# Isolation and Identification of the *Hanseniaspora opuntiae* MK 460485 as an efficient strain for ethanol production

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**In the last few years, ethanol is being increasingly one of most necessary and popular high-demand materials in the food industry, agriculture, medicine. It is most commonly produced from biomass such as sugarcane, corn, switchgrass, etc. The aim of this study was the isolation and identification of indigenous yeasts of the grapefruit for possible bioethanol production. 200 gr of each sample (Flame Seedless, Sultanina, Fakhri, Muscat Ottonel, Pinot Noir from Vitis vinifera species) was soaked in 500 ml water at 25°C for 14 days. After the fermentation of grape samles, 42 yeasts isolates were observed by culture in the culture medium of YPD. Among these isolated yeasts six of them were selected through their responses to high osmotic conditions and high ethanol concentration. One of the isolates was more capable of produce higher amounts of ethanol and to resistant against high osmotic conditions and high ethanol concentration, compared with other studied isolates. In continue, this strain of yeast been studied based on biochemical and morphological properties and genetically identified by the sequence of D1/D2 domain of the 26S rRNA gene and phylogenetic analysis, that was a new strain of the family of Hanseniaspora we named it as *Hanseniaspora opuntiae* MK 460485. This strain showed significant growth potential in high concentration of ethanol, glucose and in wide range of temperatures and pH.**

**Keywords:** microbial biotechnology; ethanol producers; yeast identification; Hanseniaspora opuntiae strain; resistance to osmosis; fermentation; ethanol.
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# Выделение и идентификация *Hanseniaspora opuntiae* MK 460485 как эффективного штамма для производства этанола

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**Исследовали выделение и идентификацию местных дрожжей из 6 различных ферментированных сортов винограда Ирана и изучали наиболее эффективный штамм для возможного производства биоэтанола. В экспериментах использовали по 200 г винограда из каждого сорта (Flame Seedless, Sultanina, Fakhri, Muscat Ottonel, Pinot Noir из вида Vitis vinifera), в каждый образец добавляли по 500 мл воды и выдерживали при температуре 25°С в течение 14 суток для обеспечения процесса ферментации в культуральной среде YPD. После ферментации путем культивирования выделяли 42 дрожжевых**

**изолята, из которых 6 были отобраны по их толерантности к высокому осмотическому давлению и высокой концентрации этанола. Среди изученных изолятов, изолят Af оказался наиболее устойчив к высоким осмотическим условиям глюкозы (больше 60 г/л) и высокой концентрации этанола (больше %16 о/о) по сравнению с другими изученными изолятами. Этот штамм дрожжей изучали на биохимические и морфологические свойства, он был генетически идентифицирован по последовательности домена D1/D2 гена 26S рРНК. Филогенетические анализы показали, что данный штамм является новым штаммом семейства *Hanseniaspora*, который был назван *Hanseniaspora opuntiae* MK 460485. Этот штамм дрожжей продемонстрировал значительный потенциал роста в высоких концентрациях этанола (больше %16 о/о), глюкозы (больше 60 г/л), при широком диапазоне значений температуры (25–42°C) и pH (3–7).**
**Ключевые слова:** микробная биотехнология; продуценты этанола; идентификация дрожжей; штамм *Hanseniaspora opuntiae*; осмоустойчивость; ферментация; этанол.

## Introduction

In the last century, one of the most important issues facing all the countries of the world, especially the developing and developed ones due to the high population growth and industrialization, is the energy crisis which shows the importance of studying and discovering new processes involved in the production of renewable and clean compounds as alternative energy sources to the reduction of environmental pollution [1]. Among the renewable resources that are nowadays considered by many European and American countries are Biofuels [2]. Commercialization of bioethanol as an eco-friendly fuel has been recently intensified because of its market stability, low cost, sustainability, alternative fuel energy composition, greener output and colossal fossil fuel depletion [3].

The major and significant limitations causing reduced alcohol yields and quality are generally fermentation process design, co-contamination, limited availability of raw materials and other things [4]. In addition to the sugar source price which is an important parameter for optimizing alcohol yields and economy of production [5], selecting the potent microorganisms is another crucial factor in fermentation. Yeasts are the most commonly used microorganisms which can produce ethanol concentrations as high as 18% of the fermentation broth [6]. Various types of yeast strains are available in the market worldwide and are usually used in traditional fermentation processes to produce different types of alcohol [7, 8]. The well-known selected strains for this purpose are *S. cerevisiea*, *S. eliypsoideus*, *S. carlbergensis*, *S. fragilis*, and *Chisovacaromycesom pombe*. Approximately 80% of ethanol is produced by anaerobic fermentation of various sugar sources by *S. cerevisiae* [5, 9]. In order to achieve efficient ethanol fermentation, it is necessary to use an efficient yeast strains that can tolerate high ethanol concentrations.

Considering the importance of using ethanol in recent years and the important role of yeasts in its production, there is a need for research in various fields to increase its production, such as isolation or generation of strains by genetic engineering, with high and varied physiological abilities, including resistance to high sugar concentration as substrata and ethanol as the final product in fermentation medium, the ability to ferment a wide range of sugar sources and growth at high temperatures [10, 11].

The purpose of this study was yeast strains isolated from different fermented grape species, investigation of their physiological characteristics and, finally, select the best strains for their use in the ethanol industry.

## Materials and Methods

### *Isolation and purification and maintenance*

Grape samples (Flame Seedless, Sultanina, Fakhri, Muscat Ottonel, Pinot Noir from Vitis vinifera species) were collected from the Tehran Central Fruit & Vegetable Market (Azadegan Expy., Behesht Zahra Rd., Tehran, Iran ). Amount of 200 gm of samples were soaked in 500 ml water at 25°C for 14 days. After 14 days incubation, in order to isolate microorganisms from pieces of fermented fruit, every 10 ml of each fermented suspension was transferred to 90 ml normal saline containing 0.1% Tween 80 and shacked in 150 rpm. After 24 h, 50 ml of each sample were centrifuged for 5 minutes at 2000 rpm and the supernatants were diluted from 10-1 to 10-10. The amount of 200 µl of each dilution was spread into a plate containing YPD media supplemented with chloramphenicol and was incubated aerobically at 30°C for 24–48 h. Growth yeasts were

isolated on the basis of colonies morphological characteristics and purification was performed by colonies subculturing in YPD medium. The obtained isolates were maintained by subculturing on slants using SDA medium in a refrigerator at 4°C for future use. To longtime maintenance of isolates, a dense suspension of each isolate was prepared in SDB containing 10% glycerol and stored at –20 and –80°C.

***Screening of isolates for ethanol producer***

In this study, the colorimetric method which was obtained by reaction of ethanol with potassium dichromate was used to determine of ethanol production rate in fermentation media by selected isolates. This procedure was performed based on the mechanism of formation of green chromate ions from alcohol and potassium dichromate treatment as a limiting reaction in the presence of $H_2SO_4$ at pH 4.3. Then the absorbance of treated samples was determined using UV Spectrophotometer at 578 nm. In order to screen we select a more effective isolate from the ethanol producers to resist high concentrations of ethanol and high osmotic pressure in the presence of 60% glucose that is directly linked to the ethanol production ability.

To evaluate the tolerance of isolates in presence of different ethanol and glucose concentrations, the effects of concentrations 12%, 14%, 15%, 16%, and 17% of ethanol and also 50% and 60% of glucose were investigated on the isolates. Tubes of culture media containing different concentrations of ethanol and glucose were inoculated with 2% of 0.5 MacFarland suspensions of 24-hour cultures. After 24 hours of incubation, the growth rate of each isolate was estimated by absorbance measuring in 600 nm.

***Identification of selected ethanol producer isolates***

Isolates were identified according to their morphological and physiological characteristics as described by Yarrow et al. [12] and Kurtzman et al. [13]. The macroscopic and microscopic morphological characteristics of the colony grown from fresh cultures were investigated in terms of color, form, shape and size on the SDA medium and also Gram, Lactophignole blue and spore stain.

To determine the Physicochemical characterization, the fermentation of different carbohydrates by yeast isolates from sugars glucose, sucrose, maltose, molluscum, lactose and galactose, detecting thermotolerance at 25; 30; 32; 37; 40, and 44°C, growth at different pH 3–7, osmotolerence observation at 6%, 9%, 12%, 15%, 18%, and 20% of NaCl and also filamentation and ascosporic formation were investigated for selected isolated.

***Molecular identification***

The yeast strains were identified by sequence analysis of the 28 S rDNA D1/D2 domain. The genomic DNA extraction and purification were carried out by using the method of Makimura et al. [14]. The sequences of the rDNA D1/D2 domain were amplified and sequenced as described by Lu et al. [15].

The phylogenetic tree was generated by the neighbor-joining method. Identification of the selected strain was carried out by sequencing the 26S rDNA. The LSU D1/D2 gene of 26S rDNA was amplified by PCR with the NL1 (5’-GCATATCAATAAGCGGAGGAAAAG-3’) and NL4 (5’-GGTCCGTGTTTCAAGACGG-3’) primers [16]. The BLASTN program was used to search for gene homology [17]. The phylogenetic analyses were based on an analysis of 540 base pairs of a combined alignment of D1/D2 sequences and performed using the neighbourjoining method [18] with the program MEGA3 [19].

## Results

***Isolation and screening for ethanol production***

From the grapes samples, approximately 42 yeast strains were isolated on agar plates. Therefore, 12%, 14%, 15%, 16%, and 17% (v/v) ethanol and 5% and 6% glucose were added to the YPD medium separately to obtain yeast strains that can tolerate high ethanol concentrations. Among these, 6 strains were screened for their ability to grow in YPD broth containing 15% ethanol and 6% of glucose (Table 1). Then six selected isolates were evaluated for the second stage of screening to measure the amount of ethanol produced by colorimetric method.

*Table 1. Initial screening of isolates based on tolerance to different concentrations of glucose and ethanol*

| Isolate | Ethanol concentrations (v/v) | | | | | Glucose concentrations (g/l) | |
|---|---|---|---|---|---|---|---|
| | 17 | 16 | 15 | 14 | 12 | 60 | 50 |
| Aa | – | – | – | – | – | – | – |
| Ab | – | – | – | – | + | – | ± |
| Ac | – | – | – | + | + | + | + |
| Ad | – | – | – | – | – | – | – |
| Ae | – | – | – | + | + | – | – |
| Af | – | + | + | + | + | + | + |
| Aj | – | – | – | – | + | – | ± |
| Ah | – | – | – | + | + | – | ± |
| Ba | – | – | – | + | + | – | – |
| Bb | – | – | – | – | – | – | – |
| Bc | – | – | – | – | ± | – | – |
| Bd | – | – | – | – | – | – | – |
| Be | – | – | – | – | – | – | – |
| Bf | – | – | – | – | – | – | – |
| Bj | – | – | – | + | + | ± | ± |
| Bh | – | ± | + | + | + | ± | + |
| Bk | – | – | – | + | + | – | ± |
| Bl | – | – | – | – | – | – | – |
| Bm | – | – | – | – | – | – | – |
| Bn | – | – | – | – | – | – | – |
| Bo | – | – | – | – | – | – | – |
| Ca | – | – | – | – | – | – | – |
| Cb | – | – | – | – | – | – | – |
| Cc | – | – | + | + | + | + | + |
| Ce | – | – | – | – | – | – | – |
| Cf | – | – | – | – | – | – | – |
| Cg | – | – | – | – | + | – | – |
| Ch | – | – | – | – | + | – | ± |
| Cj | – | – | – | – | – | – | – |
| Ea | – | – | – | – | + | – | – |
| Eb | – | – | – | – | + | – | – |
| Ec | – | – | + | + | + | + | + |
| Ee | – | – | – | – | – | – | – |
| Ef | – | – | – | – | – | – | – |
| Eg | – | – | + | + | + | + | + |
| Fa | – | – | – | – | – | – | – |
| Fb | – | – | – | – | – | – | – |
| Fc | – | – | – | – | – | – | – |
| Fe | – | – | – | – | + | – | ± |
| Ff | – | – | – | – | + | – | – |
| Fg | – | – | + | + | + | ± | + |
| Fh | – | – | – | – | + | – | – |

*Key: + Strong growth ± Weak growth – Lack of growth*

The standard curve of ethanol was drawn by colorimetric method for selected strains (Figure 1). Based on this curve, the ethanol production chart was obtained for these strains (Figure 2). Based on the results it was found that although ethanol production was observed by all the selected strains, strain Af, produced high ethanol

concentrations at 30°C after 68 h fermentation, as shown in Figure 2. Therefore, the Af strain was selected for further studies.

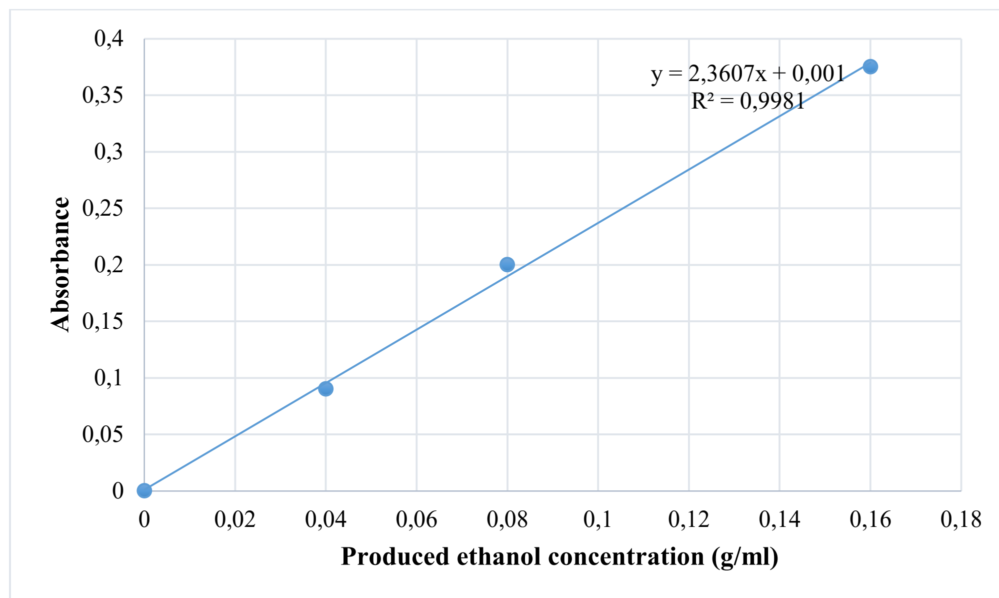



*Figure 1. Standard curve of ethanol concentration produced in fermentation mediums based on colorimetric method*

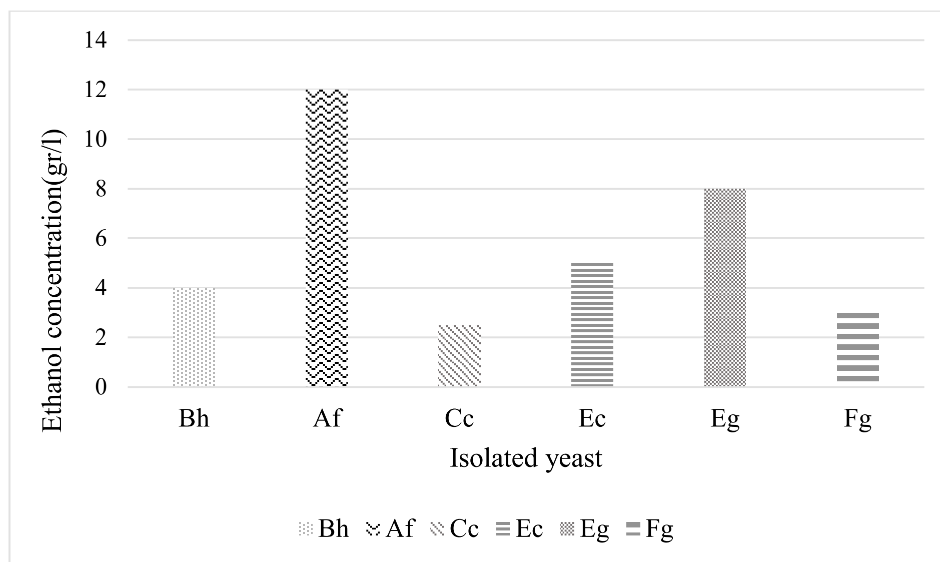
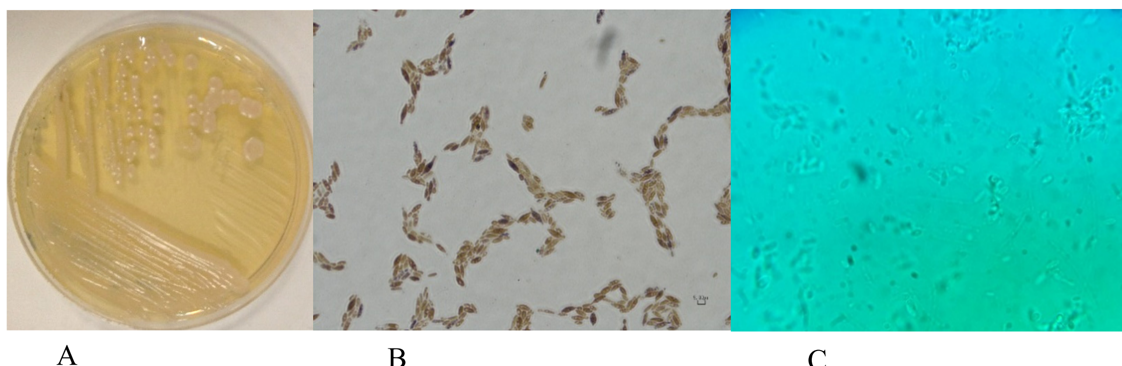



*Figure 2. The concentration of ethanol, produced by each selected isolate in fermentation mediums*

### *Morphological and physiological characteristics*

On YPD agar media, the streak culture of the colonies of the Af isolate had a cream-white and smooth surface after 24 h at 30°C. The colonies were observed as singles, pairs or in groups, cream colored, butyrous, smooth, glossy, and flat to slightly raised at the center and vegetative cells were gram positive spindle shape (Figure 3). Physiological characteristics of selected strain were studied. This strain could ferment glucose, sucrose and maltose. Maximum growth temperature of strain was 42°C, and pH 3–7 (Table 2).



A B C

*Figure 3. Macroscopic and microscopic morphological characteristics of selected isolates:* A *– medium-sized, cream colored, slim and bulgy colonies with spindle shape cells;* B *– gram staining;* C *– lactophenol staining*

*Table 2. Biochemical tests performed on selected isolate*

| Physical characteristics of selected strain | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| pH | | | | | Temperatures (°C) | | | |
| 3 | 4 | 5 | 6 | 7 | 25 | 30 | 37 | 42 |
| + | + | + | + | + | + | + | + | + |

| Biochemical characteristics of selected strain | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Ethanol concentrations (%) | | | | | Osmotolerance in high glucose concentration (%) | | Acid production | Carbohydrate fermentation | | | | | |
| 20 | 18 | 15 | 12 | 9 | 50 | 60 | | Galactose | Lactose | Melebiose | Maltose | Sucrose | Glucose |
| – | – | +- | + | + | + | + | + | + | – | – | – | + | + |

| Formation of differentiated structures | |
|---|---|
| Ascosporic | Hyphae |
| – | – |

### *rDNA gene sequence analysis*

Molecular taxonomic analysis was compared using the results of the D1/D2 domain sequencing analysis of Af isolate with the sequence of similar species in the Gene Bank database. Similarity searches on public nucleotide databases using that sequence revealed 100% identity with *H. opuntiae*. The sequence was deposited in Gene Bank under the accession number MK460485. The MEGA Ver 6 software was used to estimate the divergence between pairs. Molecular data showed that the strain had the least difference in divergence (d) with *H. opuntiae* (*d* = 0/00) and the highest difference was found among other species of *Hanseniaspora* with *H. thailandica* (*d* = 0.258) (Figure 4).

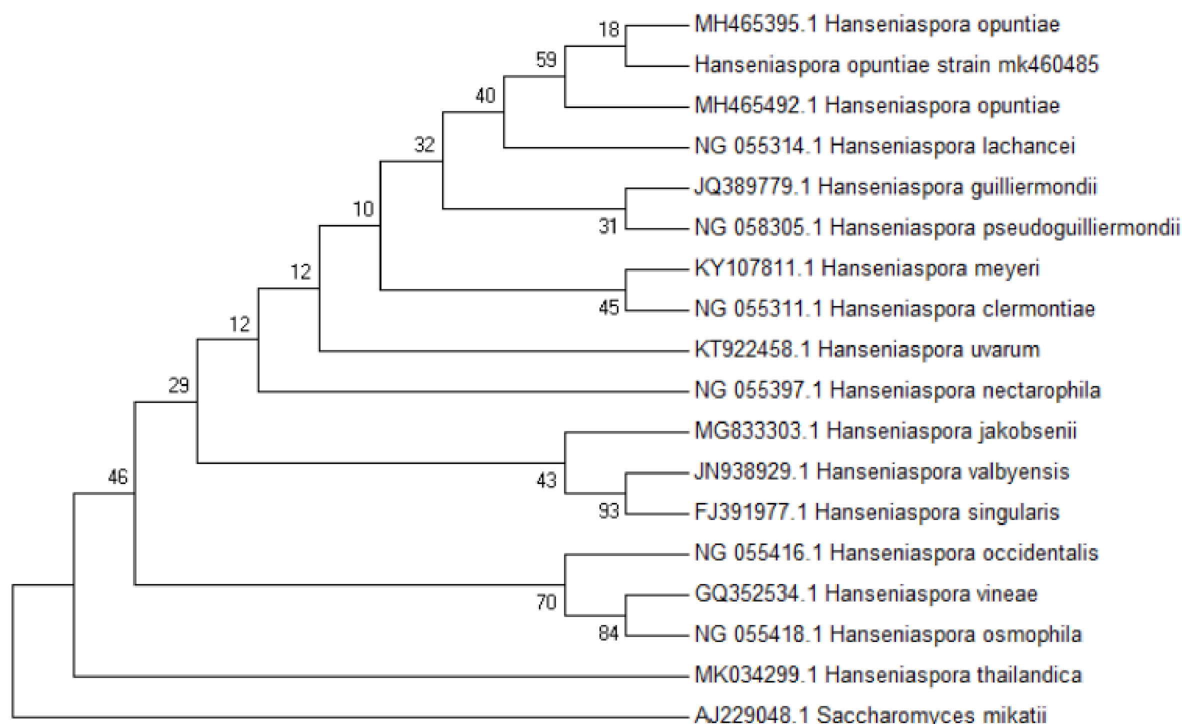



*Figure 4. Phylogenetic relationship between H. opuntiae obtained in this study and other sequences of published strains in the Gene Bank. Accession numbers for sequence is as shown in the phylogenetic tree*

## Discussion

Over the recent years, bioethanol (C2H5OH) has developed as a renewable and biodegradable bio-energy, clear-colorless liquid and ecofriendly potential fuel [7]. Any plants producing a large amount of readily fermentable sugars can be considered as an ideal substrate for bioethanol production [20]. Many fruits contain adequate sugars and nutrients that are important for yeasts growing the wide spectrum of yeast species which are associated with fruits [21]. For instance, grape, sugarcane, and date which abundantly cultivated in Iran are examples of fruits and vegetables that can be potential source for yeast bioethanol producers. In the present study, from 42 yeast strains isolated of fermentation broth of 5 different grapefruit samples, after primary and secondary screening, six isolates have high tolerance to high concentrations of glucose and ethanol and one isolate has the great potential to produce higher concentrations of ethanol in the liquid medium than others, 12 g/l ethanol in broth medium. This isolate morphologicaly was related to *Hanseniaspora* genus. Recently, an inclusive research demonstrated that the D1/D2 region of the 26S rDNA with 600 bp length is a prevailing standard, rapid and accurate tool in yeast identification at the species level compared with the classical method [22].

Based on molecular analysis of 591 bp length this strain belonged to *Hanseniaspora* genus with 100% similarity to *H. opuntiae*. Various species of *Hanseniaspora* genus recorded among frutophilic species were frequently isolated from many grapes and mature fruits [23–25] and their association with the first stages of alcoholic fermentation has been reported during the last century [26, 27]. *H. opuntiae* was found to be primarily associated with Cactaceae in the Hawaiian islands [28] and was also isolated from grape berries in Australia (strain CBS 9791) and in Greece [29].

The efficiency of yeast strains is determined by their ability to utilize various sugar substances, ethanol tolerance capacity in ethanol different concentrations, growth at wide range of temperatures specially 370°C and alcohol production capacity of yeast strains [30]. Yeast strains associated with fruit surfaces can convert wide range of sugars such as fructose and glucose into alcohol and they are also able to tolerate high concentration of alcohol. A yeast strain that used in industrial applications requires specific physiological properties [31]. Thus, yeast strains belonging to the genus *Hanseniaspora* have been used in various fermentation processes. Therefore, the yeast isolated from grape samples and identified as *Hanseniaspora* sp. MK460485 was evaluated for the

production of ethanol and results show that this strain can be considered as a promising microorganism for the production of bioethanol. Pratt-Marshall et al. (2003) and Reddy and Reddy (2006) in separate studies showed that sugar concentrations increasing had a highly inhibiting effect on yeast growth and their capability to ethanol production [32, 33]. This reduction is due to production of other compounds than ethanol and also rising of intracellular toxic ethanol concentration [34] which can stop the fermentation process and finally ethanol formation [35]. Several authors have used different *Hanseniaspora* strains in the fermentation processes. Escalante et al. [36] evaluated the fermentative activity of *H. uvarum* using grape juice to produce fermented beverages. Andorra et al. [37] tested *H. guilliermondii* for ethanol production. Pina et al. [38] studied the tolerance of non-Saccharomyces strains to produce ethanol, in which both of them belonged to the genus *Hanseniaspora*. The obtained strain, *Hanseniaspora* sp. MK460485, showed a high ability to tolerate 60% glucose and 15% ethanol. Also, this strain growth can be seen in wide range of pH 3 to 7 and temperatures of 25 to 42°C. Temperature is the most important factor affecting ethanol production during fermentation process, which has a direct effect on the biochemical reactions, metabolism [39] and the formation of some metabolites such as ethanol, glycerol, acetic acid (LafonLafourcade, 1983) of yeasts. Also, pH has a significant impact on the fermentation, because of its effects on the growth of yeasts, the fermentation rates and the formation of byproducts. Pramanik (2005) reported that the maximum ethanol concentration produced by *S. cerevisiae* was achieved at pH 4.25–5.0 [40]. Russell (2003) recorded that yeast prefers an acid pH and its optimum pH is 5.0–5.2 [41]. Narendranath and Power (2005) found that the optimum pH for yeast growth and ethanol production by *S. cerevisiae* was pH 4.9 [42].

Iran is one of the fruit-producing countries in the world and it has an advanced production and processing industry. Rotten fruits caused by inappropriate storage and waste of processing fruits that are useless and should be thrown away can be used as appropriate substrata for the growth of many microorganisms and can be a good source for growth of microorganisms and could be transformed into very important products such as bioethanol "biofuel". Recent studies have shown strains of the genus *Hanseniaspora* that also isolated in this research are normal flora of fruits and can be considered as potential producers for bioethanol during the fermentation process.

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