysian Local Plants: Sentang & Sesenduk. *Asia Pacif. J. Ener. Environ. (APJEE)*, 2: 141–144

Kachalkin, A.V., D.A. Abdullabekova, E.S. Magomedova, G.G. Magomedov and I.Y. Chernov, 2015. Yeasts of the vineyards in Dagestan and other regions. *Microbiology*, 84: 425–432

Khan, Z. and A.K. Dwivedi, 2013. Fermentation of Biomass for Production of Ethanol: A Review. *Univ. J. Environ. Res. Technol*., 3: 1–13

Kurtzman, C.P. and C.J. Robnett, 1998. Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Anton. Van Leeuwenh*., 73: 331–371

Lafon-Lafourcade, S., 1983. Wine and Bandy. In: Rehm, H.J., Reed, G. (Eds.). Food and Feed Production with Microorganisms. *Biotechnol. Verlage Chemie Weinheim.*, 5: 81–163

Limtong, S., C. Sringiew and W. Yongmanitchai, 2007. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marixianus*. *Biores. Technol.*, 98: 3367–3374

Mehaia, M.A. and M. Cheryan, 1991. Fermentation of date extracts to ethanol and vinegar in batch and continuous membrane reactors. *Enz. Microbiol. Technol*., 13: 257–261

Miller, G.L., 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Anal Chem.*, 31: 426–428

Narendranath, N.V. and R. Power, 2005. Relationship between pH and medium dissolved soids in terms of growth and metabolism of *Lactobacilli* and *Saccharomyces cerevisiae* during ethanol production. *Appl. Environ. Microbiol*., 71: 2239–2243

Okon, A.A. and T.U. Nwabueze, 2010. Optimization of divalent cation in *Saccharomyces pastorianus* medium conditions for ethanol production. *Afr. J. Biotechnol*., 9: 5423–5429

Pramanik, K., 2003. Parametric Studies on Batch Alcohol Fermentation Using Saccharomyces Yeast Extracted from Toddy. *J. Chin. Inst. Chem. Engrs*., 34: 487–492

Pratt-Marshall, P.L., J.H. Bryce and G.G. Stewart, 2003. The effects of osmotic pressure and ethanol on yeast viability and morphology. *J. Inst. Brew*., 109: 218–228

Reddy, L.V.A. and O.V.S. Reddy, 2006. Rapid and enhanced production of ethanol in very high gravity (VHG) sugar fermentation by *Saccharomyces cerevisiae*: Role of finger millet (*Eleusine coracana* L.) flour. *Process Biotechnol*., 41: 726–729

Russell, I., 2003. Understanding yeast fundamentals. *In*: *The Alcohol Textbook-a Reference for the Beverage, Fuel and Industrial Alcohol Industries*, pp: 85–119. Jacques, K.A., T.P. Lyons and D.R. Kelsall (eds.). UK: Nottingham

Sarkar, N., S.K. Ghosh, S. Bannerjee and K. Aikat, 2012. Bioethanol production from agricultural wastes: An overview. *Renewab. Ener*., 37: 19–27

Shiyuan, Y., W. Morris and K.P. Sarad, 1987. Fermentation of Ethanol of Pentose Containing Spent Sulphite Liquor. *Biotechnol. Bioeng.*, 29: 1144

Shobayashi, M., S.I. Mitsueda, A. Mariko, T. Fujii, K. Iwashita and H. Iefuji, 2005. Effects of culture condition on ergosterol biosynthesis by *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.*, 69: 2381–2388

Sols, A., C. Gancedo and G. Delafuente, 1971. *The Yeasts*, Vol. 2, p: 271. Rose, A.H. and J.S. Harrison (eds.). Academic Press, London

Thanonkeo, P., P. Laopaiboon, K. Sootsuwan and M. Yamada, 2007. Magnesium ions improve growth and ethanol production of *Zymomonas mobilis* under heat or ethanol stress. *Biotechnology*, 6: 112–119

Torija, M.J., N. Rozes, M. Poblet, J.M. Guillamon and A. Mas, 2003. Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *Int. J. Food Microbiol*., 80: 47–53

Tosun, A. and M. Ergun, 2007. Use of experimental design method to investigate metal ion effects in yeast fermentations. *J. Chem. Biotechnol*., 82: 11–15

Walker, G.M., 2000. *The Role of Metal Ions in Optimizing Yeast Fermentation Performance, In: Nutrition Aspects II*. Synergy between yeasts and bacteria. Lalleman Technical Meeting 2000 Perugia, Italy

Wang, L., M. Sharifzadeh, R. Templer and R.J. Murphy, 2013. Bioethanol production from various waste papers: Economic feasibility and sensitivity analysis. *Appl. Energ*., 111: 1172–1182

Yue, G., J. Yu, X. Zhanga and T. Tan, 2012. The influence of nitrogen sources on ethanol production by yeast from concentrated sweet sorghum juice. *Biom. Bioen.*, 39: 48–52

Zhao, X.Q., C. Xue, X.M. Ge, W.J. Yuan, J.Y. Wang and F.W. Bai, 2009. Impact of zinc supplementation on the improvement of ethanol tolerance and yield of self-flocculating yeast in continuous ethanol fermentation. *J. Biotechnol*., 139: 55–60

Zohri, A.A. and E. Mostafa, 2000. Ethanol production from dates in Saudi Arabia on industrial scale. *Microbiology*, 28: 76–81

(Received 19 December 2016; Accepted 20 March 2017)