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IDENTIFICATION OF YEAST STRAINS AND FILAMENTOUS FUNGI IN THE HAI HAU TRADITIONAL ALCOHOL YEAST CAKE

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Abstract. This study aims to determine the various strains of yeast and filamentous fungi in traditional alcohol yeast cake from Hai Hau district, Nam Dinh province. By traditional laboratory techniques such as isolation on YPD agar, observation of colony morphology as well as cell characteristics by microscopy, combined with molecular biology method to sequence ITS region 03 strains of yeast *Saccharomyces cerevisiae* CN1, CN2, and NM3 were identified: 01 pseudo-strain yeast *Saccharomycopsis fibuligera* NM02 and 01 strain of filamentous fungi *Rhizopus microsporus* NS04. The density of yeast strains cultured on YPD agar supplemented with 50 mg/L Tetracycline was 4.5×10^8 CFU/g, while the density of *R. microsporus* NS04 filamentous fungi was 2.0×10^6 CFU/g. With the agar plate diffusion method, yeast strains of *S. cerevisiae* CN1, CN2, and NM3 that are unable to biosynthesize extracellular enzymes have been identified. Yeast pseudo-strain *S. fibuligera* NM02 is capable of producing amylase (with starch substrate hydrolysis ring diameter > 9 mm), cellulase (16 mm), and protease (18 mm). The filamentous fungi *R. microsporus* NS04 has a higher ability to produce extracellular enzymes such as amylase (> 9 mm), cellulase (33 mm), and protease (39 mm).

Keywords: extracellular enzymes, filamentous fungi, Hai Hau, pseudo-yeast, yeast, alcohol yeast cake.

1. Introduction

The technology of traditional alcohol with yeast cake creates Vietnamese famous brands, including Hai Hau - Nam Dinh sticky rice alcohol. In light of this, sticky rice is cooked, allowed to cool on trays, and mixed with traditional alcoholic yeast cake. After solid fermentation and adding of purified water, the liquid fermentation is carried

out in a closed container. Most producers in Hai Hau district, Nam Dinh province apply the fractional method of distillation to remove part of the impurities in obtained alcohol such as methanol, acetaldehyde, and fusel oil and combine with the maturation process to create high quality products [1].

Traditional alcohol has unique cultural characteristics of the region. However, the fermentable efficiency, sanitary conditions, and the quality of alcohol in many locations are unstable [1]. One of the causes of this instability is the different species of used microorganisms in traditional alcohol yeast cake. The using of pure and highly active microorganisms in the fermentation process improves the flavor and aroma of the finished alcohol [2]. Microorganisms play an important role in the production of alcohol, directly affecting the productivity and quality of the product. Some common filamentous fungi species such as *Rhizopus*, *Amylomyces*, *Mucor*, and *Aspergillus* can produce extracellular enzymes, and break down starch and other high molecular components in solid and liquid fermentation. The yeast, which metabolizes fermentable sugars during the liquid fermentation, includes *Saccharomyces cerevisiae*, *Hansenula* spp., *Endomycopsis* spp. [3].

Different types of traditional alcohol yeast cake have been studied by Korean researchers who have identified differences in the composition of volatile substances such as methanol, high alcohol, and esters in the finished alcohol [4]. A similar conclusion was also published by scientists from China [5]. Increasing the recovery efficiency was performed by Cambodian and Philippine experts through improving cooking, fermentation, and distillation techniques [6].

The purpose of this research was to isolate and identify yeast and filamentous fungi strains in Hai Hau alcohol yeast cake, to determine the activity of their extracellular enzymes. Experimental results can be used as a scientific database to develop new alcoholic beverages from Hai Hau sticky rice alcohol.

2. Content

2.1. Materials and methods

2.1.1. Materials

The studies were conducted at the Department of Biological Functional Substances, Institute of Biotechnology, Vietnam Academy of Sciences and Technology.

The research object was to use alcohol yeast cake from Hai Hau district, Nam Dinh province for producing traditional alcohol by people themselves. The cake production process includes the steps of grinding rice into flour, mixing rice flour with water and alcohol yeast cake starter, shaping, incubation for propagation, and drying.

The used equipment includes a microscope Nikon YS100 (America), centrifuger Hittich MIKRO 185 (Germany), shacking incubator Wise Cube (China), autoclave Tomy ES 315 (Japan), electronic weigher (Vietnam), counting chamber Neubauer (Germany) and some other tools for analyzing and identifying microorganisms by PCR method.

Basal culture medium YPD agar (yeast peptone dextrose) g/l includes yeast extract 10, peptone 10, glucose 20, agar 20, pH 7.

The amylase activity of the strains was expressed by the difference in clear zone diameter and the diameter of the hole D-d (mm) on agar medium, supplemented with 1% starch. The cellulase activity - supplemented with 1% Carboxyl Methyl Cellulose (CMC) and protease activity to produce casein hydrolytic enzymes - supplemented with 1% casein substrate. The culture medium was sterilized at 121 ℃ for 20 minutes before being poured into a petri plate.

2.1.2. Methods

Isolation of filamentous fungi and yeasts: The sample of 10 g traditional alcohol yeast cake was resuspended into 90 ml of sterilized water and 10-fold serial dilutions were performed (10^{-2} - 10^{-6}). The volume of 100 µL from each diluted sample was spread on yeast peptone dextrose (YPD) agar medium for 7 days at 25 °C. Colonies with different morphotypes were picked on YPD agar medium and then incubated under the same condition.

Macroscopic study: The morphology of yeasts and filamentous fungi and their appearance on YPD medium was examined based on their cultural characteristics (colony form, elevation, margin, appearance, pigmentation, optical property, and texture).

Method for determining microbial colony density (CFU/g): Filamentous fungal colonies were counted directly on a petri plate.

Method for determining yeast cell density (CFU/mL): Determined by using a Neubauer chamber.

Conservation method: Pure strains of the selected strain after identification are preserved in saline water with 30% glycerol supplement, stored at -80 °C.

Methods for determining extracellular enzyme activity: The pure strain was grown in YPD medium and shaken at 150 rpm at 30 °C for 1 day, 2 days, 3 days, and 4 days; a sample of 50 µL of fermentation liquid was aspirated and centrifuged at 5,000 rpm for 15 minutes; the supernatant was tested for extracellular enzyme activity using the agar plate diffusion method.

DNA extraction from pure yeast strains (Kit Pathogen Ultra Nucleic Acid Isolation by Thermo ScientificTM, USA).

Total DNA was extracted according to the method of Kurtzman & Fell (1998) with some suitable modifications for the laboratory [7]. The volume of 1 mL liquid fermentation (24 h for yeast, 72 h for filamentous fungi) was taken into an Eppendorf tube and centrifuged at 10,000 rpm for 5 minutes; the supernatant was removed and the residue was collected. After that, 100 µL TE buffer, and 5 µL RNAase were added and followed by incubation at 37 ℃ for 30 minutes; after adding 500 µL solution 1 (DNA kit) the procedure was continued with incubation at 70 °C for 10 minutes and cooling the obtained sample. After 700 µL chloroform was added and centrifuged at 10,000 rpm for 5 minutes, the volume of 400 µl supernatants was transferred to new eppendrof tubes, containing 800 µL solution (including 80 µL of solution 2 and 720 µL deionization water). The obtained sample was centrifuged at 10,000 rpm for 5 minutes. After the supernatant was removed, the residue was mixed with 150 µL of solution 3 (DNA kit); a volume of 450 µL absolutely anhydrous alcohol was added and the sample was

centrifuged at 10,000 rpm for 10 minutes. DNA pellets were washed with 500 mL of 70% ethanol, and tubes were centrifuged at 10,000 rpm for 15 minutes. The ethanol was discarded, and the pellets were dried at room temperature for 45 - 60 minutes. Finally, the DNA was resuspended in 20 µL of elution buffer and stored at -20 °C until sent for analysis. In order to check DNA extraction, an electrophoresis on agarose gel was performed.

PCR method: For PCR amplification, 1 µL of the genomic DNA was used as a DNA template. A pair of two fungal universal primers for internal transcribed spacer region (ITS) including ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'TCC TCC GCT TAT TGA TAT GC-3') were used for molecular identification.

DNA sequence: The DNA sequences of the potential fungi were compared with reference sequences available in NCBI GenBank (http://www.ncbi.nlm. hih.gov/blast).

Construction classification tree: A phylogenetic tree based on ITS regions was constructed using MEGA7 Software based on Neighbor Joining (NJ) method according to Kimura (1980) using Saitou's and Nei's methods. (1987).

Statistical analysis: The experimental data were processed using the Microsoft Excel 2013 software.

2.2. Results and discussion

2.2.1. Isolation and determination of the density of yeast, filamentous fungi

The results from the isolation process of microorganisms in Hai Hau traditional alcohol yeast cake samples on YPD medium supplemented with Tetracycline antibiotic (50 mg/L) and YPD without antibiotic were shown in Figure 1, the cell density of yeast and filamentous fungi was presented in Table 1.

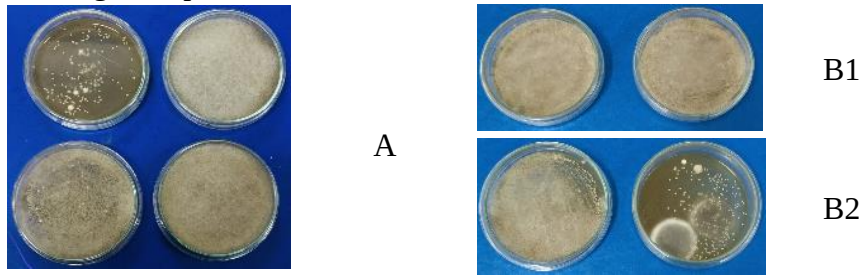


Figure 1. Morphological characteristics of strains isolated and grown in the A: YPD with Tetracycline antibiotic; B1 and B2: Antibiotic-free YPD medium

For the alcohol yeast cake samples on the YPD plate with antibiotic supplementation, it was shown that yeast cell density, molds began to appear after 3 days of breeding and then grew stronger in later days (Figure 1A). The density of yeast and mold cells after 3 days of breeding was 4.5×10^8 and 2.0×10^6 CFU/g, respectively. For alcohol yeast cake samples isolated on non-antibiotic YPD plates, the yeast density reached 4.9×10^8 and 2.0×10^6 CFU/g after 3 days of breeding, respectively (Table 1), and then grew stronger in subsequent days. (Figures B1 and B2).

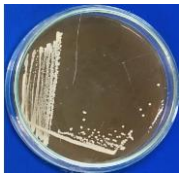
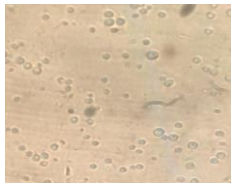
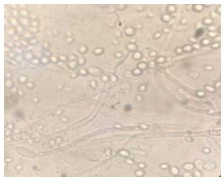
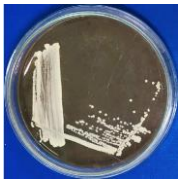
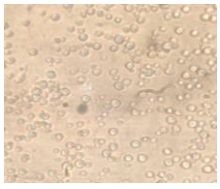
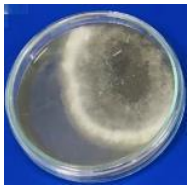
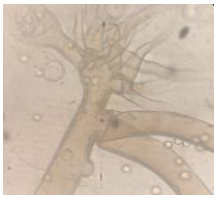
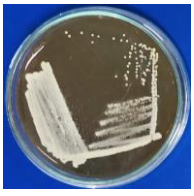
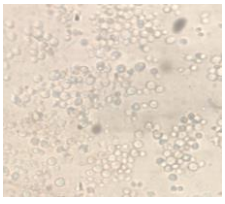
By observing the morphology of colonies on YPD agar medium and cell structure under a microscope 05 characterized colonies were selected, including 04 colonies similar to yeast and 01 colony of filamentous fungi.

Table 1. Density (CFU/g) of yeast, Filamentous fungi isolated from Hai Hau alcohol yeast cake

Microorganism	**Culture medium**	
	YPD (with antibiotics)	*YPD (non-antibiotics)*
Yeast (CFU/g)	4.5×10^8	4.9×10^8
Filamentous fungi (CFU/g)	2.0×10^6	2.0×10^6

The colonies continued to be cultured on YPD agar medium to obtain pure forms and followed a description of obtained morphology characteristics. The results are presented in Table 2.

Table 2. Morphological characteristics of selected strains of yeast, pseudo-yeast, and filamentous fungi

Strain	**The figure of colonies, cells**		**Morphological characteristics**
CN1			The colony was round, milky white, with a characteristic aroma; the cells were spherical, reproducing by budding (Strain yeast *S. cerevisiae* CN1)
NM02			The colonies were round with a characteristic aroma; the cells were spherical and attached to filaments (Strain *Saccharomycopsis* sp. NM02)
CN3			The colonies were small, round, and milky white with characteristic aroma, and the cells were egg-shaped, reproducing by budding (Strain yeast *S. cerevisiae* CN3)
NS04			Grown as filamentous, branching hyphae lacked cross-walls. Colonies were initially white, then darker, and gradually turned black. The sporangiophores were round (Strain *Rhizopus* sp. NS04)
NM3			The colonies were round, had smooth surfaces, were milky white, had a characteristic aroma, and had oval cells, reproducing by budding (Strain yeast *S. cerevisiae* NM3);

According to the morphological description and preliminary classification of Kurtzman & Fell, *Saccharomyces cerevisiae* has vegetative cells as spherical, ovoid, oval, or elliptical, reproducing by budding; colonies are round, and have a smooth surface and a characteristic aroma of yeast. Pseudo-yeast *Saccharomycopsis* has yeast-like cells, and filaments and on some filaments, there are egg-shaped cells. The filamentous fungi *Rhizopus* has aerial hyphae without septa and substrate hyphae that penetrate deeply into the environment. When mature, they form black, filamentous colonies with many spores [7].

From the descriptions of the colonies and cell morphology, it showed that the microbial strains isolated from Hai Hau alcohol yeast cake can belong to the yeast species *Saccharomyces cerevisiae*, pseudo-yeast *Saccharomycopsis* sp., and the filamentous fungi *Rhizopus* sp.

2.2.2. Determination of extracellular enzyme activity of the isolated strains

The ability of the yeasts and filamentous fungi to produce extracellular enzymes such as cellulases, proteases, and amylases was evaluated by the D-d (mm) on agar medium with corresponding substrates such as CMC, casein, and starch.

The tested results for extracellular enzyme activity using the agar plate diffusion method are presented in Table 3.

Table 3. Extracellular enzyme production ability of isolated strains

Strains	**Substrate**	**Diameter D-d (mm) of enzyme degradation after fermentation time (days)**			
		1	*2*	*3*	*4*
Yeast *Saccharomyces cerevisiae* CN1	CMC	-	-	-	-
	Casein	-	-	-	-
	Starch	-	-	-	-
Pseudo-yeast *Saccharomycopsis* sp. NM02	CMC	11	12	14	16
	Casein	12	14	16	18
	Starch	+[a]	+	++[c]	++
Yeast *Saccharomyces cerevisiae* CN3	CMC	-[b]	-	-	-
	Casein	-	-	-	-
	Starch	-	-	-	-
Filamentous fungi *Rhizopus* sp. NS04	CMC	28	29	31	33
	Casein	29	30	32	39
	Starch	+[a]	+	++	++
Yeast *Saccharomyces cerevisiae* NM3	CMC	-	-	-	-
	Casein	-	-	-	-
	Starch	-	-	-	-

[a]Activity: 9 < D <10 mm; [b] Non-activity: D ≤ 9 mm; [c]Activity: 10 ≤ D <11 mm.

The results obtained from Table 3 show that the yeast strains with sample symbols CN1, CN3, and NM5 were not capable of producing extracellular enzymes, while the pseudo-yeast strain (symbol NM02) had produced cellulases, proteases, and amylases with differences of diameters D-d after one day of fermentation being 11, 12 and 10 mm, respectively. The filamentous fungi strain (symbol NS04) in addition to the ability to synthesize amylase also could produce cellulase and protease with strong activity; the difference of diameters D-d after 1 day of fermentation reached 28 and 29 mm, respectively, and after 4 days reached 33 and 39 mm. The results presented in Table 3 showed that fermentation time had an important influence on the ability of pseudo-yeast and filamentous fungi strains to produce extracellular enzymes.

2.2.3. Identification of selected microorganisms by PCR method

Filamentous fungal strain (NS04) and yeast pseudo-yeast (NM02) were identified by DNA sequencing of the ITS region (1-4). The obtained results are shown in Figure 2 and Figure 4. The received sequence was analyzed and compared with known sequences on GenBank and built a phylogenetic tree (Figure 3 and Figure 5).

GAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATC
ATTAACTAATTGTATTGGCACTTTACTGGGATTTACTTCTCAGTATTGTTTGCTTCTAT
ACTGTGAACCTCTGGCGATGAAGGTCGTAACTGACCTTCGGGAGAGACTCAGGACA
TATAGGCTATAATGGGTAGGCCTGTTCTGGGGTTTGATCGATGCCAATCAGGATTAC
CTTTCTTCCTTTGGGAAGGAAGGTGCCTGGTACCCTTTACCATATACCATGAATTCAG
AATTGAAAGTATAATATAATAACAACTTTTAACAATGGATCTCTTGGTTCTCGCATC
GATGAAGAACGTAGCAAAGTGCGATAACTAGTGTGAATTGCATATTCGTGAATCAT
CGAGTCTTTGAACGCAGCTTGCACTCTATGGATCTTCTATAGAGTACGCTTGCTTCAG
TATCATAACCAACCCACACATAAAATTTATTTTATGTGGTGATGGACAAGCTCGGTT
AAATTTAATTATTATACCGATTGTCTAAAATACAGCCTCTTTGTAATTTTCATTAAAT
TACGAACTACCTAGCCATCGTGCTTTTTTGGTCCAACCAAAAAACATATAATCTAGG
GGTTCTGCTAGCCAGCAGATATTTTAATGATCTTTAACTATGATCTGAAGTCAAGTG
GGACTACCCGCTGAACTTAAGCATATCAATA. NS04

Figure 2. ITS sequence of regions 1-4 of strain NS 04

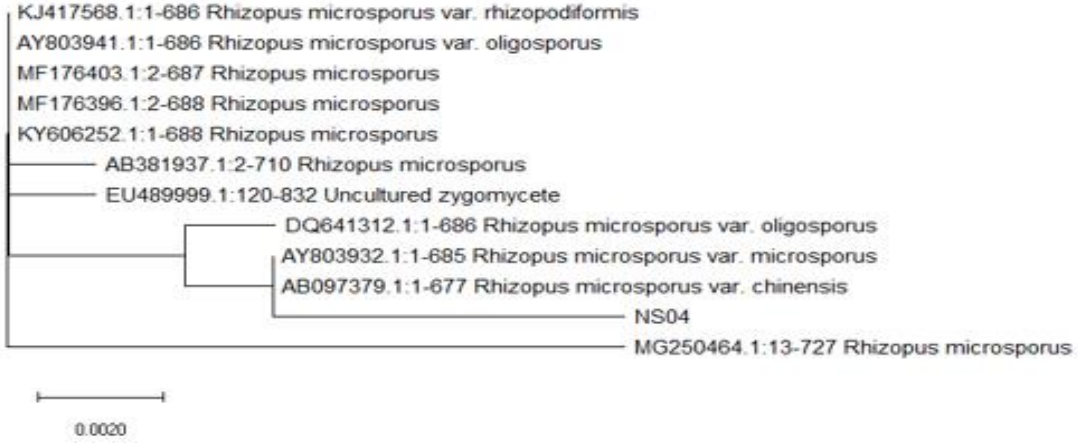


Figure 3. A phylogenetic tree of isolates of Rhizopus microsporus (NS04) constructed with the dataset based on ITS gene sequences

The strain NS04 formed a close relationship with strains of *Rhizopus microsporus* with high statistical support, while other species of *Rhizopus* were placed in the lower clades (Figure 3); therefore isolated strain (NS04) was identified as *Rhizopus microsporus* NS04.

TTTTTTAATTACAACTAGTCGATTTTACAAACTAAAAGTTTAAAACTTTCAGCA
ACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAATTGCGATAAGTAATGT
GAATTGCAGATTTTCGTGAATCATCGAATCTTTGAACGCATATTGCGCTCTATAGTAT
TCTATAGAGCATGCCTGTTTGAGCGTCATTTCTCTCTTAAACCTTTGGGTTTAGTATT
GAAGGTTGTGTTAGCTTCTGTTAACTCCTTTGAAATGACTTGGCAATTGATTGAGTTT
TCCATATATTTGCTTAAGGATTTAATATTAGGTTCTACCAACTTATTAAATACCCTTT
TGCGAAGGACTTACTCCTGTATCAAGGCCTTATAACCTGTCATTA. NM02

Figure 4. ITS sequence of regions 1-4 of strain NM02

Figure 5. A phylogenetic tree of isolates of Saccharomycopsis fibuligera (NM02) was constructed with the dataset based on ITS gene sequences

The strain NM02 formed a close relationship with strains of S*accharomycopsis fibuligera* with high statistical support, while other species of *Saccharomycopsis* were placed in the lower clades (Figure 5), therefore isolated strain (NM02) were identified as S*accharomycopsis fibuligera* NM02.

The results of isolating and identifying microorganisms in Hai Hau traditional alcohol yeast cake showed differences in species compared to the research results by Hanoi Technical Institute for Beer - Alcohol - Beverage on traditional alcohol yeast cake in Ha Giang province, in which the mold species *Aspergillus oryzae, A. niger*, *Mucor* sp., and the yeast *S. cerevisiae* have been identified [8]. The microflora in Mai Ha district, Hoa Binh province alcohol yeast cake was quite diverse with three main groups: molds *Rhizopus*, *Mucor*, and *A. oryzae*, yeast *S. cerevisiae* and pseudo-yeast *Saccharomycopsis* [9].

Research results showed that the species of yeast, pseudo-yeast, and filamentous fungi that were isolated in Hai Hau alcohol yeast cake were overlapped and different from traditional alcohol yeast cake of other localities such as Ha Giang, and Hoa Binh. This can help hypothesize that the different microbial strains in traditional alcohol yeast cake are decisive for the sensory properties, chemical composition, and characteristic flavor of Hai Hau alcoholic drink.

3. Conclusions

With traditional analytical methods, by cultivating yeast and filamentous fungi on YPD agar medium, isolating, and observing colony morphology and the cells under a microscope, combined with techniques using ITS region DNA sequencing and building a classification tree, the filamentous fungal strain *Rhizopus microsporus* NS04 and the pseudo-yeast strain *Saccharomycopsis fibuligera* NM02 in Hai Hau alcohol yeast cake were identified to species. In Hai Hau alcohol yeast cake there are also 03 yeast strains *Saccharomyces cerevisiae* CN1, CN3, and NM03 which were classified on colony and cell morphology, reproducing by budding method. The density of yeast cultured on YPD agar medium supplemented with 50 mg/l of Tetracycline was 4.5×10^8 CFU/g, and the density of filamentous fungi was 2.0×10^6 CFU/g. The yeast strains *Saccharomyces cerevisiae* CN1, CN3, and NM03 were not capable of producing extracellular enzymes. However, the pseudo-yeast strain *Saccharomycopsis fibuligera* NM02 was capable of producing enzymes that hydrolyze starch, cellulose, and casein after 4 days of fermentation. The filamentous fungal strain *Rhizopus microsporus* NS04 was capable of producing highly active extracellular enzymes such as amylase, cellulose, and protease with differences of diameters D-d of starch, cellulose, and casein of > 9; 33 and 39 mm, respectively. Obtained experimental results can be used as a scientific database to develop new alcoholic beverages like whisky from sticky rice.

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