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Indigenous Yeasts of the Rotten Date Fruits and their Potentiality in Bioethanol and Single-cell Protein Production

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Abstract

Isolation and identification of the indigenous yeasts of the rotten date fruits for possible production of bioethanol and single-cell protein was the aim of this study. Results showed that a considerable amount of date fruits is subjected to unfavorable conditions of storage that induce their rot and spoilage. From the rotten date fruits, ten yeast isolates were obtained and genetically identified by the sequence of D1/D2 domain of the 26S rRNA gene and phylogenetic analysis. The identity of these yeasts was: *Hanseniaspora guilliermondii, H. uvarum* (2 strains), *H. opuntiae, Pichia kudriavzevii* (2 strains), *Issatchenkia orientalis, Wickerhamomyces anomalus, Yarrowia lipolytica and Zygosaccharomyces rouxii.* The ability of these strains to ferment 20% of the spoilage date fruit juice evaluated on laboratory scale. Results proved that *P. kudriavzevii* KKUY-0151 and *H. uvarum* KKUY-0153 yielded 67.48 and 67.37 g/L respectively, of bioethanol as the highest producers. However, *H. uvarum* KKUY-0078, *H. guilliermondii* KKUY-0009 and *Z. Rouxii* KKUY-0157 produced the highest fresh biomass weight 31.76, 30.96 and 30.69 g/L, respectively as a single cell protein production. The study is a pioneer to investigate the endemic yeasts of the rotten date fruits. It concludes that some of the indigenous yeasts of the rotten date fruits are promising organisms in recycling the substrate into valuable products such as bioethanol and single-cell protein.

Keywords: Date fruit; Yeasts; Bioethanol; Single-cell protein; 26S rRNA gene sequencing; Phylogenetic analysis

Introduction

Arab world is famous for date palm (*Phoenix dactylifera* L.), which is an important crop cultivated on its lands. Among the Arab countries, Saudi Arabia is the main producer of the dates. It produces about 13% of the world production (El-Juhany, 2010). There are more than six million of date palm trees distributed all over the Saudi land and the date manufacturing is one of the most popular industries in it. Date tree yields 400-600 kg/year of fruits and its production extends for to 60 years (Shinwari, 1993; Zohary and Hopf, 2000). However, date palm fruits and trees are subjected to many insects attacking (El-Juhany, 2010), that act as vectors for many toxigenic fungi, such Aspergilli, which contaminate the dates and date products with harmful aflatoxins (Shenasi *et al.,* 2002). We noticed that there is a considerable amount of the date fruits showed many rotten and spoilage signs in the store-houses of the farmers and merchants. The rot of dates could be due to the pests and microorganisms infection, inappropriate transporting, lack of god stores, and longtime of marketing.

Spoilage and rotten dates are abundant renewable agro-industrial waste material produced in Saudi Arabia and its low cost is an important factor for the economic viability of many valuable products such as bioethanol and single-cell protein. Yeasts are important microorganisms in food manufacturing and fermentation. Also, many of them are infectious agents (Lachance and Starmer, 1998). Different yeasts have been isolated from both natural and artificial environments (Lee *et al.*, 2001). However yeasts are considered as an important group of microorganisms in the biosphere, their biodiversity in the natural environment have not been paid a considerable attention (Lachance and Starmer, 1998). The identification of yeasts depending on the sequence of 26S rDNA became a consistent, accurate and rapid technique compared with the conventional and physiological method (Kurtzman and Robnett, 1998; Fell *et al.,* 2000). We assume that the indigenous yeasts of the rotten date fruits that could adapt themselves in high concentration of sugars could play an important role in either fermentation of the rotten dates into bioethanol or production of great quantities of biomass as single-cell protein. So the identification of this group of yeasts is a

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good deal for the bioethanol and single-cell protein biotechnology.

Therefore, the aim of this study was to isolate the indigenous yeasts of the rotten date fruits. Molecular tools including PCR amplification and sequencing of the D1/D2 region of the 26S rRNA gene as well as the phylogenetic analysis were applied for the identification of the yeast isolates at species levels. The recovered yeasts were tested for their potentiality to ferment the rotten date fruit to bioethanol and production of single-cell protein to fulfill the gap in renewable biofuel as well as protein sources in fishery and poultry sectors.

Materials and Methods

Collection and Examination of Spoilage Dates

Different dates' markets in Abha were visited to collect the rotten dates. Two date cultivars (Berhi and Nabt Ali) were selected, because we noticed that these two cultivars are subjected to the rot and spoilage more than the others. From each cultivar, 25 samples (500 g/sample) were collected and stored in the laboratory at 4°C for further uses. Different date samples were examined by the ordinary light microscope to detect to what extent they degraded and infected with microorganisms.

Isolation of Yeast from Different Sources

Yeasts were isolated from the collected rotten date samples using dilution plate and direct touch method as described by Kurtzman and Fell (1998) on Yeast Extract-Malt Extract Agar (YMA) medium (Wickerham, 1951). The medium was sterilized by autoclaving at 121°C for 15 min. The sterilized agar medium was cooled to approximately 45°C and adjusted its pH to 3.7 and poured into petri dishes. For each sample, five plates were inoculated 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 hours and then transferred into 4°C to be stored.

Identification of the Isolated Yeasts

The isolated yeasts were subjected for identification using the sequence analysis of D1/D2 domain of 26S ribosomal DNA. The detailed procedures are explained in the following section.

DNA Extraction

DNA of yeasts was extracted based on the protocol of Hesham *et al.* (2006a). Briefly, 50 mL of 48 h cultures was collected via centrifugation. The pellets were dissolved in 400 µL of breaking buffer (1% SDS, 100 mM NaCl, 10 mM Tris with pH at 8.0, 1 mM EDTA with pH at 8.0) and 600 µL of phenol–chloroform (v/v = 25:24). The biomass was homogenized and incubated at 60°C for 10 min. The supernatant was extracted twice, firstly in phenol-chloroform-isoamyl alcohol (v/v/v = 25:24:1, pH = 8.0) and secondly in chloroform-isoamyl alcohol (v/v = 24:1, pH = 8.0). The extracts were mixed and the DNA was precipitated in 0.1 volumes of sodium acetate (pH = 5.2) and 0.6 volumes of isopropanol. This mixture was kept on ice for 30 min and centrifuged at 15,000 g for 20 min to recover the nucleic acids. The obtained pellets were washed with 70% ice-cold ethanol and centrifuged at 15,000 g for 20 min under cooling (4°C). Finally, the pellets were dissolved in 60 µL of TE buffer.

Table 1: Identity of the isolated yeasts from the rotten date fruits, number of isolates, accession number and similarity percentage with the references strains in the GenBank

Code of isolates	Identification	GenBank Accession number	Similarity with the reference of GenBank
KKUY-0009	*Hanseniaspora guilliermondii*	JQ690243	100%
KKUY-0034	*Pichia kudriavzevii*	JQ690250	97%
KKUY-0051	*Wickerhamomyces anomalus*	JQ690261	98%
KKUY-0054	*Yarrowia lipolytica*	JQ690248	100%
KKUY-0078	*Hanseniaspora uvarum*	JQ690236	99%
KKUY-0111	*Issatchenkia orientalis*	JQ690265	98%
KKUY-0151	*Pichia kudriavzevii*	KC110833	99%
KKUY-0153	*Hanseniaspora uvarum*	KC110835	100%
KKUY-0152	*Hanseniaspora opuntiae*	KC110834	100%
KKUY-0157	*Zygosaccharomyces rouxii*	KC110836	100%

PCR Amplification of the D1/D2 Region of the 26S rRNA Gene

The D1/D2 domain of the 26S rRNA gene was amplified using the primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') according to the method described by Kurtzman and Robnett (1998). 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 condition and agarose gel electrophoresis analysis were as described by Hesham *et al.* (2006b).

Sequencing of the D1/D2 Region of the 26S rRNA Gene and Phylogenetic Analysis

After the purification of PCR products, the amplified D1/D2 fragments were sequenced by ABI 3730 automated sequencer (Macrogen Company, Korea). The obtained sequences of 26S rRNA gene of our yeast strains were aligned with known 26S rRNA gene sequences in the GenBank database using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/ BLAST). The percent homology scores were generated to identify yeast strains. The isolates identification was confirmed by the

construction of phylogenetic trees using MEGA version 4.0 program.

GenBank Accession Numbers

The 26S rRNA gene sequences of the isolated yeasts reported in this study have been deposited in the DDBJ, EMBL, and GenBank nucleotide sequence database under special accession numbers (Table 1).

Potential production of bioethanol and single-cell protein by the yeast strains

Preparation of the spoilage dates juice: Two date cultivars (Arihy and Nabt Ali) were mixed in ration 1:1 (W:W) without stones and stored at 4°C. Samples were ground using high speed blender, then was diluted with distilled water to 2:8 (W:V). The mixture was sterilized at 121°C for 30 min. The produced date fruit 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.

Screening of Ethanol Production by the Isolated Yeast Strains

The ten isolated and identified yeasts were tested for their potentiality to ferment the spoilage date juice (SDJ) on Lab scale. 200-mL bottles containing 100 mL of SDJ were inoculated with 5 mL of desired yeast strain (10^8 mL). The inoculated bottles were plugged with sterilized rubber plugs and incubated at 25ºC and 150 rpm for 72 h in a rotatory incubator. All treatments were in triplicate and arranged in a complete randomized design and the mean and standard error were calculated. At the end of the incubation time, 5 mL of the fermented SDJ was withdrawn into a glass tube and centrifuged at 10000 rpm for 15 min and 1 mL of the supernatant was used to determine the ethanol concentration. Ethanol was estimated enzymatically using the ethanol estimation kit (K620-100) provided by BioVision company. The procedures provided with the product were employed. Briefly, the method depends on the presence of alcohol oxidase, which oxidizes ethanol to produce H_2O_2 and then reacts with the probe to generate color (λ max= 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. The concentration of ethanol in the samples was expressed as g/L.

Screening the Single-cell Protein (SCP) Production by the Isolated Yeasts

The same ten isolates were screened for SCP production from spoilage date juice (SDJ). Five mL of desired yeast strain (10^8 mL) was inoculated inton250-mL conical flasks containing 100 mL of sterilized SDJ. The inoculated flasks were plugged with sterilized cotton plugs for aerobic cultivation and were incubated at 25°C and 150 rpm for 72 h in rotatory incubator. All treatments were in triplicate and arranged in a complete randomized design. The mean and the standard errors were calculated. At the end of the incubation time, 10 mL of the culture medium of SDJ containing the residue biomass of the yeast was transferred into a clean, weighed (W) and dry class tube to calculate the fresh and dry biomass of the yeast to indicate the productivity of the single cell protein. The tubes containing the culture medium were centrifuged at 10000 rpm for 15 min and supernatant was decanted. The pellets were air dried and weighted for calculation the fresh weight and re-dried in an oven at 80°C for 24 h to calculate the dry weight of the biomass.

Statistical analysis

All experiments were repeated twice with three replicates for each treatment. One-way analysis (ANOVA) was applied and the least significant difference (LSD) ($P \leq 0.5$) was detected (Gomez and Gomez, 1984).

Results

Examination of the rotten dates: Visiting many storehouses, in which the stock of date fruits is stored showed that large quantities of dates fruits having many rotten signs like fermentation odor, degradation of tissues and growth of insect larvae (Fig. 1 A and B). There were different appearances of spoilage of dates such as maceration, blacking, growing of microorganisms and degradation (Fig. 1 C-E). Examination of the spoilage date with the light microscope revealed that most of the internal tissues were collapsed by action of the microorganisms. This leads to release of the lignified structures such as sclerotia (Fig. 2A). Also, examination of the collapsed tissues confirmed the presence of degrading microorganisms including different types of yeasts and bacteria (Fig. 2B).

Molecular Identification of the Date-associated Yeasts

The common ten yeast strains, which were isolated predominantly from the rotten date fruits were subjected for identification using PCR amplification and sequencing of the D1/D2 region of the26S rRNA gene as well as the phylogenetic analysis. As shown in Fig. 3, the size of the amplified 26S rRNA gene was 600 bp for all the yeast isolates, which is the expected size of 26S rRNA regions. The result of the sequence analysis confirmed the identity of these isolates with a homogeneity score of 97 to 100% with the reference strains located in the GenBank database. The isolates were given accession numbers from the

Fig. 1: Appearance of spoilage and rotten signs on dates including growth of microorganisms (A), growth of insect larvae (B), disintegration of tissues (C) and blacking (D and E)

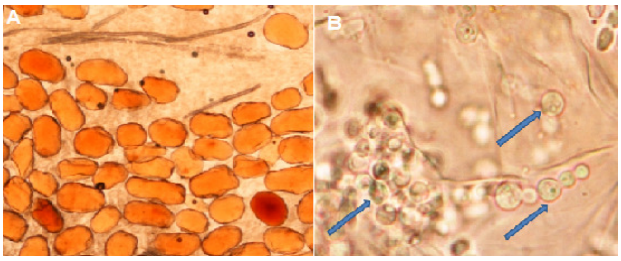

Fig. 2: Disintegration of the date tissues as a result of infection with microorganisms (A) and growth of yeasts and bacteria inside date tissues (B)

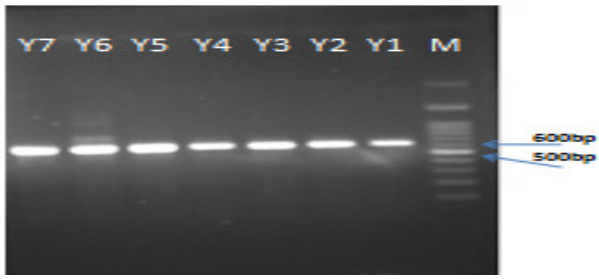

Fig. 3: Amplified DNA of the D1/D2 domain with primer pair NL1 and NL4. Lane M represents 100 bp marker; Lanes Y1-Y7 are PCR products amplified from some of isolated yeasts

GenBank (Table 1). Phylogenetic analysis of the isolates with the related species confirmed that the isolates were belonged to six genera: *Hanseniaspora*, *Pichia*, *Yarrowia*, *Issatchenkia*, *Wickerhamomyces* and *Zygosaccharomyces* (Fig. 4). The first genus was represented by four strains including *H. guilliermondii* KKUY-0009, *H. uvarum* KKUY-0078 *H. uvarum* KKUY-0153 and *H. opuntiae* KKUY-0152. The genus *Pichia* was represented by *P. kudriavzevii* KKUY-0034 and *P. kudriavzevii* KKUY-0151. The other four genera were represented by one strain per each and they were *Yarrowia lipolytica* KKUY-0054, *Issatchenkia orientalis* KKUY-0111, *Wickerhamomyces anomalus* KKUY-0051 and *Zygosaccharomyces rouxii* KKUY-0157.

Screening of Ethanol Production by the Isolated Yeast Strains

Screening of ethanol production from the spoilage date juice (20%, w:v) revealed that the yeast strains, isolated from the rotten date fruits vary in their ability to ferment the date juice, were able to produce ethanol in different concentrations. Fig. 5 shows that *P. kudriavzevii* KKUY-0151 and *H. uvarum* KKUY-0153 produced the highest quantities of ethanol (67.48 and 67.37 g/L, respectively). However, both *Y. lipolytica* KKUY-0054 and *Z. rouxii* KKUY-0157 produced the lowest ethanol concentrations. The other six isolates produced moderate quantity of ethanol ranged from 66 to 48.44 g/L.

Screening the Single-cell Protein (SCP) Production by the Isolated Yeasts

The biomass (single cell protein, SCP) yield of the 10 yeast isolates grown aerobically for 72 h was detected as fresh and dry weight (g/L) after centrifugation of the yeast culture at 10000 rpm for 15 min. Results showed that *H. uvarum* KKUY-0078, *H. guilliermondii* KKUY-0009 and *Z. rouxii*KKUY-0157 produced the highest fresh biomass among the tested isolates. They produced 31.76, 30.96 and 30.69 g/L of the fresh weight (Fig. 6). The productivity of *W. anomalus* KKUY-0051, *I. orientalis* KKUY-0111 and *H. opuntiae* KKUY-0152 was lower than the previous group, where their fresh weight ranged from 29.04 to 27.22 g/L. The other four strains produced a relatively low biomass yield (24.32 -22.16 g/L). Regarding the dry biomass, its production showed a similar trend of the fresh weight, where the first group produced the highest yield (11.96-9.97 g/L).

Discussion

Association between yeasts and other microorganisms in the degraded tissue of the rotten date fruits supports our hypothesis that the spoilage of date fruits is a complicated and successive process. It starts with the growth of some microorganisms that having the capacity to degrade date fruits to produce the fermentable sugars, followed by invading with yeasts, which ferment the sugars into different compounds like ethanol. We built up this hypothesis, because we noticed a permanent association between yeast and bacteria present in the collapsed date fruits and fermentation odor. Papalexandratou *et al.* (2011) studied the spontaneous fermentation of coca and mentioned that many yeast species were found during the early stage of fermentation. They reported that *Pichia kudriavzevii* and *Pichia manshurica* were the dominant ethanol-producing

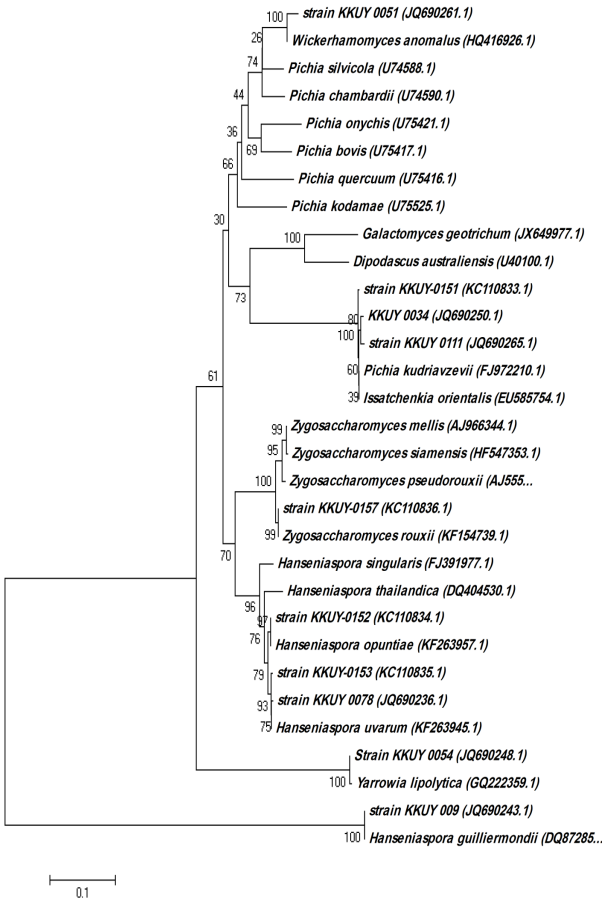

Fig. 4: Phylogenetic relationship between yeast strains and other 26S rRNA sequences of published strains. Accession numbers for sequences are as shown in the phylogenetic tree

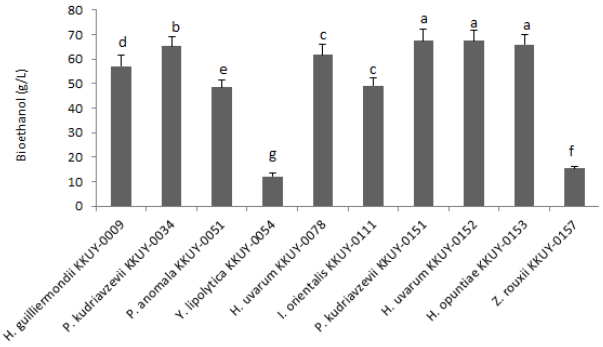

Fig. 5: Production of ethanol by the rotten date-inhabiting yeasts. Columns followed by the same letter are not significantly different ($P \leq 0.5$) and the bars represent the standard errors.

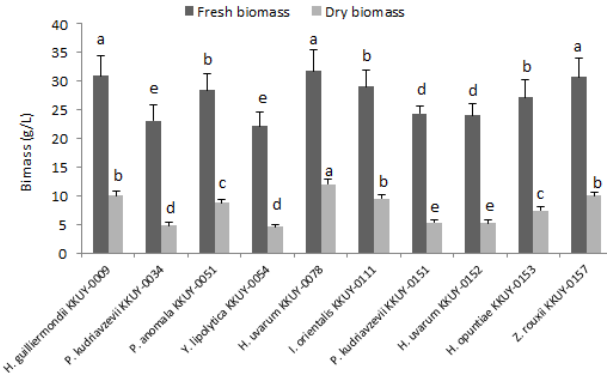

Fig. 6: Production of single-cell protein (fresh and dry biomass) by the rotten date-inhabiting yeasts. Columns followed by the same letter are not significantly different ($P \leq 0.5$) and the bars represent the standard errors

yeast species followed by *Saccharomyces cerevisiae*. El-Juhany (2010) claimed that date palm fruits and trees are suffering from attacking by many insects that could act as vectors of many microorganisms. Many dates and date products were contaminated by Aspergilli (Emam *et al.*, 1994; Shenasi *et al.*, 2002).

From the rotten date fruits, we isolated ten different isolates of yeasts belonging to five genera: *Hanseniaspora, Pichia, Yarrowia, Issatchenkia* and *Zygosaccharomyces.* Our results are greatly supported by those of other investigators, who isolated many of yeast species from vegetables, fruits, soil and other natural sources (Hashem, 2005; Amoa-Awua *et al.,* 2006; Stringini *et al.,* 2008). *Zygosaccharomyces rouxii* was isolated previously from food products such as sugar syrups, honey and fruit juices (Leandro *et al.,* 2011). *Hanseniaspora uvarum* was frequently isolated from many natural sources (Romano, 2002). It was recorded among frutophilic species of *Hanseniaspora* yeasts (Ciani *et al.,* 2006). The association of wide spectrum of yeast species with fruits is because fruits contain adequate sugars and nutrients that are important several yeasts (Starmer and Lachance, 2011). Also, the fruits act as transitional substrates for of insects and their premature stages, which carry and distribute the yeasts to new other substrates (Morais *et al.,* 2006). Recently, an inclusive researches have been made to identify the yeast flora in a great number of foods using molecular techniques especially PCR amplification of ribosomal RNA genes (BaleirasCouto *et al.,* 2005). These researches revealed that the sequence of D1/D2 region is a prevailing tool in yeast identification (Kurtzman and Robnett, 1998).

Results showed that *P. kudriavzevii* KKUY-0151 and *H. uvarum* KKUY-0153 produced the highest quantities of ethanol. However, both *Y. lipolytica* KKUY-0054 and *Z. rouxii* KKUY-0157 produced the lowest ethanol concentrations. Our results were supported by those of Al-Talibi *et al.* (1975), who found that the ethanol yield by *Saccharamyces cerevisiae* fermented 20% pure sugar solution reached 9.96%. Mehaia and Cheryan (1991)

reported that the ethanol production by *S. cerevisiae* was 48.27% and 47.0% of the total sugar concentration when 9.8 and 13.83% sugars date juice was used in batch fermentation, respectively. Zohri (1999) studied the utilization of the date juice at 18% sugar for ethanol production by *S. bayamus* at 30°C in submerged ferment or and found that the yield of ethanol reached 9.161%. Most of yeast strains, isolated in this study, were mentioned as fermenting organisms of many substrates. The more frequently encountered yeasts in the early formation phase are non-*Saccharomyces* species such as *Hanseniaspora*, *Candida* and *Metschnikowia*, which initiate the fermentation during the first 3-4 days (Paraggio, 2004; Romano, 2002). Lachance (1995) identified the yeasts *Hanseniaspora, Kluyveromyces,* and *Torulaspora* in the naturally fermented juice from *Agavtequilana* that gradually gave the fermentation to *Saccharomyces, Zygosaccharomyces, Candida* and *Brettanomyces* species.

Regarding the single-cell protein production, *H. uvarum* KKUY-0078, *H. guilliermondii* KKUY-0009 and *Z. rouxii* KKUY-0157 produced the highest fresh and dry biomass among the tested isolates. The other strains produced a relatively low biomass yield. Yeasts are the pioneer microorganisms in single-cell protein production and they were accepted by consumers. Yeasts are seldom toxic or infectious and can be used in human foods. They contain essential amino acids such as lysine (6 to 9%), tryptophan and threonine. They contain sulfur-containing amino acids such as methionine and cysteine and vitamins (B group) (Boze *et al.,* 2008). Many types of yeast were previously used in production of single-cell protein such as *Saccharomyces cerevisiae, Kluyveromyces marxianus, Candida utilis*, *Saccharomycopsis lipolytica* and *Cryptococcus laurentii* (Garcia-Garibay and Gomez-Rulz, 1999; Ceccato-Antonin and Tauk, 1994). Our finding represents a new addition of yeasts that could be used in the single-cell production.

Our results concluded that considerable amounts of the date fruits in Saudi Arabia are subjected to bad storage conditions that make the fruits are appropriate for the growth of many microorganisms and spoilage. The spoilage and rotten fruits become useless and should be thrown away. However, this substrate could be transformed into very important products such as bioethanol "biofuel" and single-cell protein for poultry and fishery feeding. This could be done by using fermenting yeasts. We assume that the indigenous yeasts are more appropriate to carry out the fermentation of the rotten date fruit. Our results approved this assumption, where we isolated ten yeast strains, some of them produced high quantities of ethanol and biomass yields. We recommend the application of these yeasts in fermentation of the very economic substance of the rotten date fruits into bioethanol and single-cell protein on a large scale to minimize the capital cost of the production of such important products.

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