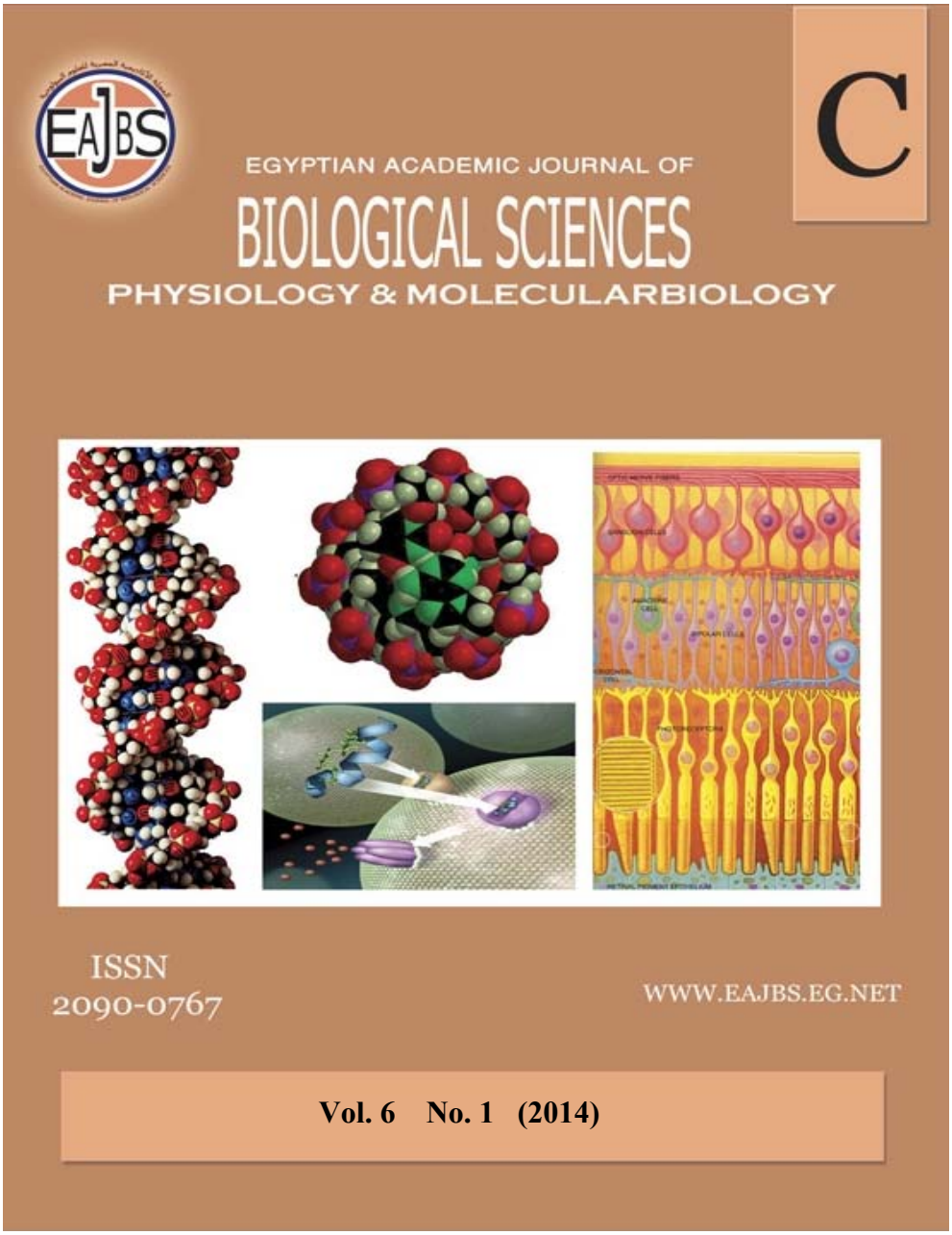

Egyptian Academic Journal of Biological Sciences is the official English language journal of the Egyptian Society for Biological Sciences ,Department of Entomology ,Faculty of Sciences Ain Shams University .
Physiology & molecular biology journal is one of the series issued twice by the Egyptian Academic Journal of Biological Sciences, and is devoted to publication of original papers that elucidate important biological, chemical, or physical mechanisms of broad physiological significance.

www.eajbs.eg.net

Egypt. Acad. J. Biolog. Sci., 6(1): 1 – 12 (2014)

Egyptian Academic Journal of Biological Sciences
C. Physiology & Molecular Biology

ISSN 2090-0767

www.eajbs.eg.net

Intermediate and pharmaceutical compounds associated with fermentation of spoilage date fruits by *Hanseniaspora guilliermondii* KKUY-0045

Mohamed Hashem[1, 2 *]; Abd El-Latif Hesham[2, 3]; Saad A. Alamri[2] and Sulaiman A. Alrumman[2]

1- Faculty of Science, Botany and Microbiology Department, Assiut University, Assiut, Egypt

2- Faculty of Science, Biology Department, King Khalid University, Abha, Saudi Arabia

3- Faculty of Agriculture, Genetics Department, Assiut University, Assiut, Egypt

[*] Email: drmhashem69@yahoo.com

ARTICLE INFO

Article History
Received: 20/9/2013
Accepted:26/10/2013

Keywords:
Fermentation
Hanseniaspora guilliermondii
GC-MS
Pregnane and nonacosane
26S rDNA sequencing
Phylogenetic analysis

ABSTRACT

The aim of this study was to determine the intermediate chemical compounds during the fermentation of spoilage date juice (SDJ) by newly isolated yeast strain *Hanseniaspora guilliermondii* KKUY-0045. This yeast strain was identified based on sequence of the variable D1/D2 domain of the large subunit (26S) ribosomal DNA and comparing it with similar sequences in the Genbank database. Phylogenetic analysis using rDNA sequences indicated that strain KKUY-00045 shared one cluster with *Hanseniaspora guilliermondii*. In 7-L fermenetor, this strain produced 87.74 g/L of ethanol after 3 days of incubation. The chemical analysis of the cell free extract of the yeast strain grown on the SDJ by using GC-MS revealed the presence of some important chemical compound such as pregnane and nonacosane that could be used in the medical and pharmaceutical industries. The study recommends extensive studies to discover the chemicals associated with fermentation process and their industrial role.

INTRODUCTION

Saudi Arabia is famous for the wide cultivation of date palm trees. It produces about 13% of the world production of dates. There are more than six million of date palm trees distributed all over Saudi landscape. Date manufacturing is one of the most popular industries in Saudi Arabia. However many insects are known to attack date palm fruits and trees (El-Juhany, 2010). Mycotoxigenic fungi, particularly aflatoxigenic Aspergilli, have been associated with dates and date products (Emam *et al.,* 1994; Shenasi *et al.,* 2002). The rot of dates is due to increased infestation of pests and diseases, improper transporting, handling, and lack of cold stores, lack of marketing and export.

Spoilage dates is abundant renewable agro-industrial waste material produced in Saudi Arabia and its low cost is an important factor for the economic production of bioethanol by fermentation. Bioethanol can be produced different renewable feed starch sources. However, sugarcane industrial wastes, agricultural low cost fruits, vegetables, starch materials cellulosic materials, agricultural by products of starch industries, such as potato pulp, tapioca pulp, papaya fruit pulp from raw starch hydrolyzate are attractive resources for economical production of ethanol (Balasubramanian *et al.,* 2011). We introduce the spoilage dates as one of the most economic and renewable source for bioethanol production, because it contains high amount of sugars which doesn't need to any chemical pretreatments comparing to the other agrowastes. The investigation of secondary compounds, which occasionally formed during fermentation, is of great importance. These compounds might have medicinal or pharmaceutical importance. In addition to their extreme usefulness, production of such compounds will reduce the production cost of the main target process "bioethanol". Some active compounds such as methyl-diols and secondary alcohol derivatives were mentioned as by-products produced by yeasts (Fuganti and Grasselli, 1985, Hashem and Darwish, 2010).

The objective of this study was to determine the intermediate and pharmaceutical compounds formed during the fermentation of the spoilage date fruits by isolated yeast. 26S rDNA sequencing and phylogenetic analysis was applied for molecular identification of the yeast at specie level.

MATERIALS AND METHODS

Preparation of the spoilage dates juice (SDJ):

Two date cultivars (Arihy and Nabt Ali) that were the most spoilage cultivars were selected in this study. Samples of these two cultivars were mixed in ration 1:1 (w:w) without stones and stored at 4°C. Fresh samples were ground using high speed blender, then was diluted with distilled water to 2:8 (w:v) and sterilized at 121 °C for 30 min. The produced juice was passed through double layer of muslin cloth to exclude all large undigested particles. The filtrate was considered as the prepared date juice (SDJ) and stored in refrigerator for further use.

Yeast isolation and purification:

Yeast strain was isolated from the collected spoilage date fruits using dilution plate method as described by Kutzman and Fell (1998) on malt extract yeast extract agar (YMA) medium .The medium was sterilized by autoclaving at 121°C for 15 min. The sterilized agar medium was cooled to approximately 45°C and its pH was adjusted to 3.7 and then poured into the petri dishes. Samples were inoculated onto the dishes and incubated at 25°C for 48-72 h. Then, the growing yeasts were isolated and purified. For purification purpose, the isolated yeasts were streaked twice on YMA plates for single pure colonies. The pure colonies were inoculated onto YMA slants and incubated at 25 °C for 48 -72 h and then transferred into 4°C to be stored.

Molecular identification of the isolated yeast:

DNA extraction and PCR amplification of D1/D2 domain of 26S rDNA region:

The extraction of total yeast genomic DNA was performed according to procedures described by Hesham et al. (2006). The D1/D2 domain of the 26S rDNA region was amplified using the primers NL1 (5'- GCATATCAATAAG CGGAGGAAAAG-3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') (Kurtzman and Robnett 1998). PCR 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 amplification was carried out by PCR under the following conditions : initial denaturation at 95 °C for 5 min, followed by 36 cycles at 94 °C for 2 min, 52 °C for 1 min, 72 °C for 2 min; final extension at 72 °C for 7 min, and holding at 4 °C. PCR products (5 µL) were analyzed using 1.5% 0.59 TBE agarose gel electrophoresis. The gel was stained with ethidium bromide, visualized under UV light, and photographed.

Sequencing of the D1/D2 domain of the 26S of rDNA and phylogenetic analysis:

DNA for sequencing was amplified with forward and reverse primers (NL1-NL4). The amplified D1/D2 fragment was purified and sequenced using an ABI 3730 automated sequencer (Macrogene Company, Korea). The yeast 26S rDNA sequences obtained were then aligned with known 26S rDNA sequences in the GenBank database using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) and percent homology scores were generated to identify isolated yeast. To determine the taxonomic position of the isolate, a phylogenetic tree was constructed with MEGA version 4.0 using a neighbor-joining algorithm, plus the Jukes-Cantor distance estimation method with bootstrap analyses for 1,000 replicates was performed.

Nucleotide sequence accession number:

The 26S rDNA sequences of the isolated yeast reported in this study has been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number JQ690239 (*H. guilliermondii* KKUY-0045).

Fermentation of the spoilage date fruits by *H. guilliermondii* KKUY-0045:

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 were used as the batch fermenter in this study. The fermenter was cleaned and steam sterilized at 121°C for 15 minutes. 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 was transferred to the fermenter. the seed culture was grown at 25 °C for 24 h in 250 mL flask containing 100 mL of a YPD medium. The temperature of fermentation was maintained at 30 ± 1 °C. The pH of the fermentation broth was regulated at 4.5 within pH 0.1 unit by the peristaltic pump which injected a fine stream of sulfuric acid or sodium hydroxide. The fermentations were carried out at atmospheric pressure. 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 6 days of fermentation to monitor the ethanol concentration.

Ethanol determination (Quantitative estimation of ethanol):

Concentration of ethanol in samples was estimated enzymatically using Ethanol estimation kit (K620-100) provided by BioVision company. The procedures provided with the product was employed. Briefly, this method is based on presence of alcohol oxidase that oxidizes ethanol to generate H_2O which reacts with the probe to generate color (λmax= 570 nm). A standard curve was constructed using the standard ethanol provided within the kit. The amount of ethanol in a given sample was determined from the standard curve after measuring its absorbance at 570 nm.

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis:

During fermentation, different samples of fermentation medium (10 mL) were taken at different time intervals (2, 4 and 6 days). The samples were centrifuged at 10000 rpm for 15 min under cooling and the supernatant was filtered through cellulose membrane filter (0.45 μm) to exclude any yeast cells, and then was extracted by chloroform solution. An aliquot of one μL extract (chloroform extract) of cell free extract was injected into the GC–MS (6890 N/5975B). The HP-5MS column was 30 m in length, 0.25 mm i.d., and 0.25 mm in thickness. The carrier gas was helium with average velocity 36 cm/sec, and flow 1 mL/min. The operating condition of GC oven temperature was maintained as follows: initial temperature 40 °C for 9 min, 150 °C for 8 min, at 15 °C/min up to final temperature 310 °C with isotherm for 3 min at 25 °C/min. The injector and detector temperatures were set at 250 and 280 °C, respectively, according to the standard method 8270 EPA (Cakir *et al*., 2004). Identification of the components of the prepared extract was assigned by comparison of their retention indices, relative to a series of n-alkane indices on the capillary column and GC–MS spectra from the Wiley 6.0 MS data.

RESULTS AND DISCUSSION

Molecular identification of the yeast:

In order to identify and determine the correct phylogenetic position of the yeast strain KKUY-0045, molecular genetic identification were performed by amplification and sequencing of the D1/D2 region of the 26S rRNA gene. The obtained sequence data were compared with the sequences of 26S rRNA regions in Genbank by means of BLAST search of the National Center for Biotechnology Information (NCBI) databases. Alignment of the 26S rRNA gene sequences of the yeast with sequences obtained by doing a BLAST search revealed up to 100% similarity to *H. guilliermondii* (Fig. 1a).

```
KKUY       1  ACTGGATACTTAGTACGGCGAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTTT  60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.     1  ACTGGATACTTAGTACGGCGAGTGAAGCGGTAAAAGCTCAAATTTGAAATCTGGTACTTT  60

KKUY      61  CAGTGCCCGAGCTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCTT  120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.    61  CAGTGCCCGAGCTGTAATTTGTAGAATTTGTCTTTGATTAGGTCCTTGTCTATGTTCCTT  120

KKUY     121  GGAACAGGACGTCATAGAGGGAGAGAATCCCGCTTGGCGAGGATACCTTTTCCCTGTAAT  180
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   121  GGAACAGGACGTCATAGAGGGAGAGAATCCCGCTTGGCGAGGATACCTTTTCCCTGTAAT  180

KKUY     181  ACTTTTTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATC  240
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   181  ACTTTTTCAAAGAGTCGAGTTGTTTGGGAATGCAGCTCAAAGTGGGTGGTAAATTCCATC  240

KKUY     241  TAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAA  300
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   241  TAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAAGTACAGTGATGGAAAGATGAAAA  300

KKUY     301  GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATC  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   301  GAACTTTGAAAAGAGAGTGAAAAAGTACGTGAAATTGTTGAAAGGGAAGGGCATTTGATC  360

KKUY     361  AGACATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCA  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   361  AGACATGGTGTTTTTTGCATGCACTCGCCTCTCGTGGGCTTGGGCCTCTCAAAAATTTCA  420

KKUY     421  CTGGGCCAACATCAATTCTGGCAGTAGGATAAATCATTAAGAATGTAGCTACCTCGGAGT  480
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   421  CTGGGCCAACATCAATTCTGGCAGTAGGATAAATCATTAAGAATGTAGCTACCTCGGAGT  480

KKUY     481  GTTATAGCTTATTGGAATACTGCTAGCTGGGATTGAGGACTGCGCTTCGGCAAGGATGTT  540
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   481  GTTATAGCTTATTGGAATACTGCTAGCTGGGATTGAGGACTGCGCTTCGGCAAGGATGTT  540

KKUY     541  GGCATAATGGTTAAATGCCGCCCGCTGAAAAGGGGGACCAAATTGCGAAATCTTA  595
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||
H.gui.   541  GGCATAATGGTTAAATGCCGCCCGCTGAAAAGGGGGACCAAATTGCGAAATCTTA  595
```

Fig. 1a: Sequence alignment of the isolate KKUY-0045 against D1/D2 of 26S rRNA gene sequence data of *Hanseniaspora guilliermondii* showing no base substitutions.

To confirm the position of our strain in phylogeny, a number of sequences were selected from Genbank database for the construction of a phylogenetic tree using MEGA4 program. As shown in Fig. 1b, the phylogenetic tree indicated that strain KKUY-0045 and *H. guilliermondii* shared one clade cluster. Therefore, the strain KKUY-0045 was identified as *H. guilliermondii*.

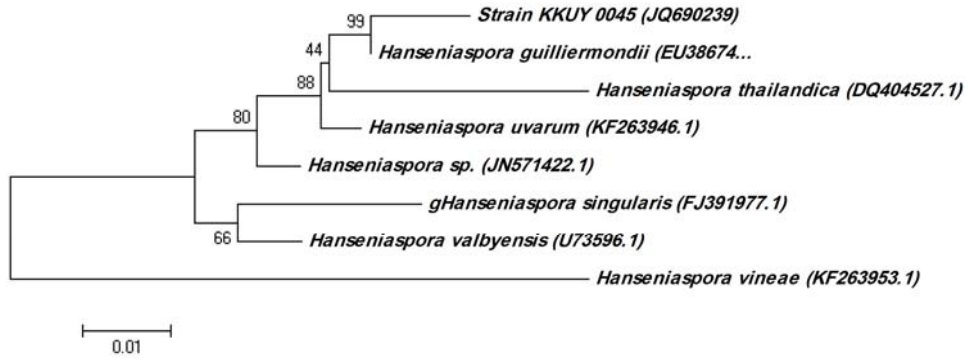

Fig. 1b: Phylogenetic relationship between *Hanseniaspora guilliermondii* KC110834 strain KKUY-0045 and other 26S rRNA gene sequences of published strains. In the phylogenetic tree, KKUY-0045 and *Hanseniaspora guilliermondii* were clustered together as one clade segments corresponding to an evolutionary distance of 0.01 are shown with bars. Accession numbers for sequences are as shown in the phylogenetic tree.

In the last few years, an extensive research effort have been made to characterize the yeast microbiota in a vast number of food preparations through techniques based on molecular biology with particular emphasis on PCR amplification of ribosomal RNA genes and spacer regions as internal transcribed spacers (ITS) and non-transcribed spacers (NTS) followed by restriction analysis with restriction enzymes (Esteve-Zarzoso *et al.,* 1999; Baleiras Couto *et al.,* 2005; Raspor *et al.,* 2006). The sequence of the large subunit ribosomal DNA (LSU rDNA), especially D1/D2 region, has revealed to be a powerful tool in yeast identification (Kurtzman and Robnett 1998) becoming more accessible due to new efficient nucleic acids sequencing techniques. The sequencing of the D1/D2 of the large-subunit 26S ribosomal DNA is now widely accepted as a standard procedure for yeast identification. Moreover, a 600 bp length of the D1/D2 domain of the 26S rDNA contains sufficient variation to define individuals at the species level (Kurtzman and Robnett, 1998; Scorzetti *et al.,* 2002; Frutos *et al.*, 2004). It was found that molecular method based on the sequences of the 26S rDNA, D1/D2 domain is rapid and precise compared with the physiological method for the yeast identification, and has also been applied to study the phylogeny of different yeast groups (Kurtzman and Robnett, 1998; Hesham *et al.*, 2006; Hesham and Mohamed, 2011).

Ethanol production by the *H. guilliermondii* KKUY-0045:

Fig. 2 shows the ethanol production by *H. guilliermondii* KKUY-0045 during 6 days of fermentation in a 7-L fermentor. Concnetration of ethanol produced by this strain increased gradually until 3 days to record the maximum production as 87.74 g/L. Then the concentration begain to decline deliberately to the end of the fermentation time. Gradual increasing of ethanol production by other yeast strains up 72 h was reported by Limtong *et al.* (2007). They used four isolates of *Kluyveromyces marxianus* for ethanol production from sugar cane juice and found that the maximum ethanol concentrations was formed by the four yeast isolates after 72 h of starting the fermentation at 30°C. Our results are in close agreement of those authors. We suggest that the similarity in ethanol productivity by *K. marxianus* and our strain is due to their taxonomically intimacy and their similarity in physiology and required cultural conditions.

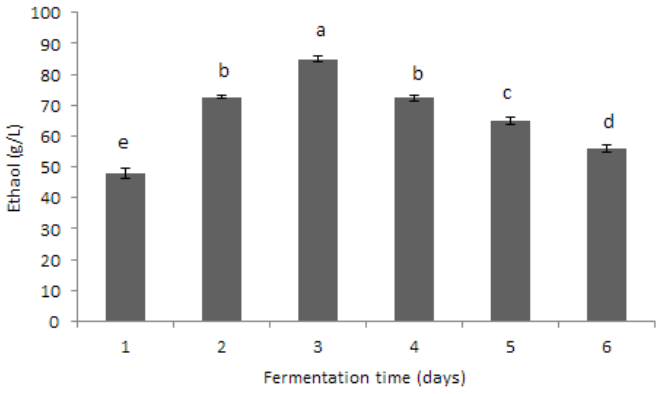

Fig. 2: Fermentation of spoilage date fruits by *H. guilliermondii* KKUY-0045 during 6 days of incubation in 7-L Fermentor at 30 °C.

GC-MS analysis of the fermented date juice by *H. guilliermondii* KKUY-0045:

The results of GC-MS analyses of the cell free extract of *H. guilliermondii* KKUY-0045 at different time intervals (2, 4, and 6 days) are reported in Tables 1-3 and Figs. 3-5. Pregnane were detected as the top compounds in 30.093% and 28.027%, respectively. There are five compounds including: Methyl diethyl borane, 1-Iodooctadecane, Octacosane, Hexadecane and 1,2,4-trimethyl cyclohexane were detected in moderate concentrations (2-6%). Eight compounds were detected in concentrations 1-2%, however the other 27 compounds were detected in very low concentration (less than 1%). After 4 days, the number of the detected volatile compound increased to 56 compounds. Methyl diethyl borane and 14B-Pregnane were recoded as the leader compounds that contributed 35.19 and 21.595%, respectively. It is noticed that the concentration of 14B-Pregnane decreased compared to in the previous 2 days (Table 1 and Fig. 3). There are other six compounds were detected in moderate concentration (2- 4.66%).

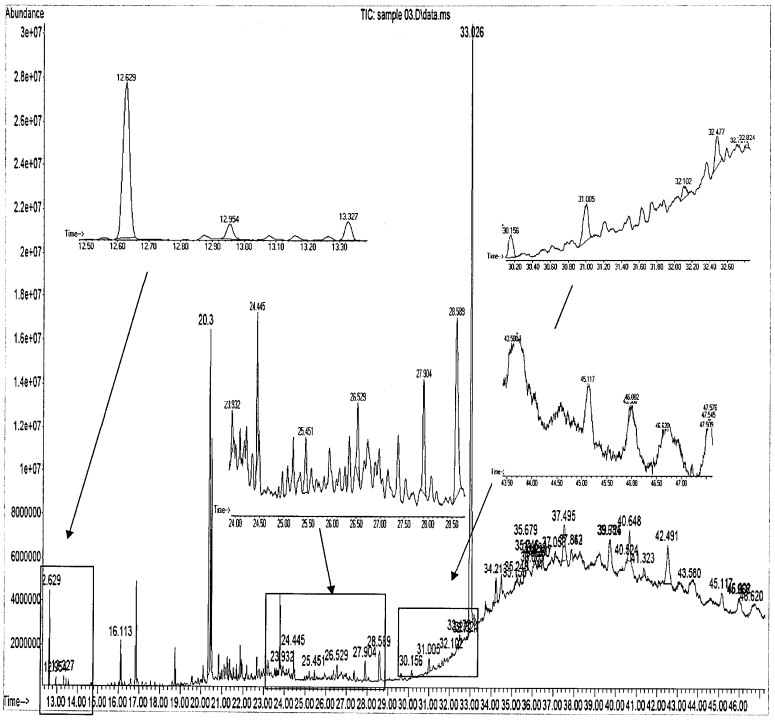

Fig. 3: GC-MS chart the fermented date juice by *H. guilliermondii* KKUY-0045 after 2 days of incubation at 25 °C.

Table 1: GC-MS analysis of the fermented date juice by *H. guilliermondii* KKUY-0045 after 2 days of incubation at 25 °C.

	Compounds	Conc. %	RT(min)
1	(9.alpha, 13.alpha)-5-chloro-5,6,8,14-tetadehydro-3-hydroxy-2,4,6-trimethoxy-17methyl-B-homomorphinan-7-one	30.093	33.027
2	14B-Pregnane	28.267	20.393
3	Methyl diethyl borane	6.216	34.215
4	1-Iodooctadecane	4.342	42.491
5	Octacosane	4.255	40.649
6	Hexadecane	3.511	37.495
7	1,2,4-trimethyl cyclohexane	2.255	35.684
8	Nonadecane	1.953	39.721
9	Hahnfett	1.455	36.369
10	(Z.Z.) -4-ethyl-3-methyl-5-(5-4-aminophenyl-2-methylene)-3,4-di,ethyl-5H-pyrrolyl-2-methylene)-3-pyrrolin-2on	1.437	12.631
11	7.Beta.,9.beta.:8alpha.,10alpha.-bis(dimethylmethylenedioxy)-7,8,9,10-tetrahydrobenzo[a]pyrene	1.315	32.482
12	1-Hexadecanol	1.282	28.591
13	2,6,10,14-Tetramethyl hexadecane	1.271	45.122
14	Tetradecanonic acid methyl ester	1.17	39.689
15	Docosane	1.121	36.094
16	Butyl phthalate	0.974	16.117
17	Octadecane	0.858	41.324
18	2-Hexyl-1-Decanol	0.857	26.531
19	Cyclododecanone	0.714	35.243
20	3,7,11-Trimethyl-1-dodecanol	0.696	27.906
21	2-Bromo dodeane	0.662	37.847
22	4,4',6,6'-Tetra-butyl-o o'-biphenol	0.400	40.525
23	Eicosane	0.376	36.26
24	1-Tetradecanol	0.352	30.158
25	2,10-Dimethyl octacosanoic acid	0.344	24.445
26	Butyl citrate	0.34	35.16
27	17-Pentatriacontene	0.333	23.393
28	Tricosane	0.301	47.576
29	Nonyl cyclopropane	0.291	35.746
30	1-Bromoeicosane	0.247	25.452
31	2,3,4-Trimethyl hexane	0.245	37.863
32	Diisooctyl adipate	0.238	36.011
33	2,4-Di-tert-butylphenol	0.234	45.968
34	9-phenanthrene methyl anthrancene-9-carboxylate	0.209	32.721
35	1,54-Dibromotetra pentacontane	0.195	47.509
36	Tetratriaconate	0.169	47.55
37	7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione	0.163	32.104
38	Butyl phthalate	0.161	13.331
39	1-Chloroheptacosane	0.141	37.052
40	Isobutyl phthalate	0.136	12.958
41	1,3-Bis(1,1-dimethylethyl)-benzene	0.107	32.825
42	Dodecyl3-mercaptopropionate	0.079	46.004
42	1-(2-hydroxybenzoyl)-3methyl-5-(trifluoromethyl)-3,4-dihydro-1H-pyrazol-5ol	0.032	46.621

RT=Retention time, Conc. =concentration.

They were Docosane, Hahnfett, Tetradecyl-oxirane, 2,4-ditert-butyl-6-nitrophenol, 1-Iodohexadecane and Nonadecane. Cyclododecane, Nonacosane, 9.alpha, 13.alpha)-5-chloro-5,6,8,14-tetadehydro-3-hydroxy-2,4,6-trimethoxy-17methyl-B-omomorphinan-7-one, 7.beta., 9.beta.: 8alpha.-bis (dimethyl methyl enedioxy) -7,8,9,10-tetrahydrobenzo [a]pyrene, 7-Methyl-6-tridecene and 1,2,4-Trimethylycyclohexane were detected in low concentration (1-less than 2 %). However the other 43 compounds were detected in very low concentration (less than 1%) (Table 2 and Fig. 4).

Table 2: GC-MS analysis of the fermented date juice by *H. guilliermondii* KKUY-0045 after 4 days of incubation at 25 °C.

No.	Compounds	Conc. %	RT (min)	No.	Compounds	Conc. %	RT (min)
1	Methyl diethyl borane	35.192	33.069	29	2-Ethyl hexanol	0.36	23.107
2	14B-Pregnane	21.595	20.408	30	Dicotyl adipate	0.355	38.268
3	Docosane	4.655	35.72	31	Tributyl acetylcitrate	0.333	46.751
4	Hahnfett	3.594	36.114	32	1,3-Bis(1,1-dimthylethyl)-benzene	0.311	27.366
5	Tetradecyl-oxirane	3.342	40.696	33	1-Tetradecanol	0.29	23.21
6	2,4-ditert-butyl-6-nitrophenol	3.274	42.569	34	Chloroxylenol	0.254	34.361
7	1-Iodohexadecane	2.811	37.572	35	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	0.25	23.932
8	Nonadecane	2.548	39.752	36	1-Hexadecanol	0.249	22.992
9	Cyclododecane	1.925	34.485	37	4-Methoxy carbonyl-3,5-diphenyl-1-pyrroline	0.24	23.672
10	Nonacosane	1.793	39.243	38	Octadecyl tichlorosilane	0.21	43.083
11	(9.alpha, 13.alpha)-5-chloro-5,6,8,14-tetadehydro-3-hydroxy-2,4,6-trimethoxy-17methyl-B-homomorphinan-7-one	1.638	28.601	39	Tetratriacontane	0.204	46.077
12	7.beta.,9.beta.:8alpha.-bis(dimetylmethylenedioxy)-7,8,9,10-tetrahydrobenzo[a]pyrene	1.584	24.445	40	Tricosane	0.198	46.777
13	7-Methyl-6-tridecene	1.461	23.776	41	17-pentatriacontene	0.196	22.78
14	1,2,4-Trimethyl cyclohexane	1.378	35.175	42	2-Hydroxy-3,5,5-trimethyl-cyclohex-2-enone	0.193	23.625
15	2,6,10,14-tetamethyl hexadecane	0.85	37.858	43	2,10-Dimethyloctacosanoic acid	0.171	22.894
16	1-Chlorooctadecane	0.764	40.546	44	28-Nor-17.alpha.(H)-hopane	0.169	23.335
17	4-Ethyl-3-,4-dimethylcyclohexanone	0.764	35.258	45	3-[(1Z)-2-Carboxy-1-propenyl]-2,2-dimethylcyclopropanecarboxylic acid	0.132	9.144
18	6,6-dimethyl-2-(2-methylpropoyl)bicycle[3.1.1]heptan-3-one	0.711	30.998	46	1,4-Diaza-2,5-dioxo-3-isobutylbicycl[4.3.0]nonane	0.13	14.659
19	4-(2-Hydroxyethyl)phenol	0.674	27.907	47	N-[amino (imino) methyl] tryptophan	0.13	35.881
20	Tetradecane	0.627	43.711	48	1-Tridecanol	0.124	23.241
21	Isobutyl phthalate	0.617	12.625	49	Heptacosane	0.104	36.768
22	1,54-Dibromo tetrapentacontane	0.518	45.195	50	1,3-diacetyl-cyclopentane	0.094	13.326
23	2,4-Di-tert-butylphenol	0.505	41.407	51	Triacontane	0.088	46.097
24	4-Ethyl tetadecane	0.475	43.788	52	1-Butyl 2-isobutyl phthalate	0.064	12.952
25	3,4-Dimethy-benzenamine	0.45	26.733	53	4-methyl heptadecane	0.059	36.929
26	2,3,4-trimethyl hexane	0.437	38.102	54	1-Chloroheeptacosane	0.044	36.799
27	2-Bromo dodecane	0.433	23.542	55	Bis (2-ethylhexyl) phthalate	0.031	31.834
28	Tetracosane	0.381	43.659	56	1-Iodotridecane	0.021	47.612

RT=Retention time, Conc. =concentration.

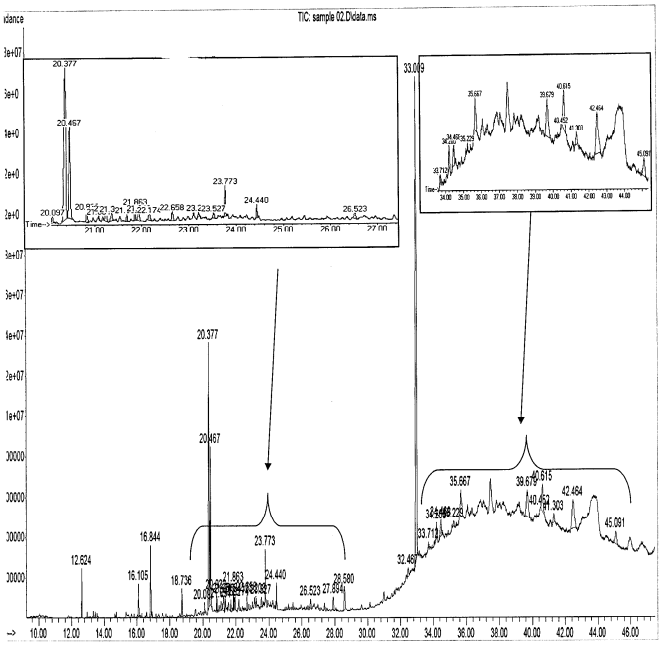

Fig. 4: GC-MS chart the fermented date juice by *H. guilliermondii* KKUY-0045 after 4 days of incubation at 25 °C.

After 6 days, only 35 compounds were detected. Among them, Di-(2-ethyl hexyl) phthalate and 14B-Pregnane are the leader compounds, which were detected in 30.765% and 18.48%, respectively. Eight compounds were detected in concentrations of 2-9%. They were 1-Tetradecanol, 1-Hexadecanol, Tricosane, 1-chloroheptacosane, 7.beta., 9. beta.: 8alpha., 10. alpha.-bis (dimetylmethylenedioxy)-7,8,9,10-tetrahydrobenzo [a]pyrene, Eicosane, 2,4-Ditert-butylphenol and Methyldiethylborane. There were another eight compound detected in low concentration (1 – less than 2%), however the other 17 compounds were detected in very low concentration (less than 1%) (Table 3 and Fig. 5).

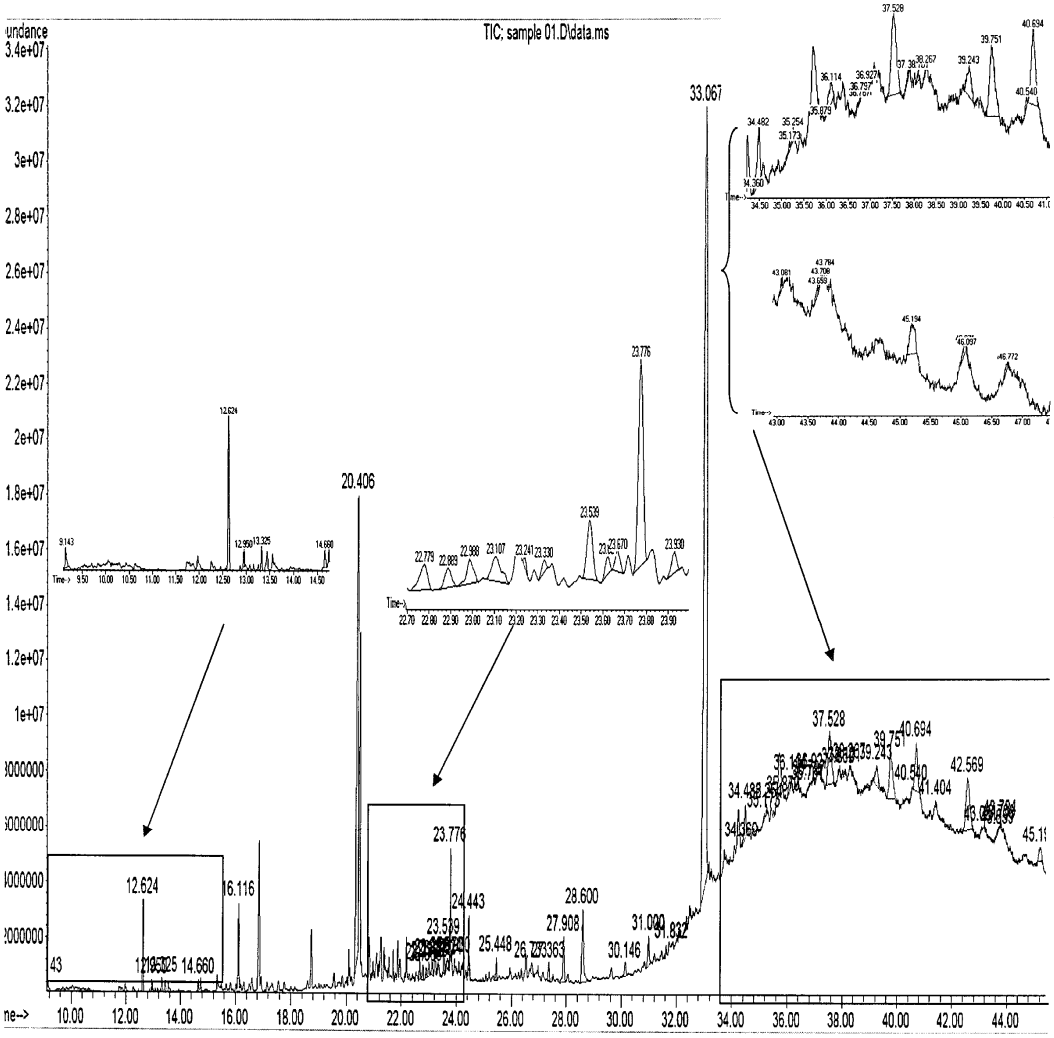

Fig. 5: GC-MS chart the fermented date juice by *H. opuntiae* KKUY-0153 after 6 days of incubation at 25 °C

Table 3: GC-MS analysis of the fermented date juice by *H. guilliermondii* KKUY-0045 after 6 days of incubation at 25 °C.

	Compounds	Conc. %	RT(min)
1	Di-(2-ethyl hexyl) phthalate	30.765	33.012
2	14B-Pregnane	18.48	40.618
3	1-Tetradecanol	9.942	20.377
4	1-Hexadecanol	4.721	20.471
5	Tricosane	3.09	35.668
6	1-chloroheptacosane	2.998	39.472
7	7.beta.,9.beta.:8alpha.,10.alpha.-bis(dimetylmethylenedioxy)-7,8,9,10-tetrahydrobenzo[a]pyrene	2.369	41.308
8	Eicosane	2.354	42.465
9	2,4-Ditert-butylphenol	2.346	16.844
10	Methyl diethylborane	2.273	34.47
11	Nanodecane	1.601	40.452
12	Hahnfett	1.584	33.712
13	1,54-dibromotetra pentacontane	1.45	34.205
14	1-butyl 2-isobutyl phthalate	1.417	23.776
15	Benzaldoxine	1.332	28.581
16	(17.Alpha.H,21.beta.H)-hopane	1.172	45.091
17	Cyclodecane	1.009	16.107
18	1,3-bis(1,1- dimwthyl ethyl)-benzene	1.006	12.626
19	Hexadecane	0.782	18.738
20	2,6,10,14-Tetramethyl hexadecane	0.72	21.944
21	1-Bromo-hexadecane	0.686	21.099
22	2-(Tetradecyloxy)-ethanol	0.682	21.254
23	Tributyl acetylcitrate	0.654	27.896
24	Octadecane	0.642	21.867
25	1,4-Diaza-2,5-dioxo-3-isobutyl bicyclio[4.3.0]nonane	0.625	23.527
26	a,a.9-trimethyl-2,3-dihydro-1H-pyrrol[1,2-a]indol	0.593	22.178
27	1-Cyclopentyl-2-n-hexyloctane	0.592	32.472
28	1-Iodohexadecane	0.573	21.368
29	GLycerol 1,3-dihexandecanoate-(DELTA.-9,12) -octadecadienoate	0.555	35.712
30	4-methoxy carbonyl-3-,5-diphenyl-1-pyrroline	0.509	26.526
31	Pentadecane	0.479	20.834
32	Dedocyl 3-mercaptopropionate	0.467	24.445
33	1,2-Benaenedicarboxylic acid, bis(2-methylpropyl) ester	0.455	22.66
34	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	0.427	23.21
35	2,4-ditert-butyl-6-nitrophenol	0.355	20.102
36	1-Iodotridecane	0.298	21.695

RT=Retention time, Conc. =concentration

We observed that there are many compounds detected during the three periods of fermentation but in various concentrations. They are: 14B-Pregnane,1- chloroheptacosane, 1-Hexadecanol, 1- Iodohexadecane, 1-Tetradecanol, 2,4- ditert-butyl -6-nitrophenol, Cyclodecane, Eicosane, Hahnfett, Hexadecane and Tricosane. These observation make us to assume that production of the volatile compounds is greatly depending on the strain of yeast itself and the time of fermentation. Where some of the produced volatiles could be transformed into another or degraded by the yeast.

Almost of the volatile compounds detected here were previously reported as yeast derivative volatile components, and their olfactory characters were already described (Ames and Elmore, 1992; Ames and McLeod, 1985; Münch and Schieberle, 1998; Wang *et al.*, 2011). Comuzzo *et al.* (2006) identified more than 160 volatile compounds in the headspace of the commercial powders

(some not previously reported in literature). However there are some compounds were not previously detected.

For example, 14 β -Pregnane is an important compound. Pregnane is, indirectly, a parent of progesterone. It is a parent hydrocarbon for two series of steroids stemming from 5α-pregnane and 5β-pregnane. 5β-Pregnane is the parent of the progesterones, pregnane alcohols, ketones, and several adrenocortical hormones and is found largely in urine as a metabolic product of 5β-pregnane compounds. Nonacosane has been reported to be a component of a pheromone of *Orgyia leucostigma* and evidence suggests it plays a role in the chemical communication of several insects, including the female *Anopheles stephensi* mosquito (Brei *et al*., 2004).

CONCLUSION

A new powerful yeast strain for bio-ethanol production was acquired. The yeast strain was identified as *Hanseniaspora guilliermondii* based on the sequencing of 26S rDNA region and the phylogenetic analysis. *H. guilliermondii* produced 87.74 g/L of ethanol after 3 days of incubation in 7-L fermenetor. GC-MS analysis for the intermediate compounds revealed that, the pregnane and nonacosane compounds that could be used in the medical and pharmaceutical industries were present.

ACKNOWLEDGMENT

This study was supported by King Khalid University through the grant No. KKU-SCI -11 /005

REFERENCES

Ames, J. M. and Elmore, J. S. (1992). Aroma components of yeast extracts. Flav. Fragr. J., 7: 89-103.

Ames, J. M. and McLeod, G. M. (1985). Volatile components of a yeast extract composition. J Food Sci., 50: 125–135.

Balasubramanian, K, Ambikapathy, V. and Panneerselvam, A. (2011). Studies on ethanol production from spoiled fruits by batch fermentations. J. Microbiol. Biotech. Res., 1 (4): 158-163.

Baleiras Couto, M. M., Reizinho, R.G. and Duarte, F.L. (2005). Partial 26S rDNA restriction analysis as a tool to characterize non-Saccharomyces yeasts present during red wine fermentations. Intern. J. Food Microbiol., 102: 49-56.

Brei, B., Edman, J.D., Gerade, B. and Clark, J. M. (2004). "Relative abundance of two cuticular hydrocarbons indicates whether a mosquito is old enough to transmit malaria parasites". J. Med. Entomol., 41 (4): 807–809

Cakir, A., Kordali, S., Zengin. H., Izumi, S. and Hirata, T. (2004). 'Composition and antifungal activity of essential oils isolated from Hypericum hyssopifolium and Hypericum heterophyllum'. Flav. Fragr. J., 19: 62-68.

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(8): 3998-4010.

Emam, O.A., Farag, S.E.A. and Hammad, A.I. (1994). Comparative studies between fumigation and irradiation of semi-dry date fruits. Nahrung, 38:612-20.

Esteve-Zarzoso, B., Belloch, C., Uruburu, F. and Querol, A. (1999). Identification of yeasts by RFLP analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Intern. J. System. Bacteriol., 49: 329-337.

Frutos, R.L., Fernandez–Espinar, M.T. and Querol, A. (2004). Identification of species of the genus Candida by analysis of the 5.8S rRNA gene and the two ribosomal internal transcribed spacers. Ant. van. Leeuwenh., 85: 175-185.

Fuganti, C. and Grasselli, P. (1985). Stereochemistry and synthetic application of products of fermentation of a, b-unsaturated aromatic aldehydes by baker's yeast. In: Enzymes in organic synthesis, vol. 111. Ciba Found. Sympos., 112–125.

Hashem, M. and Darwish, S.M.I. (2010). Production of bioethanol and associated by-products from potato starch residue stream by Saccharomyces cerevisiae. Biom. Bioen., 34: 953 – 959.

Hesham, A., Wang, Z., Zhang, Y, Zhang, J., Lv, W. and Yang, M. (2006). Isolation and identification of a yeast strain capable of degrading four and five ring aromatic hydrocarbons. Ann. Microbiol., 56: 109-112.

Hesham, A. and Mohamed, H. (2011). Molecular genetic identification of yeast strains isolated from Egyptian soils for solubilization of inorganic phosphates and growth promotion of corn plants. J. Microbiol. Biotechnol., 21: 55-61

Kurtzman, C. P. and Fell, J. W. (1998). The yeasts-a taxonomic study. 4th ed. Elsevier Science Publ. B.V. Amsterdam, the Netherlands.

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

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

Münch, P. and Schieberle, P. (1998). Quantitative studies on the formation of key odorants in thermally treated yeast extracts using stable isotope dilution assays. J. Agricult. Food Chem., 46: 4695-4701.

Raspor, P., Mikliå M., D., Polanc, J., Smole M. S. and Åadeÿ, N. (2006). Yeasts isolated from three varieties of grapes cultivated in different locations of the Dolenjska vine-growing region, Slovenia. Int. J. food microbiol., 109(1/2): 97-102.

Scorzetti, G., Fell, J.W., Fonseca, A., and Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. FEMS Yeast Res., 1497: 1-23.

Shenasi, M., Aidoo, K.E. and Candlish, A.A.G. (2002). Microflora of date fruits and production of aflatoxins at various stages of maturation. Int. J. Food Microbiol., 79:113-119.

Wang, L., Yang, Z., Wang, S., Wang, S. and Liu, J. (2011). Antioxidant and antibacterial activities of Camptotheca acuminate D. seed oil. Afr. J. Microbiol. Res., 5(32): 5854-5862.

ARABIC SUMMARY

المركبات الكيمائية و الصيدلانية الوسطية أثناء عملية تخمر عصير التمور التالفة بواسطة خميرة هاسينوسبورا جيولرمندای

محمد هاشم [1،2] ـ عبد اللطيف هشام [1،3] ـ سعد العمري [1] ـ سليمان آل رمان [1]

1- قسم الاحياء ، كلية العلوم ، جامعة الملك خالد ، المملكة العربية السعودية

2- قسم النبات و الميكروبيولوجيا ، كلية العلوم ، جامعة اسيوط ، جمهورية مصر العربية

3- قسم الوراثة ، كلية الزراعة ، جامعة اسيوط ، جمهورية مصر العربية

كان الهدف من هذه الدراسة هو تعريف المركبات الكيميائية الوسيطة المتكونة أثناء عملية تخمير عصير التمور التالفة بواسطة عزلة الخميرة هاسينوسبورا جيولرمندای KKUY-0045. وقد تم تعريف هذه السلالة على أساس تحليل تتابع الحمض النووى الريبوزى (26S) D1/D2 ومقارنتها مع مثيلاتها في بنك الجينات. ومن خلال شجرة القرابة الوراثية لهذه التتابعات تبين أن هذه السلالة تتشابه و تشغل نفس الموقع مع نوع هاسينوسبورا جيولرمندای وعليه فانها تعتبر سلالة من هذا النوع. و من خلال عملية التخمير لعصير التمور التالفة فى مخمر سعة سبعة لترات تبين أن هذه السلالة قادرة على انتاج كمية كبيرة من الايثانول تقدر بـ 87.74 جرام / لتر بعد 3 أيام من عملية التحضين. وكشف التحليل الكيميائي لرشيح المزرعة النامية على عصير التمور التالفة باستخدام GC-MS عن وجود عدد كبير من المواد الكيمائية المتكونة اثناء عملية التخمير والتى تختلف باختلاف عدد ايام التحضين و لوحظ بعض المواد الهام مثل مركب البريجنان ومركب نوناكوزان التي تتميز باستخداماتها الطبية والصيدلانية. وتوصي هذه الدراسة باجراء دراسات موسعة لاكتشاف و تحديد نوعية المواد الكيمائية المتشابهة اثناء عملية التخمرات الكحولية ودور هذه المركبات فى الاستخدامات الصناعية و الطبية المختلفة.