

IDENTIFICATION AND CHARACTERIZATION OF YEAST ISOLATES FROM MAPRANG (*Bouea macrophylla* Griffith) GROWING IN VIETNAM

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ABSTRACT

Fifteen yeast strains isolated from maprang (*Bouea macrophylla* Griffith) were characterised using standard microbiological procedures. The species were identified belonging to three genera *Saccharomyces*, *Hanseniaspora* and *Pichia* and their abilities for wine production were tested by analysing the alcohol produced and remaining sugar. The best biochemically active strain was used along with commercial yeast (*S. cerevisiae*) to produce wine from maprang juice. Fermenting activity of isolated yeasts was higher than commercial yeast. The isolated yeast strain namely 3.5B and 5.5A showed the best fermenting activity such as fast fermentation by Durham test and highest alcohol percentage (11.5 and 11.83% v/v). Based on the 28S rRNA sequences, yeast strain 5.5A and 3.5B showed 99% similarity to *S. cerevisiae* (KP723678.1 and KF728774.1, respectively). Using isolated yeast strain 5.5A for wine fermentation at pH 4.0, total soluble solid content 24% and inoculated with yeast density of 10⁷ cells/mL, the alcohol content produced was 14.5% v/v and the wine had favorable color and flavor. Our work constitutes a foundation for strain selection for future applications as starter cultures in fruit wine fermentations. This work is the first ever on yeast diversity from maprang which is the special fruit of Vinh Long province, Vietnam.

Key words: Characterisation, yeast identification, maprang, yeast strain, wine production, 28S rRNA analysis

INTRODUCTION

Bouea macrophylla, commonly known as maprang and gandaria in English, is a species of flowering plant native to Southeast Asia, and in Vietnamese called as *thanh trà*. In Vietnam, there is a special area of maprang tree growing. Due to the high farming technique, the fruit showed promise for greater commercial production in Vietnam. Maprang grown in Vietnam contains high amount of water (86.6%), protein (40%), carbohydrate (13%), β -carotene (25%) and vitamin C (38%) (Thuy *et al.*, 2018). Maprang is a soft fibrous fruit that yields a highly nutritious juice, which is good for digestion and also can be utilised for the preparation of other value-added products. Among the fruits that have the potential for the production of wine, maprang presents appropriate characteristics such as sugar content, pH, strong flavor and best color (Thuy *et*

al., 2018). However, these fruits are highly perishable, and susceptible to bacterial and fungal contamination as a result they fail to reach the market due to spoilage.

Wine-making is a process of fermentation that involves production of ethanol. Yeasts play a key role in the fermentation process. The term wine is only used for the fermentation of grapes, however, many other fruits can be used in the same process (Dorneles *et al.*, 2005), as well as other species of yeasts associated to these fruits (Vadkertiová *et al.*, 2012). The most commonly used strain for ethanol production is *Saccharomyces cerevisiae*. However, there is limited information available about the effect of various factors on the fermentation profile of maprang wine even by using commercial yeast or isolated yeast strains from these fruits. Fermentation by isolated yeasts has many advantages such as good flavor, high alcohol concentration and low sugar content. This work focused on the characterisation and identification

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of local yeast strains from the fresh pulps of maprang collected in Vinh Long, Vietnam. Screening and selection of pure yeast are important to select the high biological activity of yeast with the best fermentation ability. This study revealed the possibility of producing wine from locally available fruits using simple and adaptable technology with biochemically characterised yeast strains.

MATERIALS AND METHODS

All the research work was carried out in the Laboratory of Food Technology, College of Agriculture and Laboratory of Food Biotechnology, Biotechnology Research and Development Institute, Can Tho University, Vietnam.

Isolation of yeasts

Maprang were collected from Dong Thanh and Dong Binh commune, Binh Minh district, Vinh Long province.

Yeast Extract-Peptone-Dextrose (YPD) medium was prepared. Reagents were dissolved in 1 L of distilled water and autoclaved for 20 min at 121°C then stored at room temperature. Maprang fruit (without peeling) of 50 g were sliced and placed in prepared YPD medium (in erlenmeyer flask 100 mL) and shaken at $28 \pm 2^\circ\text{C}$ for about 2 days. Series of dilution was done (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5}) and 1 mL of appropriate dilution was plated (using the standard pour plating technique) onto YDPA medium, the petri dishes were incubated upside down at 30°C for 48 hr.

Purification of yeasts

Single colonies of representative isolates were purified following the dilution plating technique in the agar medium. Separated colonies were transferred again to the agar slants. Purification was done by streaking on plated agar and repeated two or three times or until pure cultures were obtained, as confirmed by microscopic examination. Purified yeast cultures were identified through morphological, biochemical tests and DNA sequencing.

Morphological characteristics

Morphological distinguished colonies were then selected under a dissection microscope. The isolates were grouped according to their colonial morphology and cell characteristics. The colonies were counted and re-isolated in pure culture using the medium on which they had grown. The shape, color, size of colonies and yeast cells, which were present on the nutrient medium were examined by naked eyes and under the microscope, respectively. Fifteen yeast strains were isolated and coded as

2.5A, 3.4A, 3.5A, 4.5A, 5.5A, 6.5A, 2.5B, 3.5B, 4.5B, 5.5B, 6.4B, 6.5B, 4.5C, 6.5C, 1.5. All these strains were store in YPD medium (in 5 mL agar slant tube/eppendorf tube 200 μL YPD medium and 200 μL glycerol) at 4°C to maintain the original characteristics of yeast strains, recorded and labeled carefully.

Biochemical characteristics of yeasts

Biochemical characteristics of fifteen isolated yeast strains were defined through sugar fermentation test (Kurtzman & Fell, 1998) and urease test (Christensen, 1946).

Identification of yeast isolates by DNA Sequencing

The selected yeast strain was identified by sequencing 28S rRNA. The sequenced gene consists of 801-805 nitrogen bases and this gene segment is compared with yeast 28S rRNA genes was deposited in the GenBank database on NCBI with BLASTN software.

Fermentation and yeast activity test

Maprang juice was adjusted to pH 4.5, total soluble solid content 23% and sterilized by NaHSO_3 (140 mg/L) for 2 hr. The yeast cells were inoculated at density of 10^6 cells/mL, and left to be fermented at 30°C. Analysis was made at the end of fermentation (about 10 days). From there, the most biochemically active strain was selected.

Wine fermenting by selected yeast strains

Maprang juice was prepared at different pH (3.5, 4, 4.5), total soluble solid content (TSS) (22, 24, 26%), yeast cell numbers (10^3 , 10^5 and 10^7 cells/mL) and fermented about 10 days at 30°C. Samples were analyzed at the end of fermentation (10 to 12 days).

Data collection and analysis

Observation

The shape, colony size and yeast cells cultured in the nutrient medium were observed by naked eyes and under microscope. The number of colonies/dish, the yeast cells/mL sample were counted and yeast identification was carried out.

Yeast activity test

The chemical analysis of maprang juice was carried out, including measuring pH value, residual sugar content (%) and alcohol content (%v/v) (AOAC, 2005). The total yeast counts were observed according to Ndip *et al.* (2001).

Sensory evaluation

Maprang wine (after aging for 1 month) was evaluated by 50 panelists. The observations on

color, clarity, odor and taste were recorded. The score ranging from 1-5 and the highest score (5) was expressed for excellent maprang wine quality. The typical Spidergram was performed.

Statistical analysis

Chemical analyses were repeated three times for each sample. The one way analysis of variance (ANOVA), and Duncan multiple comparison test to measure variation between treatments at a probability level of $p < 0.05$ was applied.

RESULTS AND DISCUSSION

Characterisation of yeast strains from maprang fruit

Urea assimilation test

Colony morphology varied between isolates. All colonies were off-white to cream, and butyrous, dry or rough in surface appearance. Based on classification keys, morphological, biochemical and budding characteristics and spore formation (Kurtzman & Fell, 1998) (Table 1), the fifteen yeast strains isolated from maprang belonged to the genera *Saccharomyces*, *Hanseniaspora* and *Pichia*.

The yeast strains of group 1 (3.5B, 4.5C, 5.5A – spherical shape), group 2 (3.5A, 5.5B – small oval shape), group 3 (6.4B, 6.5B, 6.5C – large oval) and group 4 (3.4A, 4.5A, 6.5A – long ellipsoidal) showed the same characteristics as the yeast shape are round, oval, ellipsoidal, haploid cells axial

budding reproduced sexually by forming spores, each spore containing 1-2, 1-3 and 1-4 round spore, sugar fermentation, no urea assimilation. These characteristics are similar to the description of morphological characteristics, a preliminary classification of the genus *Saccharomyces* by Luong *et al.* (2003), Kurtzman and Fell (1998) described similar *Saccharomyces* vegetative cells budded in various directions, round, ovate, oval or ellipsoidal shape extended, forming 1-4 round spores, ovoid, smooth surface, sugar fermentation and no urea assimilation. Thus it can be concluded 11 yeast strains 3.5B, 4.5C, 5.5A, 3.5A, 5.5B, 6.4B, 6.5B, 6.5C, 3.4A, 4.5A, 6.5A belong to *Saccharomyces* genus.

Furthermore, two yeast strains of group 5 (2.5A, 4.5B – pointed ellipsoidal) shown similar characteristic shape (ellipsoidal yeast cells and pointed at both ends) vegetative cells of bipolar budding, formation of 1-4 spheres spore, hemispheres, sugar fermentation and no ability to produce urease. These characteristics are consistent with descriptions of Kurtzman and Fell (1998) and Ebabhi *et al.* (2013) for morphological characteristics of *Hanseniaspora* genus, such as bipolar budding, sexual reproduction by forming 1-4 spheres spores, hemispheres, sugar fermentation and no ability to activate urease. On that basis, it can be concluded that the two isolated yeast strains 2.5A, 4.5B belong to *Hanseniaspora* genus.

In 15 yeast strain isolates cultured in Christensen medium, two yeast strains (1.5, 2.5B) belonged to the long ellipsoidal yeast cell group

Table 1. Summary of morphological, biochemical characteristics, and preliminary classification of isolated yeast strains

Yeast strains	Morphological characteristics			Physiological and biochemical characteristics			Genera (Preliminary classification)
	Yeast shape	Budding	Spore forming	Sugar fermentation test		Urease test	
				Glucose	Saccharose		
3.5B, 4.5C, 5.5A	Spherical	Axial/multilateral	1-2	+	+	-	<i>Saccharomyces</i>
3.5A, 5.5B	Small Oval	Axial/multilateral	1-3	+	+	-	<i>Saccharomyces</i>
6.4B, 6.5B, 6.5C	Large Oval	Axial/multilateral	1-2	+	-	-	<i>Saccharomyces</i>
1.5, 2.5B	Long Ellipsoidal	Axial/multilateral	1-4	+	-	+	<i>Pichia</i>
3.4A, 4.5A, 6.5A	Long Ellipsoidal	Axial/multilateral	1-4	+	-	-	<i>Saccharomyces</i>
2.5A, 4.5B	Pointed Ellipsoidal	Bipolar	1-4	+	-	-	<i>Hanseniaspora</i>

Notes: + : positive; - : negative.

that made the medium turn pink (Figure 1). These two yeast strains could produce urease to break down urea and also have similar characteristics such as haploid cells axial budding and sugar fermentation. The research of Ebabhi *et al.* (2013) also reported that urea hydrolysis was very vigorously registered by *Pichia* genus. These characteristics are similar to the description of morphological varieties of *Pichia* (Kurtzman & Fell, 1998). Thus it can be concluded the two yeast strains (1.5, 2.5B) belong to *Pichia* genus.

Screening and identification of yeast strains from maprang collected in Vinh Long, Vietnam

Propagation of isolated yeasts

The number of yeast cells during propagation in maprang juice was collected, the yeast cell numbers of 11 yeast strains were about 10^7 cells/mL maprang juice after one propagative day. After two days of propagation, 10-40% budding cells could be observed, dead cells did not exceed 4% and yeast cell number of 10^7 cells/mL. The obtained results showed that two days of propagation is appropriate for yeast activation (incubation at 30°C with

shaking at 140 rpm). This result is consistent with the previous results (Thuy *et al.*, 2013a; Thuy *et al.*, 2013b), a good yeast culture for wine making consists of 12-14 million of yeast cells per mL of yeast culture, from 10 to 15% of budding yeast cells and low number of dead cells (below 4%).

Selection of the highest fermentation activity yeast strain from the isolated strains

Eleven yeast strains were selected (3.4A, 3.5A, 3.5B, 4.5A, 4.5C, 5.5A, 5.5B, 6.4B, 6.5A, 6.5B, 6.5C) to carry out the fermentation process along with the commercial *Saccharomyces cerevisiae* (TM) yeast strain to determine the highest fermentation efficiency yeast strain.

The height of CO₂ (cm) produced in Durham test tubes

By Durham tube testing, the yeast strain 5.5A (*Saccharomyces*) isolated from maprang collected at Dong Binh commune, Vinh Long) had the shortest time (8 hr) to push the maximum height (3.2 cm) of Durham tubes compared to the other yeasts (Table 2). It can be concluded that the yeast strain 5.5A has the highest fermentative activity.

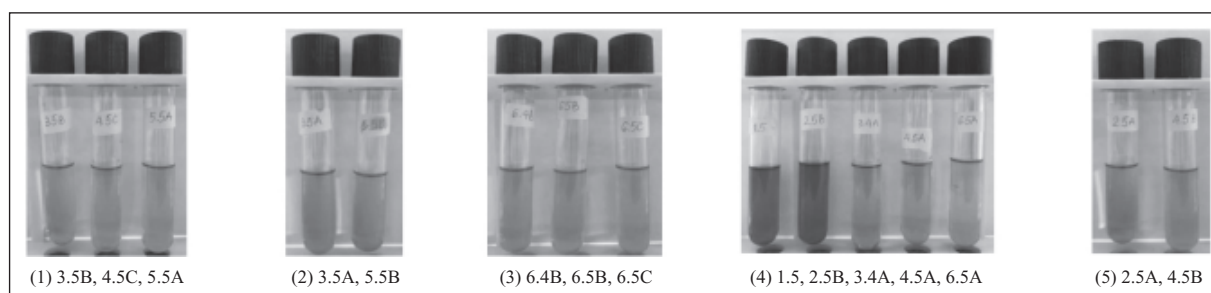


Fig. 1. Color changes of Christensen medium after inoculation and incubation of 15 yeast strains.

Table 2. The height of CO₂ (cm) produced in Durham test tubes of isolated yeast strains in comparison to commercial yeast strain after 10 days of fermentation

Yeast strain	The height of CO ₂ (cm)					
	Fermentation time (hrs)					
	2	4	6	8	10	12
3.4A	0.1	0.5	1.2	1.8	2.5	3.0
3.5A	0	0.3	0.7	1.7	2.2	2.8
3.5B	0.2	1.3	2.2	2.9	3.2	–
4.5A	0.2	0.6	1.1	1.9	2.6	3.1
4.5C	0.3	1.0	1.7	2.2	2.9	3.2
5.5A	0.1	1.4	2.6	3.2	–	–
5.5B	0.2	0.8	1.6	2.1	2.6	3.1
6.4B	0.3	0.5	1.3	1.9	2.5	2.8
6.5A	0.3	1.1	1.9	2.6	3.2	–
6.5B	0.2	0.7	1.4	2.5	3.2	–
6.5C	0.1	0.5	1.2	2.0	2.7	3.2
TM	0.3	1.2	1.8	2.3	3.0	3.2

(Note: The height of CO₂ column does not change).

This result is consistent with the previous results (Thuy *et al.*, 2011; Thanh *et al.*, 2013), the fermentation ability of isolated yeasts from palm and pineapple also based on their gas production in Durham test and the selected yeast strain was fast and strong ability of gas production.

The average of alcohol percent and soluble solid content of maprang wine

After 10 days of fermentation, the alcohol percent of maprang wine are shown in Table 3. The alcohol content of maprang wine ranged from 5 to 11.83% (v/v). However, maprang wine produced by using yeast strains 3.5B, 5.5A, TM obtained the highest percentage of alcohol (11.50, 11.83 and 11.33%, respectively), lowest residue sugar (7.5, 7 and 8.5%, respectively) and showed significant differences when compared with other value obtained from remaining yeast strains.

Thus, combining the results of measuring the height of CO₂ (cm) produced in Durham test tubes, the alcohol percentage, the total soluble solid content after fermentation, it was observed that the isolated yeast strain 5.5A had the shortest time to push gas in the Durham tube (8 hr), high alcohol percent (11.83% v/v) and low TSS (7%). Therefore, this isolated yeast strain was considered to exhibit the highest fermentation activity and was selected to carry out the next experiment.

Identification of yeast strains that were selected from isolated yeast strains from maprang

After selecting the highest yeast fermentation activity, two yeast strains 3.5B and 5.5A were identified using 28S rRNA sequencing (FIRST BASE LABORATORIES, Malaysia).

The sequenced gene consists of 801 nitrogen bases and this gene segment is compared with yeast

28S rRNA genes was deposited in the GenBank database on NCBI with BLASTN software. The results showed that the 28S rRNA gene fragment of yeast strain 3.5B was 99% similarity to the 28S rRNA sequence of *Saccharomyces cerevisiae* (KF728774.1).

The 5.5A yeast strain was identified by sequencing 28S rRNA. The sequenced gene consists of 805 nitrogen bases and this gene segment is compared with yeast 28S rRNA genes deposited in the GenBank database on NCBI with BLASTN software. The results showed that the 28S rRNA gene segment of yeast strain 5.5A shared 99% similarity to the 28S rRNA gene sequence of *Saccharomyces cerevisiae* (KP723678.1).

Effect of total soluble solid content, yeast density and pH on maprang wine

The effect of total soluble solid content, pH, the initial inoculated density of yeast cells on Brix, pH and alcohol content of the maprang wine during fermentation is presented in Table 4. The alcohol content of the wine with initial pH 4.5 (TSS content 24% and 10⁷ CFU/mL) was found to be the highest (14.5% v/v). With an increase in total soluble solid content, alcohol percent does not increase linearly. Similar observations were reported by Satav and Pethe (2016). Low alcohol percentage was found at another pH. This might be due to the inhibition of growth of other microbial flora at lower pH (< 4.5) and more alcohol production by yeast. Various factors such as pH, temperature, concentration of sugars, can affect the physicochemical parameters of wine during its fermentation. pH is one of the important factors which affects the growth and metabolism of yeast (Satav & Pethe, 2016). Generally acidic pH is favourable for wine producing microorganisms and the optimum pH for

Table 3. Alcohol content (% v/v) and soluble solid content (%) of maprang wine fermented by using eleven isolated and commercial yeast strains

No	Yeast strains	Alcohol content (% v/v)	Soluble solid (°Brix)
1	3.4A	6.00 ^{cd}	13.00 ^{de}
2	3.5A	8.50 ^{bc}	10.50 ^{abcd}
3	3.5B	11.50 ^a	7.50 ^{ab}
4	4.5A	7.33 ^{bcd}	11.00 ^{bcde}
5	4.5C	5.00 ^d	14.50 ^e
6	5.5A	11.83 ^a	7.00 ^a
7	5.5B	6.83 ^{bcd}	12.00 ^{cde}
8	6.4B	8.17 ^{bc}	11.00 ^{bcde}
9	6.5A	7.17 ^{bcd}	11.83 ^{cde}
10	6.5B	9.33 ^{ab}	9.67 ^{abcd}
11	6.5C	6.33 ^{cd}	12.50 ^{de}
12	TM	11.33 ^a	8.50 ^{abc}

*Data are means of triplicates. Means in column with different letters are significantly different according to LSD test ($p < 0.05$); TM: commercial yeast (*Saccharomyces cerevisiae*).

Table 4. Effect of controlled Brix, pH, inoculated density of yeast cells on Brix level, pH and alcohol content of maprang wine after fermentation

Controlled °Brix	pH	Yeast density (cell/mL)	TSS (after fermentation)	pH (after fermentation)	Alcohol content (% v/v)
22	3.5	10 ³	7.0 ^{abc*}	3.65 [*]	10 ^{de*}
		10 ⁵	7.5 ^{cd}	3.64	10.5 ^{cde}
		10 ⁷	7.4 ^{bcd}	3.65	11 ^{bcd}
	4.0	10 ³	6.3 ^a	4.01	9.5 ^e
		10 ⁵	6.5 ^{ab}	4.02	10 ^{de}
		10 ⁷	6.4 ^a	3.98	11.5 ^{bcd}
	4.5	10 ³	7.8 ^{cde}	4.39	9.5 ^e
		10 ⁵	8.0 ^{de}	4.55	9.5 ^e
		10 ⁷	8.0 ^{de}	4.45	10.5 ^{cde}
24	3.5	10 ³	9.5 ^h	3.65	12 ^{bc}
		10 ⁵	8.5 ^{efg}	3.64	12.5 ^b
		10 ⁷	9.0 ^{fgh}	3.65	10.5 ^{cde}
	4.0	10 ³	8.5 ^{efg}	4.03	11 ^{bcd}
		10 ⁵	8.0 ^{de}	4.14	12 ^{bc}
		10 ⁷	8.1 ^{def}	4.03	14.5 ^a
	4.5	10 ³	9.3 ^{gh}	4.15	10.5 ^{cde}
		10 ⁵	9.5 ^h	4.32	12.5 ^b
		10 ⁷	9.0 ^{fgh}	4.51	12.5 ^b
26	3.5	10 ³	14.7 ^l	3.67	10.5 ^{cde}
		10 ⁵	14.5 ^{kl}	3.69	11 ^{bcd}
		10 ⁷	16.0 ^m	3.71	11.5 ^{bcd}
	4.0	10 ³	13.8 ^{ijkl}	4.05	10.5 ^{cde}
		10 ⁵	13.7 ^{jk}	4.06	11.5 ^{bcd}
		10 ⁷	13.5 ^j	4.06	12.5 ^b
	4.5	10 ³	13.0 ^j	4.11	11 ^{bcd}
		10 ⁵	14.5 ^{kl}	4.50	11.5 ^{bcd}
		10 ⁷	12.0 ⁱ	4.48	12 ^{bc}

Data are means of triplicates. Means in column with different letters are significantly different according to LSD test ($p < 0.05$).

the growth of yeast and lactic acid bacteria is around pH 4.5 (Jacobson, 2006). At all pH, it was observed that total soluble solid (%) decreased. This was due to sucrose utilisation by microorganism. Similar results were found by Satav and Pethe (2016) for fermentation of banana must. However, they reported low TSS content (6%) as compared to our results (8.1%).

Comparison of maprang wine quality producing by using isolated yeasts and commercial *Saccharomyces cerevisiae*

The fermentation process was done by using yeast strain 5.5A (*S. cerevisiae*, the highest fermentative activity) and commercial *S. cerevisiae* at similar fermentation conditions (pH 4; TSS 24% and inoculated yeast with density of 10⁵ cells/mL). After 12 days of fermentation, maprang wine quality produced by yeast strain 5.5A was compared with maprang wine produced by commercial *S.*

cerevisiae. The highest (12.67% v/v) and lowest (10.33% v/v) alcohol concentrations with corresponding residual TSS content of 5.33 and 6.87% were produced from maprang juice after fermentation with 5.5A yeast strain and commercial *Saccharomyces cerevisiae*, respectively (Figure 2). The spidergram (Quantitative Descriptive Analysis) of sensory evaluation of maprang wine with isolated yeast strain (5.5A) and commercial yeast strain (TM) is shown in Figure 3.

QDA revealed significant ($p < 0.05$) difference in sensory attributes of maprang wine produced from isolated yeast strain 5.5A and commercial *Saccharomyces cerevisiae*. It was observed that the yeast strain 5.5A (*Saccharomyces cerevisiae*) was found to be the best yeast strain producing wine with the highest acceptable scores, in term of smell, taste and color. The maprang wine was fermented from isolated yeast strain showing a special taste, translucent appearance and light yellow color.

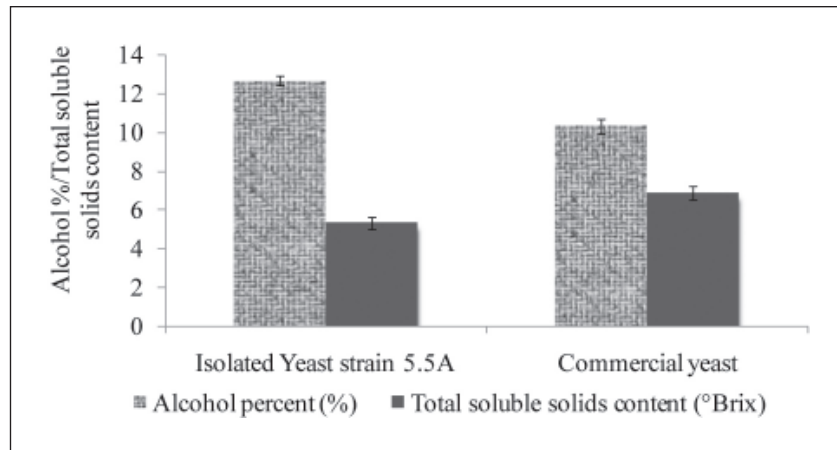


Fig. 2. Alcohol content (%) and total soluble solid (°Brix) of maprang wine by using 5.5A and commercial *Saccharomyces cerevisiae*.

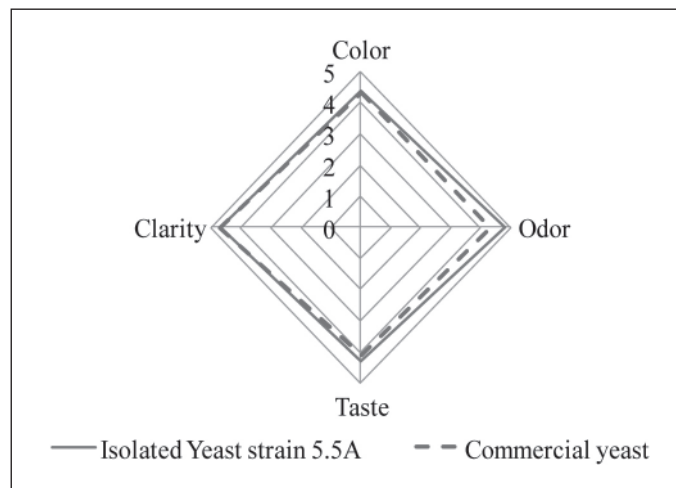


Fig. 3. Spidergram of sensory evaluation of maprang wine producing from isolated yeast strain 5.5A and commercial *Saccharomyces cerevisiae*.

CONCLUSION

This study has isolated and identified fifteen yeast strains from maprang belong to three genera of yeasts, such as *Saccharomyces*, *Haseniaspora* and *Pichia*. The isolated yeast strain 5.5A was the best yeast strain for producing maprang wine with the highest ethanol (12.57% v/v), lowest residual sugar concentrations and highest acceptable scores. The yeast isolated from maprang showed greater ethanol yield compared to the commercial yeast. The isolated yeasts 5.5A was identified as *Saccharomyces cerevisiae* from maprang and could be explored further for the production of maprang wine.

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