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ISOLATION AND SELECTION OF YEAST STRAINS CAPABLE OF FERMENTING ORANGE JUICE

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Abstract. This research aims to isolate and select native yeast strains on oranges, capable of fermenting orange juice. Six types of fresh oranges from the Hanoi market were used as samples to isolate yeasts. By morphological observation, eight isolates were confirmed to be yeast strains. Hoa Binh orange juice was used to test the capability of alcoholic fermentation for each of the isolated yeast strains. The chemical composition of the obtained drink after wine distillation was analyzed and the sensory properties of the distillate were evaluated. The result showed that the strain HB2 had the best production capacity of alcohol among the tested strains, the sensory score reached 16.0, which is in the good category. Through a combination of morphological analysis, culture characteristics on agar and liquid media, and MALDI-TOF techniques, the HB2 strain was identified as *Saccharomyces cerevisiae*.

Keywords: fermentation, isolation, MALDI-TOF, orange, selection, yeast.

1. Introduction

Vietnam is one of the cradles of citrus species cultivated today. Oranges are a precious food that has been domesticated for a long time. Famous orange areas are often alluvial, high, and relatively light soil along rivers. Northern Vietnam has famous orange growing areas along the rivers such as The Red, Lam, Lo, Thuong, Thai Binh, and others. There are also many large orange gardens covering dozens of hectares in the Mekong Delta such as in Tien Giang, Can Tho, Vinh Long, etc. [1]. Oranges are rich in essential nutrients, including vitamins (C, B_1, B_2, B_6, E, etc.), minerals (iron, magnesium, manganese, potassium, zinc, copper, etc.), amino acids (lysine, methionine,

tryptophan, tyrosine, etc.) and dietary fiber, which contribute to health benefits [2]. The fruit flesh is yellow-orange due to the carotenoid group with about more than 50 substances including typical substances such as phytoene, beta carotene, cryptoxanthin, luteoxanthin, auroxanthin, mutatoxanthin, etc., in addition, there are also a small number of flavonoids. Aromatics include a mixture of more than 200 alcohols, aldehydes, esters, hydrocarbons, ketones, and other compounds. Essential oils of orange peel consist of D-limonene (90%), decyclicaldehyde, alcohols such as linalool; D, L terpirol; nonylic alcohol; butyric acid; methyl anthranilate and caprylic ester [3].

The chemical composition of oranges is related to the nutrition and production capacity of the selected yeast strain. The isolation of yeast strains from oranges meets the concept of indigenous microorganisms with high adaptability to the medium as orange juice. Yeast is widely distributed in nature, especially in mediums containing fermentable sugar such as fruits, vegetables, molasses, honey, cereals., etc. [4].

Isolation and selection of yeast strains to produce high-quality orange wine in Tra Vinh, Vinh Long provinces, and Can Tho city have been conducted by scientists at Can Tho University. Yeast strains were identified by DNA sequencing and determined to be 99% similar to *Saccharomyces cerevisiae* [5]. Yeast species present in orange juice were screened, among which *Candida kruesi* and *Rhodotorula minuta* were the predominant species in fresh fermented orange juice, while *C. zeylanoides* and *C. parapsilosis* were the dominant species in defective orange juice [6]. Apple, orange, banana, and other fruits are locally available and thus serve as readily available raw materials for the separation of ethanol fermenting yeasts [7]. The quality of wine produced greatly depends on the types and sources of yeast strains employed in the fermentation process. *S. cerevisiae var. ellipsoideus* which produced a moderate level of alcohol and an appreciable amount of vitamin C appears to be the best organism for orange wine production [8]. Two strains of *S. cerevisiae* isolated from pineapple and orange have been isolated, characterized based on morphological and physico-chemical characteristics, and optimized on ethanol producing capability using sugar cane molasses as substrate [9].

An alternative to DNA-based molecular typing methods is provided by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), a recently developed protein fingerprinting method that allows organisms to be distinguished at the species or even subspecies level [10]. The resulting mass spectrum could be regarded as a microbial protein fingerprint. It contains many peaks that correspond to soluble proteins of high abundance, which are unique for each species [11].

The purpose of this research was to isolate, and identify yeast strains from oranges in the Hanoi market, and evaluate their ability to ferment orange juice to obtain high-quality alcoholic beverages. The findings will contribute to developing a new alcoholic drink such as orange brandy.

2. Content

2.1. Materials and methods

2.1.1. Materials

The experiments were carried out at the Faculty of Food Technology, University of Economics-Technology for Industries. The research was conducted with 6 types of orange varieties, including 5 samples of oranges grown in Vietnam (Hoa Binh, Moc Chau, Hung Yen, Ha Giang, Vinh Long) and 1 sample of orange grown in China (On Chau orange).

Materials used in the laboratory: agar, 96% by volume (v/v) ethanol, distilled water, barley malt, sucrose, and other analytical chemicals, standard substances used in gas chromatography.

Microbial culture medium includes liquid medium, made by saccharification of barley malt (10% malt extract), and solid medium (added 2% agar).

The used equipment: Biological safety cabinet ESCO SC2-4E1 (Indonesia), incubator Memmert IN55 (Germany), autoclave JSAC-40 (Korea), microscope NOVEX (Holland), Analytical electronic weigher OHAUS PX224/E (America), alcohol distillation system, pycnometer, and some other tools for analyzing and identifying microorganisms by MALDI-TOF method.

2.1.2. Methods

Isolation of yeast strains from oranges: Fresh oranges were washed and labeled. About 10 grams of chopped orange was mixed with 90 mL of distilled water. Aliquots of 0.1 mL of the solution were spread onto agar plates. The samples were cultured at 30 ^{o}C for 48 hours for colonies to grow. Colonies with similar characteristics to yeast (round colonies, clear edge, opaque white to milky white in color, convex, with a shiny to matte smooth surface, size about 2 - 3 mm) were collected to culture in liquid medium (30 ^{o}C, 24 h). The culture broth was then diluted 1000 times and transferred to Petri dishes for the second isolation (30 ^{o}C, 48 h) to obtain typical yeast colonies. The screening was continued until homogeneous colonies were obtained on the Petri dish.

Description of cell morphology and culture characteristics of isolated strains: The shape of the cell was determined using a microscope (400-fold magnification) and their appearance on solid and liquid culture medium were described as culture characteristics.

Fermentation of fruit juice: Hoa Binh oranges were pressed with a hand-held juicer, and the juice was filtered through a sieve to remove all the debris. Orange pulp was washed with distilled water to recover all remaining nutrients in the fruit's flesh. The fruit juice was mixed with distilled water in a ratio of 1:3 and sucrose was added so that the dry matter content of the solution reached 16 ^{o}Bx. The solution was sterilised at 85 ^{o}C for 30 minutes. After adding the 10% propagated yeast, the fermentation process was carried out at 30 ^{o}C for 5 days.

Monitoring the fermentation process: During the fermentation process, the dry matter concentration was determined by a refractometer, and the pH was measured by a hand-held LaMotte pH meter.

Distillation of the fermented juice: After fermentation, the wine samples were distilled. The distillate was evaluated for sensory properties according to the procedure described in TCVN 3217-79 (Sensory analysis based on the score of clarity and color, smell, and taste) [12].

Analysis of aromatic substances: The composition of aromatic substances was determined by gas chromatography GC/FID on a Clarus 500 Perkin Elmer instrument with Hydrogen gas generating equipment. Capillary column Agilent J&W DB-ALC, 30 m length × 0.32 mm ID × 1.8µm df. Analysis program:

- Injector: 200 ^{o}C, Carries gas: 0,8 ml/min, Split/Splitless: Flow 5 ml/min;
- Oven 50 ^{o}C/5min;
- Ramp 1: 5 ^{o}C/min – 105 ^{o}C/2min;
- Ramp 2: 10 ^{o}C/min – 190 ^{o}C/9min;
- Detector FID: 220^{o}C, speed of air 350 ml/min, H_2 - 40 ml/min.

Alcohol concentration was measured by a DS AntonPaar 4500. The concentrations of the volatile compounds were expressed as 100 % by volume alcohol (as absolute anhydrous alcohol).

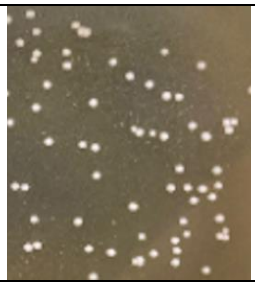
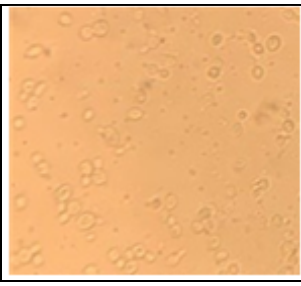
Identification of microorganisms by MALDI-TOF *methods:* The selected strains were identified by the MALDI-TOF MS JMS-S3000 SpiralTOF™ system at the National Institute for Food Safety and Hygiene Testing, Hanoi. The isolate was identifiable after matching their protein expression against the MALDI Biotyper 4.0.0.1 library.

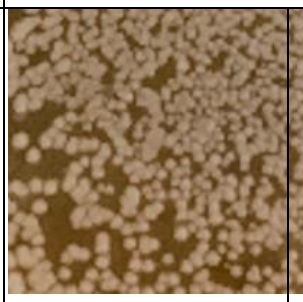
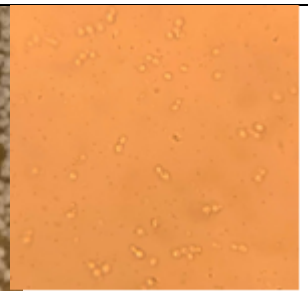
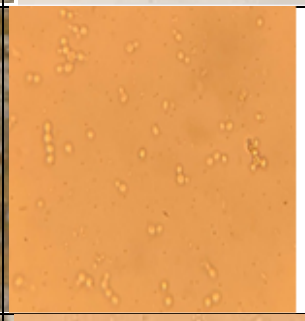
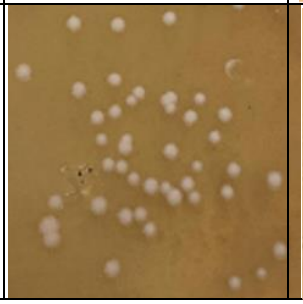
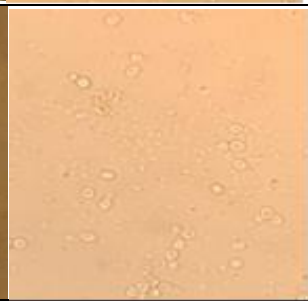
2.2. Results and discussion

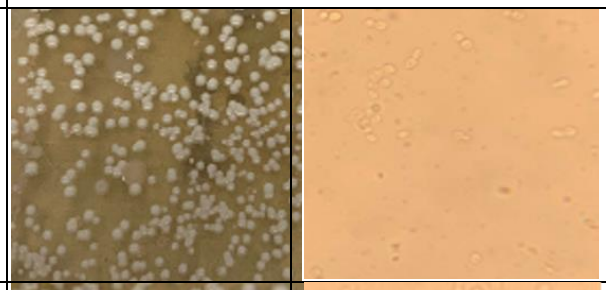
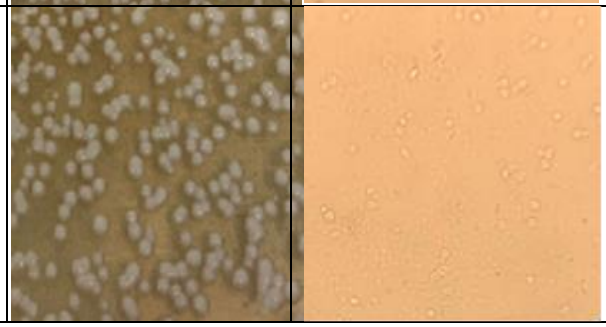
2.2.1. Isolation and description of the colony, cell morphology, and culture characteristics of isolated strains

From Hoa Binh, Ha Giang, Hung Yen, Vinh Long, Moc Chau, and On Chau oranges, after the isolation and preliminary screening, 8 pure strains of microorganisms were selected (Table 1).

Table 1. Colony characteristics and cell morphology of isolated strains

Strain	**The figure of colonies and cells**		**Morphological characteristics**	
HB1			The colonies were small, round, white, dry, convex surface, about 2.0-2.5 mm in size	The cells were spherical to elip, small size, reproducing by budding.

Strain	The figure of colonies and cells		Morphological characteristics	
HB2			Round colonies, milky white, with a smooth and shiny surface, colony size was 2-3 mm.	Cells were spherical to ellipsoidal, slightly small size, reproducing by budding.
HB3			Round colonies, small, milky white, convex surface. Size is about 2-2.5 mm	Spherical cells, slightly small size, reproducing by budding.
HB4			Round colonies, clear edge, opaque white, raised and dry surface, size about 2.5-3 mm	Spherical cells, slightly small size, reproducing by budding.
MC1			Round colonies, white edges, opaque white, convex, smooth surface, size about 2-2.5 mm	The cells were spherical to ellipsoidal, slightly small size, reproducing by budding.
MC2			Round colonies, inner edge, convex, milky white color, smooth surface, size 2.5-3 mm	Spherical cells to ellipsoidal, small size, reproducing by budding.

Strain	The figure of colonies and cells	Morphological characteristics	
MC3		Colonies were round, convex, milky white, smooth surface, colony size 2.5-3 mm	Spherical cells, slightly small size, reproducing by budding.
MC4		Round colonies, convex, milky white, smooth surface, size 2.5-3 mm	Spherical cells, slightly small size, reproducing by budding.

Analysis of the obtained results from Table 1 shows that the shapes of the formed colonies by the eight isolated strains were round with the color varying from white to opaque white, and the size of about 2 - 3 mm. This result was similar to the report of other authors from Can Tho University when isolating and selecting yeast from oranges [5].

Analysis of the cell morphology showed that all isolated strains were spherical to ellipsoidal, slightly small size, and reproduced by budding. The shape of the isolated yeast cells was similar to other reported yeast isolated from oranges [5], [13]. According to the morphological description and preliminary classification of Kurtzman & Fell, *Saccharomyces cerevisiae* has vegetative cells as spherical, ovoid, oval, or elliptical, reproduces by budding, colonies round, has a smooth surface and a characteristic aroma of yeast [14].

Observation of yeast strains growing on liquid media showed that all eight strains formed precipitation and rings around the surface of the liquid medium, similar to the description of the culture characteristics of *Saccharomyces cerevisiae* [4].

With the colony characteristics, cell morphology, and culture characteristics in a liquid medium, it can be supposed that all eight strains are likely to be *Saccharomyces cerevisiae*.

2.2.2. Fermentation of orange juice by isolated yeast strains

After 24 hours of fermentation of orange juice, the isolated strains were adapted to the environment and developed, and foam began to appear. After 2 days of fermentation, a big difference was observed. At this time, foaming took place strongly and the precipitation of biomass began. On day 3, foaming began to slow down and

yeast biomass was precipitated. Obtained results after 5 days of fermentation showed that yeast was most active on days 2 and 3 of the process when foaming was decreased and the precipitation of biomass increased. Observing all yeast strains, HB2, MC1, and HB4 produced greater foam and biomass than the remaining strains. Some monitoring indicators of fermentation are shown in Table 2.

Table 2. Dry matter concentration and pH in the fermentation medium

Strain	pH				Dry matter, %			
	Day 0	*After 3 days*	*After 4 days*	*After 5 days*	*Day 0*	*After 3 days*	*After 4 days*	*After 5 days*
HB1	4.0	3.53	3.42	3.37	16.0	5.25	4.78	4.75
HB2	4.0	3.56	3.43	3.37	16.0	5.75	4.26	4.25
HB3	4.0	3.81	3.41	3.37	16.0	5.75	4.8	4.75
HB4	4.0	3.79	3.45	3.42	16.0	5.1	5.10	5.00
MC1	4.0	3.54	3.45	3.43	16.0	5.5	5.15	5.00
MC2	4.0	3.72	3.49	3.48	16.0	5.65	5.15	5.0
MC3	4.0	3.65	3.47	3.45	16.0	5.25	5.1	5.0
MC4	4.0	3.63	3.45	3.40	16.0	5.5	4.92	4.9

After 5 days of fermentation, the stability of the monitoring parameters was observed in all strains, which was a manifestation of exhausted fermentation. The pH values of all fermentation samples decreased as compared to the starting fermentation broths (samples of day 0). The pH of HB1, HB2, and HB3 samples decreased sharply when compared to the remaining samples. Brix level decreased sharply in fermentation, which of HB1, HB2, and HB3 samples decreased more than the other samples. For the fermentation with the HB2 yeast strain, the dry matter content was reduced to 4.25%, significantly lower than that of other published strains. One reason might be that orange juice contains macromolecular compounds, which are not absorbed by yeasts, and non-fermentable ingredients. Another possible reason was the different ability of the isolated strains to ferment sugars in the juice. [5], [13].

After distillation, the alcohol and aromatic components were analyzed by gas chromatography and referred to as absolute anhydrous alcohol (Table 3).

Table 3 shows that the alcohol concentration of all eight samples was in the range of 7.59% - 7.88% by volume, while different strains have had different biosynthetic capacities of higher alcohols, esters, and acetaldehyde. The alcohol concentration obtained from the experimental samples did not inhibit the fermentation process and was suitable for yeast strain selection.

The methanol concentration in the experimental samples ranged in intervals of 52.23 - 167.19 mg/L. In comparison to specialized data, the amount of methanol obtained from the fermentation of orange juice was lower than that of other fruits.

According to Marinov, grapes and some fruits contain high levels of pectin, which is the cause of the high methanol content in wine [15].

The product consists of many aromas higher alcohols, esters, and different trace elements coming. These ingredients and fermentation ensure the exceptional organoleptic characteristics of the beverage [16].

Table 3. Component of distillate from fermented orange juice, referred to as absolute anhydrous alcohol

No.	Component	Unit	HB1	HB2	HB 3	HB4	MC 1	MC 2	MC 3	MC 4
1	Ethanol	% v/v	7.80	7.76	7.85	7.66	7.88	7.59	7.62	7.74
2	Acetaldehydes	mg/ L	132.59	124.12	155.26	93.63	120.18	145.62	119.25	242.41
3	Methanol	mg/ L	162.05	77.83	52.23	89.01	92.64	69.32	167.19	80.46
4	n-Propanol	mg/ L	197.65	129.53	116.89	186.67	123.41	132.60	112.46	105.35
5	Ethyl acetate	mg/ L	596.42	1043.39	394.82	304.42	299.99	296.27	248.79	690.07
6	Iso-butanol	mg/ L	608.41	396.64	317.29	531.17	328.93	388.11	329.26	293.54
7	3-methyl butanol	mg/ L	1570.71	1031.94	802.87	1293.20	808.83	932.40	867.31	723.28
8	2-methyl butanol	mg/ L	293.52	197.53	150.96	249.42	156.30	179.37	162.59	138.68
9	Ethyl butyrate	mg/ L	3.08	1.84	1.72	0.10	2.44	0.60	0.98	1.50
10	Butyl acetate	mg/ L	0.15	0.06	0.05	0.08	0.10	0.07	0.07	0.02
11	Isoamyl acetate	mg/ L	1.62	0.89	0.32	1.66	0.74	0.52	2.64	1.23
12	Ethyl hexanoate	mg/ L	13.33	8.77	3.93	4.73	6.38	4.37	5.43	2.84

Based on the Vietnam National Standard Method TCVN 3217-79, sensory analysis of different distillate samples is shown in Table 4.

Table 4. Sensory scores of distillate

Sample	Score	Sample	Score
HB1	14.00	MC1	13.40
HB2	16.00	MC2	14.36
HB3	12.80	MC3	13.80
HB4	11.40	MC4	14.60

The sample with the highest sensory score was HB2 (16 points, good category) due to its characteristic aroma, mildly bitter taste, and colorlessness. The composition of

chemical compounds in appropriate proportions was the reason for the good flavor and sensory value of the product. The sample with the lowest sensory score was HB4 (11.40 points, average category), with a light yellow color. In alcohol production technology, some volatile components such as methanol and acetaldehyde that have negative effects on consumers' health can be removed from the distillate by fractional distillation [15], [17].

Based on the achieved results, strain HB2 was considered to have the best production capacity. The concentration of some aromatic substances such as ethyl acetate, iso-butanol, 3-methyl butanol, and 2-methyl butanol in the distillate produced by strain HB2 were 1043.39, 396.64, 1031.94, and 197.53 mg/L, respectively.

2.2.3. Identification of selected strain by MALDI-TOF

HB2 strain with the best production capacity was identified by the MALDI-TOF technique, the protein spectrum results are presented in Figure 1.

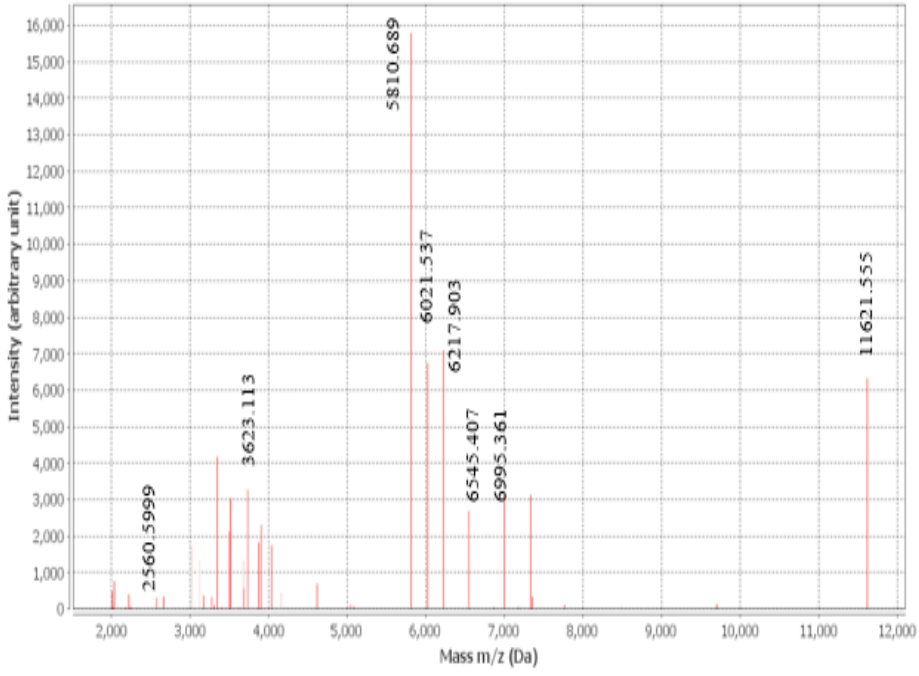


Figure 1. Protein spectrum of strain HB2

Figure 1 shows the strong release of mass ionisations in the range of m/z ratio from 3400 to 4000 Da and from 5800 to 7500 Da. The mass ionization had the highest value at 5810.689. The image shows typical ionisations of *S. cerevisiae* such as m/z values of 2560.599, 3623.113, 5810.689, 6021.537; 6217.903; 6545.407; 6995.361; 11621.555. This is a common protein spectrum of the studied *S. cerevisiae* strains. The level of ionizations in the protein spectrum is also considered as the diversity of the current *S. cerevisiae* strain.

Based on MALDI-TOF analysis, the selected strains HB2 were identified as *Saccharomyces cerevisiae* HB2 with 99.9% confidence.

3. Conclusions

From six orange samples from the Hanoi market, eight strains of microorganisms with morphological characteristics similar to yeast were isolated. The alcohol concentration, aromatic substances, and sensory properties of the distillate from the alcoholic fermentation of the Hoabinh orange juice were evaluated. Strain HB2 was determined to have the best production capacity (ethanol production of 7.76% (v/v); lower methanol production to 77.83 mg/L; etc.). By MALDI-TOF analysis, the strain HB2 was identified as *Saccharomyces cerevisiae* HB2. This strain shows the potential to be a starter culture for ethanol fermentation of Hoabinh orange juice to produce Hoabinh orange brandy in the future.

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