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CHARACTERISTIC AND AMYLASE ACTIVITY OF YEASTS ISOLATED FROM *Banh men la* STARTER TABLETS IN CENTRAL VIETNAM

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ABSTRACT

***Banh men la* is a traditional starter tablet for rice-based traditional alcoholic beverages in high-land regions of Vietnam, from minority ethnic groups. The main materials be used for *Banh me la* starter are starch, leaves and other parts of medicinal plants, or sometimes enrich microflora community by adding yeast, fungi, and other microorganisms. The aim of this study was to isolate the high amylase-producing yeast strains with crucial contribution to alcohol production from *Banh me la* starter granules. Nineteen yeast strains were successfully isolated from *banh men la* wine starter tablets collected in Quang Tri and Quang Ngai. Four *Saccharomyces* yeasts, A3(1), A3(2), 1(2) and 5(2), produced high alcohol and amylase activity levels. The optimum alcohol production and amylase activity were obtained when the yeasts were cultured on a sucrose medium at 35°C and shaken at a speed of 180 rpm. The molecular weight of amylases was determined at approximately of 43 kDa. Yeast A3(1) was the best alcohol and amylase producing strain with an alcoholic content of 45.33 g/100 mL and an amylase activity of 104.46 u/mL. The four *Saccharomyces* strains, A3(1), A3(2), 1(2) and 5(2) showed potential for the use in the production of high quality starter tablets for traditional rice-based wine production.**

Keywords: banh men la; Saccharomyces cerevisiae; wine starter tablet; yeast; alcohol fermentation.

INTRODUCTION

Fermentation is widely used by humanity as a traditional chemical process for the production of alcoholic beverages, bread, cheese, coffee, and other by-products. In this process, microorganism converts carbohydrate into an alcohol or an acid and extract energy from the process. Some species of yeast, such as *Saccharomyces cerevisiae*, perform fermentation to produce the alcohol from sugars and acquire energy [1].

The use of fermented foods and beverages has been increasing in popularity. Fermented products are becoming important subjects and many aspects have been considered and developed for scientific research. Alcoholic beverages, including beers, wines, and spirits, have long been important fermented products of human diet and cultural life. Many kinds of indigenous alcoholic beverages are produced and consumed worldwide [2].

In Vietnam, rice wine, one of the traditional alcohols, has been highly popular for centuries. Being an agriculture-based nation, Vietnamese agrarian households use the rice harvest under non-sterile and marginally controlled conditions to make rice wine. For a long time, the production of rice wine has become not only a source of income for most farmer families but also an integral part of the Vietnamese culture [2].

The alcoholic beverages obtained by yeast fermentation from carbon sources are the oldest and most economical of all biotechnologies [3]. Yeast plays an indispensable role in alcoholic fermentation. Therefore, the selection of suitable yeast strains is important in both maximizing alcohol yield and maintaining beverage quality [4].

Although rice-based traditional beverages vary tremendously according to different regions, ingredients and processes, the general manufacture is unchanged in principle. There is a biochemical modification of cereal starches in which fungi (yeasts and moulds) play essential roles. Amylases are produced by mould that can degrade starch into dextrin and sugars, and then yeasts convert sugars to alcohol [2].

There are several *S. cerevisiae* commercial strains available on the market that can be used for winemaking. However, strains isolated from winery regions are better adapted to the climate conditions, substrates, and technical skills. Some other particular characteristics of yeast strains that participate in the fermentation process also help people identify the specificity of wine products and their original regions [5].

Banh men la is a traditional starter used for production of rice-based traditional alcoholic beverages in high-land regions of Vietnam, from minority ethnic groups. The materials use for the prepareation of *Banh me la* are leaves and other parts of local medicinal plants, starch, and sometimes people add yeast, mould and other microorganisms into the starter mixtures. The aim of this study was to isolate the yeast strains with high amylase production and high alcohol producing level from the *Banh me la* starter, in order to produce a high-quality starter that can be used for traditional wine production in the future.

MATERIALS AND METHODS

Materials Collection

Banh men la starters were collected from alcohol-producing households in Dakrong, Quang Tri and Son Ha, Quang Ngai (Vietnam). These two districts locate in the central part of Vietnam and produce famous local alcohol liquors using their own *Banh men la* starters.

Yeast Isolation

The dry samples were grinded, re-suspended in a liquid potato dextrose agar (PDA) medium, diluted in serial of 10^{-3}, 10^{-4}, 10^{-5} folds, and then sub-cultured in a solid PDA medium. Plates were incubated at 28-30°C for 48 hours. Single colony was sub-cultured to a new PDA agar plate to get purified colony. Morphological characteristics such as colony color, shape, and size of the cells were examined by using an Eclipse 55i optical microscope (Nikon, Japan) to confirm the yeast colonies.

Carbohydrate Fermentation Capacity

Yeast isolates were grown in 5 mL of Hansen medium (50 g/L glucose, 3 g/L KH_2PO_4, 2 g/L

$MgSO_4.7H_2O$, 10 g/L pepton, pH5) in a thermostatic cabinet at 37°C for 24 hours with a shaking speed of 150 rpm.

In order to investigate carbohydrate fermentation capacity, 500 µL of the yeast culture was sub-cultured in a test tube containing 5 mL of medium with different carbon solutions (sterilized at 115°C° for 10 min). Two carbon solutions were used, glucose in medium 1 and sucrose in medium 2. The Durham tube was inserted into the prepared test tube and then shook, inverted, and incubated at 30°C. The height of CO_2 columns was measured at 12, 14, 16, and 18 hours during fermentation.

Starch fermentation capacity was investigated on starch medium (gelatinized rice), starch conversion ability was investigated based on the alcohol content after 12 days of fermentation. Ethanol produced after fermentation was determined by the distillation method [6].

Yeast Identification

The high fermentation capacity yeast isolates will be defined by sequencing the ITS and be blast search against NCBI nucleotide database. Yeast isolates were cultured on 5mL Hansen medium and maintained for 24 hours as described above. Cells were harvested by centrifuging at 14,000 rpm, at 4°C for 5 min and washed with autoclaved distilled water. The washed pellet was re-suspended in 500 µL CTAB (cetyltrimethyl ammonium bromide) buffer (100 mM Tris-HCl, pH 8; 2% CTAB;1.4M NaCl; 20 mM ethylenediaminetetraacetic acid; and 0.2% β-mercaptoethanol). Cell walls were broken by sonication at 60 Hz in 6-seconds interval for 5 min using a VC-130 sonicator (Sonics, Newton, CT, USA) and incubated at 65°C for 10 min. Supernatants were collected after centrifugation at 14,000 rpm and 4°C for 10 min. Genomic DNA was purified by adding 500 µL of a 25:24:1 volume mixture of phenol : chloroform : isopropanol and mixed by vortexing. The supernatant was transferred toa new 1.5 mL tube. Genomic DNA was precipitated with two volume of pure ethanol, follow by washing with 70% ethanol, and was dissolved in 50 µL distilled-water [7].

The ITS region was amplified by PCR using 40 ng genomic DNA, 10 pmol of each primer (ITS4, 5'-TCCTCCGCTTATTGATATGC-3' and ITS5, 5'-GGAAGGAGAAGTCGTAACAAGG -3') [8]and 2× Go Taq® Green Master Mix (M7502, Promega, USA) in a 20 µL reaction volume [7]. PCR amplification was performed in MJ Mini™ Thermal Cycler (Bio-Rad, USA) under the follow conditions: 95°C for 10 min; followed by 30 cycles at 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 min; last cycle was 72°C for 10 min. PCR products were visualized after separation on a 0.8% agarose gel and stained with SafeView™ Classic Nucleic Acid Stain (Applied Biological Materials Inc., Canada). Finally, PCR products were purified and sequenced using ITS4 and ITS5 primers by the Macrogen Co., LTD (Korea). The sequence was Blast search against the NCBI nucleotide database and the yeast strain was defined.

Alcohol Measurement

The effect of isolated yeasts on alcohol production using rice-based fermentation was estimated. The fermentation experiment was conducted in two stages as described by Nga [9] and the alcoholic content was measured using a hydrometer as mentioned by Spedding [10] and in TCVN [6]. In brief, a mixture for fermentation was prepared by adding 3 g of starter to 500 g of cooked rice in a 1 L glass bottle and placed at 30±2°C in dark condition for 4 days. The wort added with 400 mL of water containing yeast cells and then fermented for 9 days in the dark at 25±2°C. The fermented mixture was distilled to earn an equal volume of 300 mL of alcoholic liquor from each sample and density determination using a pycnometer at 20°C. In order to prepare a solution of 400 mL clean water containing 1 g yeast cells, each yeast isolate was cultured individually in fresh liquid Hansen medium for 24 hours and was collected by filtrating through Whatman N_o.1 filter paper. The yeast cells were dissolved in 400 mL water and mixed well in the bottle containing the 4-day fermented wort.

Effects of Culture Conditions on Amylase Production

The pre-cultured yeasts (on Hansen medium for 24 hours) was inoculated into 9 mL of sterilized

Czapek-Dox medium and grown at different temperatures (28, 30, and 35°C), a shaking speed of 180 rpm and 1% of starch as a carbon source. Amylase was determined after 3 days of fermentation by the spectrophotometry method.

The effect of shaking on amylase production was studied by growing the yeasts on the Czapek-Dox medium at a selected temperature from the previous experiment, at different speeds (160, 180, and 200 rpm). The amylase activity was measured after 3 days of fermentation.

Different carbon sources (1% of starch, glucose, and sucrose) were used to examine the effect of carbon sources on amylase production. Yeasts were grown in optimal culture conditions (selected optimal temperature, shaking speeds, and fermentation duration). Amylase activity was assayed by the spectrophotometry method.

Alpha-amylase Activity Assay

The α-amylase activity was assayed using Bernfeld's method [11] with slight modifications. One milliliter of diluted enzyme was incubated for 3 min at 20°C with 1 mL substrate solution (soluble starch). The enzyme reaction was interrupted by the addition of 2 mL 3,5-dinitrosalicylic acid (DNS) reagent. The mixture was heated for 5 min in boiling water and then cooled under running tap water. After the addition of 20 mL H_2O, the optical density of the solution containing the brown reduction product was determined spectrophotometrically at a wavelength of 540 nm against a blank prepared in the same manner without enzyme. A standard curve established with maltose (0.2-2 mg in 2 mL H_2O) was used to convert the colorimeter readings into milligrams of maltose. Alpha-amylase activity is expressed as milligrams of maltose liberated in 3 min at 20°C by 1 mL enzyme solution. One unit of α-amylase activity is defined as the amount of enzyme required to liberate 1 μmol maltose per minute under the standard assay conditions [12].

Amylase Molecular Weight Determination

Crude enzyme was precipitated by ammonium sulfate (60% saturation) at 4°C for 2 hours, centrifuged at 15,000 rpm at 4°C for 10 min. The pellet was re-suspended in PBS buffers and used for characterization of the enzyme.

After ammonium sulfate precipitation, the pellet was resuspended in PBS buffer (pH 7.4) and dialyzed overnight (around 12-15 hours). The protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the standard procedure described by Laemmli [13].

Statistical Analysis

All experiments were repeated three times. The data were analyzed in terms of means followed by Duncan's test with the values $P<0.05$ were considered statistically significant. Statistical analysis was done using the SPSS software version 15.0.

RESULTS

Yeast Isolation

In this study, seven yeast cell lines were isolated from *Banh men la* from Quang Tri province (sample codes as 1(2), 2(2), 5(2), 6(2), 7(2), 8(2), 9(2)) and 12 cell lines were isolated from *Banh men la* from Quang Ngai province (sample codes as 3(2), 4(2), 10(2), 11(2), A1(1), A1(2), A2(1), A2(2), A3(1), A3(2), A4(1), A4(2)). The morphology of 19 cell lines was described in Table 1. The yeast cell shapes are circular (11 isolates) or ovoid (8 isolates) and yeast divides by polarized budding (15 isolates) or unpolarized budding (4 isolates). Colony diameter ranged from 1 to 3.5 mm. Most of the colonies were milky white, smooth and convex, and typical of yeast *Saccharomyces*.

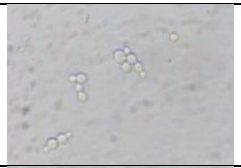
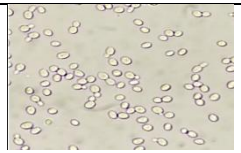
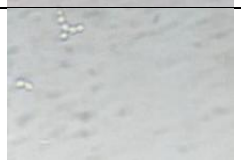
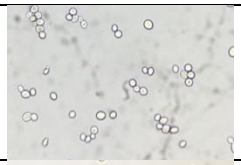
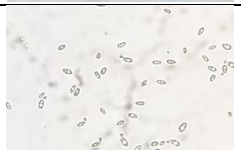
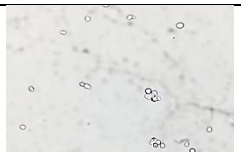
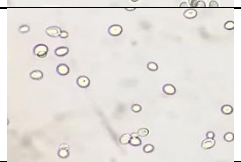
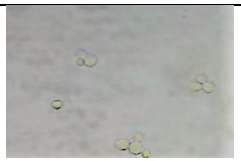
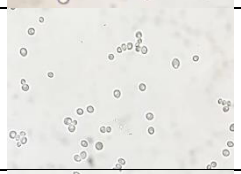
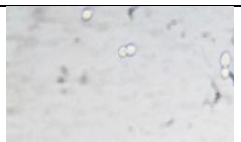
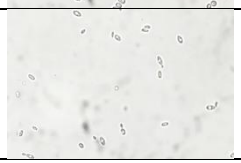
The species that predominates fermentative process for wine production is *S. cerevisiae*. During the fermentation, the yeast produces ethanol, carbon dioxide, and other secondary products important for flavor, taste, and quality of wine serving as a reference on strain isolation [5].

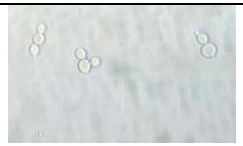
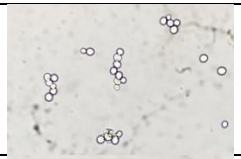
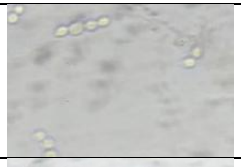
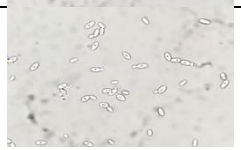
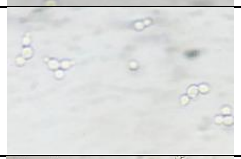
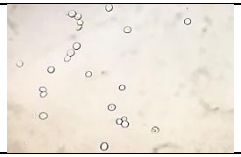
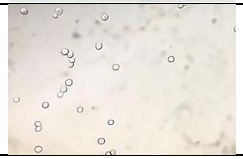
Fermentation Ability

Carbon sources fermentation ability

In this study, glucose and sucrose were both used as carbon sources to confirm the yeast isolates

Table 1. Morphology of cells and colonies of isolated yeast from *Banh men la*

Isolates	Cell shape (×40)	Cell shape	Colony shape	Isolates	Cell shape (×40)	Cell shape	Colony shape
1(2)		Circular, polarized budding	Milky white, 1-2 mm	**11(2)**		Ovoid, polarized budding	Circular, convex, milky white, smooth, 1,5-2,5 mm
2(2)		Circular, polarized budding	Milky white, 2-2.5 mm	**A3(1)**		Ovoid, polarized budding	Circular, convex, milky white, 1-2 mm
3(2)		Ovoid, polarized budding.	Circular, milky white, smooth, 1-2 mm	**A3(2)**		Circular, polarized budding	Circular, convex, milky white, 1-2 mm
4(2)		Ovoid, polarized budding	Circular, milky white, smooth, 1.5-2 mm	**A4(1)**		Ovoid, polarized budding	Circular, convex, milky white 2-2.4 mm
5(2)		Circular, unpolarized budding	Circular, convex, milky white, smooth, 1.5-2.5 mm	**A4(2)**		Circular, polarized budding	Circular, convex, milky white, 1-1.5 mm
6(2)		Ovoid, polarized budding	Circular, convex, milky white, smooth, 1-2 mm	**A1(1)**		Ovoid, polarized budding	Circular, convex, milky white, 1.5-2 mm

Isolates	Cell shape (×40)	Cell shape	Colony shape	Isolates	Cell shape (×40)	Cell shape	Colony shape
7(2)		Circular, unpolarized budding	Circular, convex, milky white, smooth, 1.5-2.5 mm	**A1(2)**		Circular, polarized budding	Circular, convex, milky white, 1-2 mm
8(2)		Circular, unpolarized budding	Circular, convex, milky white, 2-3.5 mm	**A2(1)**		Ovoid, polarized budding	Circular, convex, milky white, 2-2.5 mm
9(2)		Circular, unpolarized budding	Circular, convex, milky white, 1-2.5 mm	**A2(2)**		Circular, polarized budding	Circular, convex, milky white, 1-2.5 mm
10(2)		Circular, single, polarized budding	Circular, convex, milky white, smooth, 1-2.5 mm				

fermentation ability through the CO_2 production during fermentation. The results in Table 2 show that sucrose is a more suitable carbon source for yeast than that of glucose as a result of a conversion of 2 moles of glucose from sucrose hydrolysis. Among the 19 yeast isolates, A3(1) and A3(2) from Quang Ngai province and 1(2), 5(2) from Quang tri province had produced a high volume of carbon dioxide (2 cm) in both utilized media within the short period of 14 hours. These four isolates had significantly highest CO_2 production compared to the other 15 yeast strains isolated from *Banh men la*.

Ethanol fermentation capacity

The yeast isolates were tested for ethanol fermentation with rice as substrate. The results are showed in Table 3 indicate that all of the isolates converted starch into ethanol. The four strains with the high fermentation capacity were A3(1), A3(2), 1(2), and 5(2), and the ethanol content after distillation of the wort ranged from 43.67% to 47.67%. These results showed that the yeast producing high carbon dioxide likewise produced high ethanol yield.

Yeast Identifitation

Sequence analysis of PCR products obtained 864 bp nucleotide fragments for all 4 yeast isolates. Using the blast tool, nucleotide of 4 yeast isolates ITS sequences was found to have a similar height with *Saccharomyces cerevisiae* species genome on the GenBank (Table 4). The similar height in nucleotide sequences (>99%) indicated that all 4 isolates belong to the *S. cerevisiae* species and are name *S. cerevisiae* A3(1), *S. cerevisiae* A3(2), *S. cerevisiae* 1(1), and *S. cerevisiae* 5(1).

Effects of Culture Conditions on Amylase Activity

Effects of culture temperature

The culture temperature has a great influence on yeast growth, enzyme biosynthesis and enzyme activities [14]. Each strain has a different suitable temperature for growth. For most yeast strains, the appropriate growth temperature is usually in the range of 25-30°C [15, 16]. In this study, yeast samples were cultured in the range of temperature from 25 to 35°C and enzyme activity was assayed after 72 hours.

Table 5 showed the amylase activity of the four yeast isolates cultivated at 28°C, 30°C, and 35°C. The results indicated that 35°C is optimal incubated temperature for amylase production of the four isolates, starch hydrolysis ranged from 72.247 U/mL (isolate 1(2)) to 101.911 U/mL (isolate A3(1)), enzyme activities decreased with the reduction of incubated temperatures . Amylase activity of yeast decreased about 50% when yeasts were grown at 25°C. The A3(1) was the best strain for amylase production, with starch hydrolysis 101.911 U/mL.

Effects of shaking speed

The four strains, A3(1), A3(2), 1(2), and 5(2), were cultured in Czapek-Dox medium at 35°C with shaking speeds of 160, 180, and 200 rpm. Amylase activity was assayed after 72 hours. Table 6 showed that the amylase activity of the four yeast isolates was influenced by shaking. Amylase production was observed at 160 rpm, highest at 180 rpm and reduced when the speed of shaking was increased to 200 rpm. Statistically, yeast strains produced significantly the highest amylase activity at 180 rpm. Yeast strain A3(1) had the highest amylase activity and 1(2) showed the lowest enzyme activity.

Effects of carbon sources

The type and concentration of carbon sources have a large effect on the enzyme production of yeast. In this experiment, 1% of soluble starch, glucose, and sucrose were used as substrates for amylase production. Table 7 indicates that amylase activity was observed in soluble starch, glucose and sucrose substrates by the four yeast isolates, in which, the soluble starch is a suitable carbon source for yeast to produce amylase. Statistically evaluated, the highest starch enzyme activity was performed by yeast A3(1) and the lowest by 1(2). Difference between yeast strains did not vary.

Molecular weight determination

SDS-PAGE of total extracellular proteins were analyzed (Fig. 1). The results showed that a band

of approximately 43 kDa was observed on PAGE for all 4 strains. This result indicates that there was maybe only one extracellular amylase enzyme from these yeast strains.

Table 2. Height of CO_2 column after fermentation using glucose and sucrose substrates

Isolates	Fermented medium 1		Fermented medium 2	
	Height of CO_2 column after 12 hour fermentation (cm)	Fermentaion time for CO_2 column get 2 cm (hours)	Height of CO_2 column after 12 hour fermentation (cm)	Fermentaion time for CO_2 column get 2 cm (hours)
A1(1)	0.00^{f}	-	0.02^{c}	-
A1(2)	0.11^{ef}	18	0.22^{c}	-
A2(1)	0.00^{f}	-	0.05^{d}	-
A2(2)	0.05^{f}	-	0.28^{c}	18
A3(1)	**0.27^{e}**	**18**	**1.95^{a}**	**14**
A3(2)	**0.20^{e}**	**18**	**1.92^{a}**	**14**
A4(1)	0.00^{f}	-	0.67^{b}	18
A4(2)	0.15^{ef}	-	0.42^{b}	16
3(2)	0.00^{f}	-	0.12^{cd}	-
4(2)	0.15^{ef}	18	0.13^{cd}	-
10(2)	0.15^{ef}	18	0.05^{d}	-
11(2)	0.00^{f}	-	0.67^{b}	18
1(2)	**$1,60^{a}$**	**18**	**$1,93^{a}$**	**14**
2(2)	$0,00^{f}$	-	$0,00^{d}$	-
5(2)	**$1,27^{b}$**	**16**	**$1,97^{a}$**	**14**
6(2)	$0,10^{ef}$	-	$0,67^{b}$	-
7(2)	$0,67^{d}$	-	$0,67^{b}$	16
8(2)	**$1,07^{c}$**	**16**	**$0,57^{b}$**	**16**
9(2)	$0,77^{d}$	-	$1,93^{a}$	14

Note: Values with different letter are statistically significant at P=0.05, "-" indicate that the hight of CO_2 column in Durham test does not reach around 2 cm after 24 h fermetation

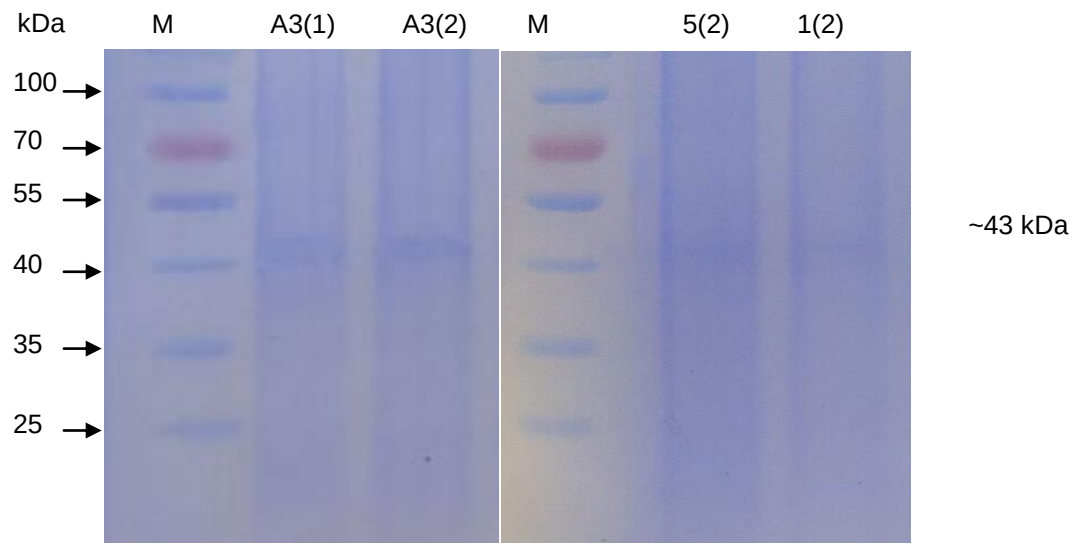


Fig. 1. SDS-PAGE of amylase. M, marker; A3(1), A3(2), 5(2), 1(2) represent to precipitated protein from culture medium of yeast isolates A3(1), A3(2), 5(2), 1(2).

Table 3. Ethanol contents after 12 days fermentation of yeast isolates using rice as substrate

Isolates	Alcoholic contents (g/100 mL)
A1(1)	31.33^{i}
A1(2)	37.17^{ef}
A2(1)	31.83^{gh}
A2(2)	33.50^{h}
A3(1)	**45.33^{ab}**
A3(2)	**43.67^{bc}**
A4(1)	35.83^{fg}
A4(2)	39.67^{de}
1(2)	**45.43^{ab}**
2(2)	40.67^{d}
3(2)	37.47^{ef}
4(2)	41.83^{cd}
5(2)	**47.67^{a}**
6(2)	33.83^{i}
7(2)	41.17^{cd}
8(2)	39.83^{de}
9(2)	39.67^{de}
10(2)	36.33^{fg}
11(2)	39.33^{de}

Note: Values with different letter are statistically significant at P=0.05

Table 4. Similarity of ITS sequences

Isolates	Reference sequences	Reference species	Identify (%)	Species
A3(1)	CP006423	*Saccharomyces cerevisiae* YJM1460	99.76	*Saccharomyces cerevisiae*
A3(2)	CP006423	*Saccharomyces cerevisiae* YJM1460	99.88	*Saccharomyces cerevisiae*
1(2)	CP006454	*Saccharomyces cerevisiae* YJM681	99.42	*Saccharomyces cerevisiae*
5(2)	CP006454	*Saccharomyces cerevisiae* YJM681	99.54	*Saccharomyces cerevisiae*

Table 5. Effects of culture temperature on amylase activity (U/mL)

Strains	Cultivation temperatures (°C)		
	28	**30**	**35**
1(2)	37.367^{b}	60.297^{b}	**72.247^{a}**
5(2)	45.011^{b}	68.001^{c}	**89.839^{a}**
A3(1)	33.364^{c}	31.423^{c}	**101.911^{a}**
A3(2)	43.676^{c}	33.728^{c}	**97.422^{a}**

Note: Values with different letter in a row are statistically significant at P=0.05

Table 6. Effects of shaking speeds on amylase activity

Strains	Shaking speeds (rpm)		
	160	**180**	**200**
1(2)	60.297^{b}	72.247^{a}	63.876^{ab}
5(2)	68.001^{c}	89.839^{a}	83.652^{b}
A3(1)	31.423^{c}	101.911^{a}	58.477^{b}
A3(2)	33.728^{c}	97.422^{a}	64.726^{b}

Note: Values with different letter in a row are statistically significant at P=0.05

Table 7. Effects of carbon sources on amylase activity (U/mL)

Strains	Carbon sources		
	Starch	Glucose	Sucrose
1(2)	79.224[a]	44.283[b]	30.816[b]
5(2)	95.359[a]	57.264[b]	39.000[c]
A3(1)	104.459[a]	34.031[b]	30.391[c]
A3(2)	100.940[a]	35.790[b]	31.908[c]

Note: Values with different letter in a row are statistically significant at P=0.05

DISCUSSION

The preparation and the use of fermentation starters as a source of inoculum are important in the manufacture of rice wine. It is recognized by winemakers that the choice of starter influences the yield and quality of the wine. A number of local processors claim that using a combination of two or three different starters yields wine of better quality with a stronger sweet alcoholic taste and more attractive flavor than is obtained with a single starter. Their experience is that each different starter has its own advantages and disadvantages. These dried starters normally include yeasts, moulds, and bacteria and convert starchy materials to fermentable sugars and subsequently to alcohol and organic acids. A variety of starter cultures is available in the markets in most Asian countries [2].

The results in this study indicated the fermentation ability of 19 yeast isolates using glucose, sucrose and soluble starch as substrates and producing CO_2 as the indicator. The four isolates, A3(1), A3(2), 1(2), and 5(2), generated high volumes of CO_2 at around 2 cm in Durham test after 24 hours of fermentation. The CO_2 volume of these yeast isolates is far lower than that of the yeast isolates present in the study of Dung et. al. [17] with the CO_2 column height at almost 3 mm after the same 24 hours of fermentation. This slow fermenting evidence can explain the reason why the production of rice wine using *Banh men la* starter takes a longer fermentation time than using Banh men (commonly used in Vietnam), 10 days [9] compare to 5-7 days [2]. On the other hand, the alcohol contents in final distilled products seem not so different with alcohol level in this study reaching approximate 45 g/100 mL (50% v/v) and the alcohol level in Vietnamese traditional rice wine is about 50-60% (v/v) [2].

Rice wines are produced predominantly at artisanal home- or cottage-level. Though each producer has his own way of making wine, depending on his individual experience and regional available raw materials, in principle, all producers use the same process. Powdered starch-based starter (about 1% - 2% of the raw starchy materials) is mixed with steamed or cooked gelatinized rice, which is then incubated under ambient conditions [2].

Rice-based wine is produced in a unique processing principle of manufacture whereby rice starches are converted into alcohol by physical, microbiological and biochemical operations [2]. The wide range in quality of rice wine in Vietnam was contributed by the manufacturing process, substrates, and starters in which different utilized starters delivered a significant effect on end-product quality, especially on the flavour and aroma.

The microorganism community in Vietnamese starters was well characterized in the studies of Dung (2007) identified 119 microbial strains including 53 molds, 51 yeasts and 15 bacteria [18]. Lee and Fujio identified 20 mold and 33 yeast strains while Thanh et. al. (2008) determined 13 species of fungi and 23 species of bacteria in starters [19]. Thanh et. al. (1999) isolated 22 microbial species, including 6 fungi species, 1 yeast-like fungus, 7 yeasts and 8 bacteria [20]. However, the presence of microorganisms in *Banh men la* and their contribution to the quality of rice wine products is lack of literature and still incomplete understanding. The reason could be a diversity of natural herb materials counterpart in *Banh men la* and/or regionals different applied techniques during preparation of *Banh men la* starter. The results in this study visualized four

out of 19 yeast isolates had a high amylase activity and high alcohol production levels. These yeast strains can be added into *Banh men la* for increasing fermentation ability and stabilizing *Banh men la* starter in rice-based wine production.

The screened microorganisms are proposed to be safe and effective starters, as opposed to traditional starters, to promote the standardization of production [21]. To the best of our knowledge, this study, for the first time, characterized yeast isolated from *Banh men la* from different regions of Central Vietnam and further screened fermentative yeast with application value in the production of rice wine.

CONCLUSION

In our study, 19 yeast strains were isolated from *Ban men la* wine starter from Quang Tri and Quang Ngai. The optimum conditions for alcohol production and amylase activity of 4 strains were obtained when yeasts were cultured on medium with rice starch as carbon source, incubated at 35°C, optimal shaking speed of 180 rpm. The molecular weight of amylases was approximately of 43 kDa. A3(1) was the best alcohol and amylase production strains, alcoholic content was 45.33 g/100 mL and amylase activity of 104.459 u/mL. This A3(1) yeast isolate can be added to starters and contribute to the stability of Banh men la starter as well as the quality of the final liquor product.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Maicas S. The role of yeasts in fermentation processes. Microorganisms. 2020;8(8):1142.
2. Dung NTP. Vietnamese rice-based alcoholic beverages. International Food Research Journal. 2013;20:1035-1041.
3. Venturini Copetti M. Yeasts and molds in fermented food production: an ancient bioprocess. Current Opinion in Food Science. 2019;25:57-61.
4. Walker GM, Stewart GG. Saccharomyces cerevisiae in the Production of Fermented Beverages. Beverages. 2016;2(4):30.
5. Guimarães T, Gomes Moriel D, Machado I, Picheth C, Bonfim T. Isolation and characterization of Saccharomyces cerevisiae strain of winery interest. Revista Brasileira de Ciências Farmacêuticas. 2006;42:119-126.
6. Technology) TVMoSa. Distilled liquors - determination of alcohol. Vietnamese National Standard TCVN 8008:2009; 2009.
7. Huy ND, Tien NTT, Huyen LT, Quang HT, Tung TQ, Luong NN, Park S-M. Screening and production of manganese peroxidase from *Fusarium* sp. on Residue Materials. Mycobiology. 2017;45(1):52-56.
8. White TJ, Bruns T, Lee S, Taylor J. 38 - Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds. PCR Protocols. San Diego: Academic Press; 1990.
9. Nga TTP. Isolation and initial application in fermeted food processing, . Master, University of Agriculture and Forestry, Hue University; 2019.
10. Spedding G. Alcohol and its measurement in Bamforth C, ed. Brewing materials and proceses: A Practical Approach to Beer Excellence. UK: Academic Press; 2016.
11. Bernfeld. Amylases, α and β. Methods in Enzymology. Academic Press. 1955;1:149-158.
12. Loc NH, Mien NTT, Thuy DTB. Purification of extracellular α-amylase from *Bacillus subtilis* by partitioning in a polyethylene glycol/potassium phosphate aqueous two-phase system. Annals of Microbiology. 2010;60(4):623-628.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of

bacteriophage T4. Nature. 1970; 227(5259):680-685.
14. Merritt NR. The influence of temperature on some properties of yeast. J. Inst. Brew. 1966;72:374-383.
15. Yalcin SK, Yesim Ozbas Z. Effects of pH and temperature on growth and glycerol production kinetics of two indigenous wine strains of Saccharomyces cerevisiae from Turkey. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]. 2008;39(2):325-332.
16. Jung PP, Christian N, Kay DP, Skupin A, Linster CL. Protocols and Programs for High-Throughput Growth and Aging Phenotyping in Yeast. PLOS ONE. 2015;10(3):e0119807.
17. Dung NTP, Tuong NH, Phong HX. Study on ethanol fermentation conditions from molasses by Thermo-tolerant Yeasts. International Journal of Business and Applied Science. 2014;1(1):13-22.
18. Dung NTP, Rombouts FM, Nout MJR. Characteristics of some traditional Vietnamese starch-based rice wine fermentation starters (men). LWT - Food Science and Technology. 2007;40(1):130-135.
19. Thanh VN, Mai le T, Tuan DA. Microbial diversity of traditional Vietnamese alcohol fermentation starters (banh men) as determined by PCR-mediated DGGE. Int J Food Microbiol. 2008;128(2): 268-273.
20. Thanh VN, Thuy NT, Chi NT, Hien DD, Ha BT, Luong DT, Ngoc PD, Ty PV. New insight into microbial diversity and functions in traditional Vietnamese alcoholic fermentation. Int J Food Microbiol. 2016;232:15-21.
21. Cai H, Zhang T, Zhang Q, Luo J, Cai C, Mao J. Microbial diversity and chemical analysis of the starters used in traditional Chinese sweet rice wine. Food Microbiology. 2018;73:319-326.