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Isolation and Characterization of Yeast Strains from Spontaneously Fermented Tofu Whey

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Abstract

In traditional tofu production in Vietnam, the coagulant used is fermented tofu whey, which is the liquid separated after the coagulation of soybean proteins and is left to ferment spontaneously. There is currently limited research on the microorganisms involved in this fermentation process. This study focuses on the isolation and characterization of yeast strains from fermented tofu whey samples collected from Mơ village at different times of the year, under varying temperature and humidity conditions. Three yeast strains (Y12.1, TA18, and TA32) were selected based on preliminary sensory evaluations for further investigation. These strains were characterized to build a basic profile, including colony morphology, cell structure, and sugar utilization. Using ITS region sequencing for molecular identification, TA18, TA32 and Y12.1 were identified as Pichia kudriavzevii, Candida tropicalis and Kluyveromyces marxianus respectively. The fermentation activities of these strains in tofu whey, including pH, acidity, yeast count, and analyses of organic acids were studied. Strain TA18 demonstrated promising fermentation and sensory characteristics. The data can be used for further studies aimed at developing microbial preparations for tofu production.

Keywords: Fermented tofu whey, yeast isolation, *Pichia kudriavzevii*, *Candida tropicalis*, *Kluyveromyces marxianus.*

1. Introduction

Tofu has a history spanning over 2000 years, originating in China and later becoming popular as a traditional dish in many Southeast Asian countries, including Vietnam [1] Tofu is one of the best sources of plant-based protein. In addition to protein, tofu contains lipids, carbohydrates, dietary fiber, isoflavones, minerals, and saponins, which can help reduce cholesterol, alleviate cardiovascular and kidney disease symptoms, and decrease the incidence of cancer and tumors [2-3].

In Vietnam, tofu is produced on both small (household) and large (industrial production line) scales. Mơ tofu is a traditional type of tofu coagulated using fermented tofu whey, known for its distinctive flavor, and produced in two traditional craft villages, Mai Động and Mơ Táo, located in Hoàng Mai District, Hanoi. In typical tofu production, soybean milk is coagulated with salts, acids, or enzymes, either individually or in combination, to create a protein gel matrix [4]. The preparation of bittern tofu using magnesium chloride ($MgCl_2$) results in an excessively firm texture, and the use of $MgCl_2$ is detrimental to health. Tofu coagulated with calcium sulfate ($CaSO_4$) exhibits an unpalatable taste, characterized by a distinct beany flavor and bitter aftertaste [1]. Using glucono-delta-lactone (GDL) produces tofu with a soft texture but a sour taste. Fermented tofu whey is a coagulant in tofu with good sensory qualities, a delicious flavor, a slightly sweet taste, and good texture; however, controlling the quality is challenging due to the variability in the microbial fermentation process [1, 5]

Several studies investigated the microbial diversity in fermented tofu whey, highlighting the dominance of lactic acid bacteria (LAB) and the presence of other microorganisms, including yeast, in smaller proportions [1, 5-6]. Nguyen Quang Duc's 2022 study also identified lactic acid as the predominant component and isolated the strain *Lactobacillus fermentum* from Mo tofu whey [7]. Research on fermenting tofu whey primarily focused on lactic acid bacteria due to their crucial role in lowering pH, leading to protein coagulation [1].

Meanwhile, the role of yeast in tofu has received less attention, despite evidence that certain yeast species can enhance flavor. For example, *Pichia amenthonina*, isolated from fermented tofu whey, has been shown to reduce beany off-flavors and contribute favorable aromas during the fermentation of tofu whey and soy yogurt [8]. Additionally, *Candida* has

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demonstrated positive effects on the sensory quality of soy sauce fermented with tofu whey as a substrate [9].

This study focuses on the isolation and characterization of yeast strains isolated from fermented tofu whey, which is the coagulant used in Mơ village tofu. The fermentation activities of these strains in tofu whey were investigated, including measurements of pH, acidity, colony-forming units (CFU), and analysis of organic acids. The data can be utilized for subsequent research focused on creating microbial formulations for the production of tofu.

2. Material and Method

2.1. Sample Collection

Samples of fermented tofu whey were collected from Mơ Village. Each sample was placed in sterile containers, transported to the laboratory on ice and stored in a refrigerator at 4 °C for isolation of yeasts. Sampling was conducted across different temperature ranges (14-19 °C, 20-24 °C, 25-29 °C, and 30-34 °C) to capture the diversity of yeast strains present under varying temperature conditions.

2.2. Isolation and Enumeration

Yeast enumeration was performed by serially diluting each sample in sterile saline solution (0.85% NaCl) and plating 100 µL of each dilution on Yeast extract Peptone Dextrose (YPD) agar plates (1% yeast extract, 2% peptone, 2% D-glucose, and 2% agar). Plates were incubated at 30 °C for 48-72 hours. Colony-forming units (CFU) were counted, and the average CFU/mL was calculated for each temperature range. Representative colonies displaying distinct morphologies were selected for further isolation. Pure cultures were obtained by streaking single colonies on fresh YPD agar plates.

2.3. Assessment of Carbohydrate Utilization

The carbohydrate utilization profiles of isolated yeast strains were determined using the API 20 C AUX strip (bioMérieux). Pure yeast cultures were prepared in a saline suspension to match the turbidity of a McFarland standard 2.0. The suspension was inoculated into the wells of the API 20C AUX strip, each containing a different carbohydrate substrate. The strips were incubated at 30 °C, and results were read after 48 and 72 hours. A color change in the wells indicated positive utilization of the corresponding carbohydrate, and the profiles were recorded for each yeast strain.

2.4. Identification of Yeast

The yeast strains were identified through molecular techniques [10]. Briefly, yeast cells were harvested from overnight cultures and resuspended in TE buffer, vortexed, and treated with lysozyme at 37 °C for 60 minutes. SDS and proteinase K were added for cell lysis. DNA was purified by phenol-chloroform extraction and ethanol precipitation. The internal transcribed spacer (ITS) region was amplified by Polymerase chain reaction (PCR) using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The PCR products were analyzed by agarose gel electrophoresis to verify size and quality. Subsequently, the PCR products were sent for sequencing by Apical Scientific (Malaysia). The obtained sequences were compared to reference sequences in the NCBI GenBank database using the BLAST tool to identify the yeast species.

2.5. Growth Curve of Yeasts during Fermentation

Fermentation experiments were conducted in 500 mL Erlenmeyer flasks containing 400 mL of tofu whey. Each flask was inoculated with a yeast strain at an initial concentration of 2×10^3 CFU/mL The flasks were incubated at 30 °C in an incubated shaker at 150 rpm. Samples (1 mL) were taken at 0, 4, 8, 12, 16, 20, and 24 hours to monitor yeast growth. Yeast growth was monitored by measuring colony-forming units (CFU) per milliliter. Each sample was serially diluted, plated on YPD agar, and incubated at 28 °C for 48 hours to determine CFU/mL.

2.6. Determination of pH and Acid Production

The pH of the fermentation medium was measured at 0, 4, 8, 12, 16, 20, and 24 hours using a calibrated pH meter. The amount of acid produced was determined by titrating 10 mL of the fermentation broth with 0.1 N NaOH until a persistent pink coloration. The acid concentration was calculated as acetic acid equivalent using the following formula:

$$\frac{V\times N\times M}{V_s}\ \text{(g/L)} \qquad (1)$$

where:

V: volume of NaOH used in titration (ml).

N: normality of NaOH (N).

V_s: volume of the fermentation broth sample (mL).

M: molecular weight of acetic acid (g/mol).

2.7. Determination of Organic Acids using High-Performance Liquid Chromatography

The determination of organic acids was performed using high-performance liquid chromatography (HPLC) with an Agilent 1200 system equipped with a refractive index detector (RID). The separation was achieved on an Aminex HPX-87H column (Bio-Rad, Hercules, CA). The mobile phase consisted of 10 mM sulfuric acid (H_2SO_4), delivered at a flow rate of 0.5 mL/min. The column temperature was maintained at 60 °C. Each sample analysis was completed within a run time of 30 minutes. For quantification, standard solutions of lactic acid, butyric

acid, and acetic acid were prepared and used for calibration.

2.8. Data Analysis

All experiments were conducted in triplicate. Data were expressed as mean plus/minus standard deviation.

3. Result and Discussion

3.1. Isolation and Diversity of Yeast Strains in Fermented Mơ Tofu Whey

A total of seven spontaneously fermented samples at different temperature ranges (14-19 °C, 20-24 °C, 25-29 °C, and 30-34 °C) were spread on YPD medium to identify the yeast type of colonies diversity in fermented tofu whey. As shown in Table 1, at temperatures between 14-19 °C, two samples were analyzed, yielding an average yeast count of 1.85×10^3 CFU/ml with 1-2 yeast types of colonies identified per sample. In the 20-24 °C range, one sample was examined, showing a lower average yeast count of 8.0×10^2 CFU/ml, with only one yeast type of colony identified. In the 25-29 °C range, three samples were tested, resulting in an average yeast count of 7.2×10^3 CFU/ml, with 1-2 yeast types of colonies identified per sample. The highest temperature range of 30-34 °C included one sample with the highest yeast count of 4.99×10^4 CFU/ml and the highest number of yeast types, with four distinct types of colonies identified in the sample.

These findings suggest that higher temperatures can enhance both the abundance and diversity of yeast during fermentation. Yiqiang Dai *et al.* (2023) [11] demonstrated that the fermentation temperature of tofu whey significantly impacts the bacterial community, diversity, physicochemical properties, and flavor compounds, ultimately affecting tofu quality. Their study found that tofu whey fermented at natural temperatures (25-37 °C) and higher temperatures (37 °C) exhibited better sensory score compared to whey fermented at lower temperatures (25 °C). Therefore, the increased yeast activity at higher temperatures likely contributes to improved tofu quality, particularly through flavor development, as yeast plays a crucial role in producing organic acids and aromatic compounds during the fermentation of tofu whey.

The isolated yeast colonies were classified into groups based on colony morphology, cell morphology, and reproduction method (Fig. 1 and Table 2). Among these, groups originating from two or more samples were selected for fermentation characterization in tofu whey, with one representative strain chosen from each group. Three representative yeast strains from three groups were Y12.1, TA32, and TA18.

Table 1. Yeast isolation and diversity in fermented Mơ tofu whey at different temperature ranges

Temperature ranges (°C)	Number of samples analyzed	Average of yeast counts (CFU/ml)	Number of yeast groups identified	Number of yeast groups per sample
14-19	2	1.85×10^3	2	1-2
20-24	1	8×10^3	1	1
25-29	3	7.2×10^3	3	1-2
30 -34	1	4.99×10^4	4	4

Table 2. Morphological and reproductive characteristics of representative yeast strains

Representative strains	Morphology		Reproduction type	Average count of the group (CFU/ml)
	Colony	Cell		
Y12.1	Circular, raised, shiny, milky white colony	Oval	Monopolar budding	1.23×10^4
TA32	Irregular edges, wrinkled surface, white	Oval, with pseudohyphae	Monopolar budding	7.77×10^3
TA18	Circular, matte surface, slightly raised center, white	Elliptical	monopolar or bipolar budding	1.51×10^4

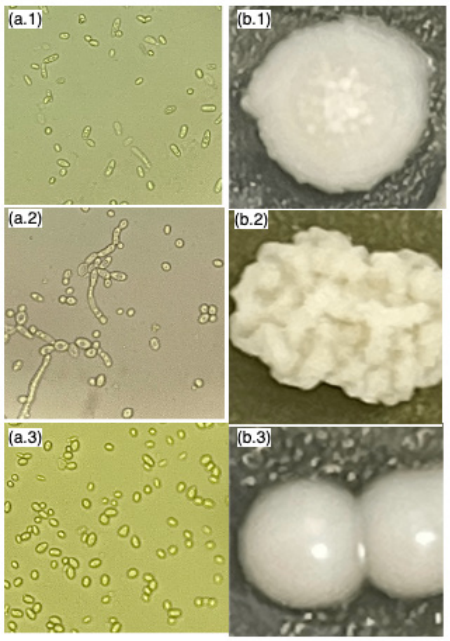

Fig. 1. Microscopic characteristics (a) and colony morphology (b) of three representative yeast strains (1: TA18, 2: TA32, and 3: Y12.1)

3.2. Characterization of Selected Yeast Strains

3.2.1. Carbohydrate utilization profiles

Three representative yeast strains from three groups were tested for their ability to ferment various carbohydrates.

The results (Table 3) suggested that strains TA32 and TA18 had extensive capabilities in utilizing carbohydrates, including disaccharides and trisaccharides such as Methyl-αD-Glucopyranoside, N-Acetyl-Glucosamine, D-cellobiose, D-maltose, and D-saccharose. This indicated that these strains had great potential for use in fermentation processes. In addition, these two strains had the ability to undergo fermentation of D-raffinose and saccharose, which were the main oligosaccharides found in soymilk [12-13]. According to a study of Sanyal and Bishi in 2021, consuming soybean products can lead to flatulence in humans because of the challenging digestion of raffinose [9].

Table 3. Sugar utilization profiles of yeast strains Y12.1, TA32, and TA18

Sugar utilization	Y12.1	TA32	TA18
Glucose	+	+	+
Glycerol	+	-	+
Arabinose	-	-	+
Xylose	-	+	-
Adonitol	+	+	+
Xylitol	-	+	-
D-galactose	+	+	-
Inositol	+	+	+
D-Sorbitol	+	+	+
Methyl-αD-Glucopyranoside	-	+	+
N-Acetyl-Glucosamine	-	+	+
D-cellobiose	-	+	+
D-lactose	-	+	+
D-maltose	-	+	+
D-saccharose	-	+	+
D-trehalose	-	+	+
D-melezitose	-	+	-
D-raffinose	-	+	+

Hence, the capacity of these two strains to metabolize raffinose has the potential to alleviate this problem, thereby enhancing the overall quality of the end product. On the other hand, strain Y12.1 was limited to fermenting only monosaccharides like glucose and D-galactose.

Table 4. Yeast strain identification and accuracy

Strain	Identification	Query cover	E value	%Identity
TA18	*Pichia kudriavzevii*	100%	0.0	99.78%
TA32	*Candida tropicalis*	100%	0.0	100%
Y12.1	*Kluyveromyces marxianus*	100%	0.0	99.8%

3.2.2. Identification of selected yeast strains

Yeast DNA was extracted, and the ITS region of the rDNA was amplified and sequenced as described in section 2.4. Comparison of the ITS sequences obtained with data from GenBank revealed that strains TA18, TA32 and Y12.1 belonged to *Pichia kudriavzevii*, *Candida tropicalis, and Kluyveromyces marxianus* (Table 4). Overall, *Kluyveromyces*, *Saccharomyces*, *Pichia*, and *Candida* are commonly used in the production of fermented dairy and non-dairy beverages worldwide [14]. It has been reported that *Kluyveromyces marxianus*, a food-grade yeast, is capable of producing aroma compounds [15]. *Pichia* and *Candida* are considered the most common genera in fermented soy sauce products using soy whey as a substrate [9] . This study also concluded that *Candida* positively influenced the sensory quality of soy sauce based on volatile compounds analysis. Another study by Yongteo Fei *et al*, 2018 [5] analyzed the microbial composition in spontaneously fermented tofu whey (used as a traditional tofu coagulant in China), showing that the most common yeast species were *Pichia* (0.9%) and *Naganishia* (2.78%). Research by Li, 2016 also isolated *Pichia amenthonina* from fermented tofu whey and demonstrated that this strain could produce aroma and eliminate the beany flavor when fermenting tofu whey and soy yogurt [8] Therefore, both strains TA18 and TA32 may play a significant role in improving the flavor of tofu whey and tofu coagulated from that whey.

Pichia kudriavzevii has been widely utilized in the food industry due to its ability to produce valuable metabolites, and it has been recognized as safe for use in certain food application [16-17]. This species has recently been classified as Generally Recognized As Safe (GRAS) and given Qualified Presumption of Safety (QPS) status, further supporting its increasing use in various food products [16]. A study by Yadav *et al* [18] also indicates that many *P.kudriavzevii* strains may be nonpathogenic because of their presence in several fermented food products. On the other hand, *Candida tropicalis* presents a higher safety risk, as it is more commonly implicated in human infections and is considered an opportunistic pathogen [19-20]. The use of *Candida tropicalis* in food production is much more limited, and its pathogenicity warrants careful consideration and strict safety assessments before any application in the food industry [19]. In our study, a preliminary safety assessment was conducted for the three yeast strains, including gelatinase, hemolysis, and coagulase tests. The results indicated that all strains were negative for these enzymes (data not shown); however, further testing is needed to comprehensively evaluate the safety of these strains. Additionally, as noted by Nguyen Quang Duc [21], heated soybean milk is typically coagulated with fermented tofu whey at 80-95 ^{o}C. This heating step likely eliminates viable yeast cells in the whey, ensuring the safety of the final tofu product.

3.3. Fermentation Process Analysis

3.3.1. Microbial count

The population of the three yeast strains in monoculture fermentation increased from approximately 3 to 8 log CFU/ml within 24 hours (Fig. 2). In contrast, natural fermentation of whey showed a yeast count of approximately 3-4 log CFU/ml after 24 hours (Table 2). This indicates that the growth of yeast during natural fermentation was restrained, possibly due to the complex interactions among various microorganisms involved in natural fermentation, which may lead to unstable quality of the fermented whey**.**

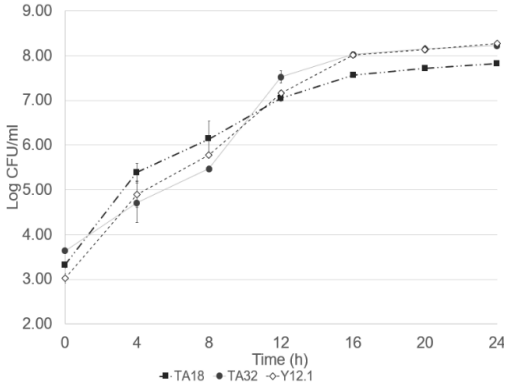

Fig. 2. Growth curve of three representative yeast strains in 24 hours of fermentation

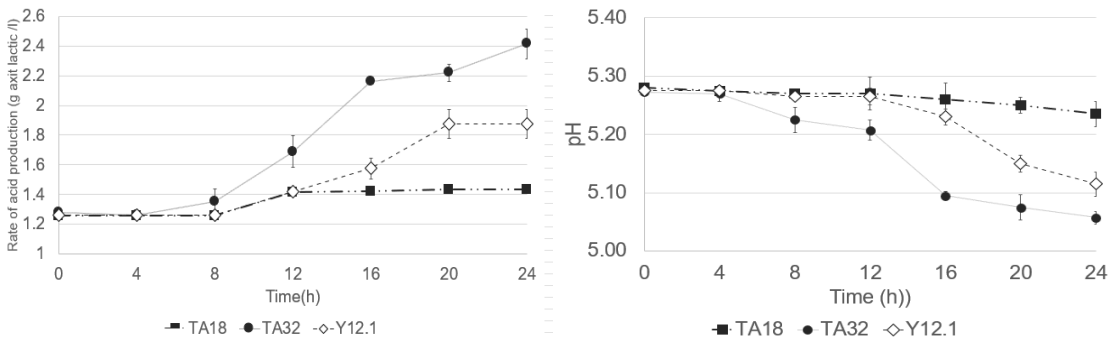

Fig. 3. Changes in acid production and pH values during fermentation of three yeast strains in tofu whey

Table 5. Organic acid components in fermented tofu whey using different yeasts.

Organic acid components	Fermenting yeast			
	Control (unfermented whey)	Y12.1	TA32	TA18
Lactic acid (g/l)	0.91	1.33	0.98	0.96
Acetic acid (g/l)	0.013	0.075	-	-
Butyric acid (g/l)	0.001	0.006	0.006	0.004

3.3.2. Determination of pH and acid production

The changes in pH and acid production during fermentation are shown in Fig. 3. None of the three yeast strains were highly effective in reducing the pH or increasing the acidity of the whey within 24 hours. Specifically, the pH of whey fermented by strain TA18 only decreased from 5.28 to 5.24. Strains TA32 and Y12.1 reduced the whey pH to 5.06 and 5.12, respectively. According to the study of Duc Nguyen Quang, the optimal pH for coagulating tofu in fermented whey is 4.1-4.4, with lactic acid bacteria playing an important role in reducing the pH during fermentation [21]. Therefore, using a monoculture of yeast may not be effective in producing tofu whey suitable for coagulating tofu. However, the natural fermentation process of Mơ Village tofu whey involves a diverse microbial community. Research by Yongtao Fei has analyzed the microbial composition in fermented tofu whey and suggested that yeast plays a crucial role in developing the aroma of traditional tofu [5]. Therefore, co-fermentation of yeast and lactic acid bacteria in tofu whey may improve the sensory quality of the tofu**.**

3.3.3. Organic acid profile

The study by Nguyen Quang Duc [21] identified three major acids in fermented tofu whey: lactic acid, acetic acid, and butyric acid, with lactic acid playing a dominant role [7]. In this study, the content of the three organic acids in tofu whey samples fermented by three strains was presented in Table 5. Lactic acid and acetic acid are the first and second most abundant acids in spontaneously fermented tofu whey, respectively, and therefore play crucial roles in the flavor and taste of traditional tofu. Luo *et al.* [22] demonstrated that acetic acid imparts a pungent sour taste, while lactic acid has a milder acidity. Tofu coagulated with whey containing high concentrations of acetic acid received the lowest sensory scores among the samples evaluated in the study by Yiqiang Dai *et al.*[11]. Butyric acid, while important for flavor in low concentrations, can produce an unpleasant odor if present in higher amounts, and it is recommended to maintain low levels during fermentation [23]. Strain Y12.1 was identified as *Kluyveromyces marxianus* in section 3.2.2. *K. marxianus* has been reported to produce both lactic acid and acetic acid during fermentation [24-26], which is consistent with the findings of this study (Table 5). After 24 hours of fermenting tofu whey with strain Y12.1, the lactic acid concentration increased from 0.91 g/L to 1.33 g/L, while the acetic acid concentration rose from 0.013 g/L to 0.075 g/L. Additionally, the results suggest minimal changes in lactic acid and butyric acid levels during the fermentation process with strains TA18 and TA32. However, acetic acid was not detected in whey fermented by TA18 and TA32, indicating that these strains may have the capacity to metabolize acetic acid. This observation is consistent with previous studies that reported *Pichia kudriavzevii* and *Candida tropicalis* possess the ability to consume acetic acid [27-28]. A preliminary sensory evaluation was conducted to assess the flavor of fermented tofu whey samples inoculated with three different yeast strains. Initial findings indicated that strain TA18 produced the most pleasant fruity flavor (data not shown), suggesting its potential application in tofu production to enhance the sensory attributes of the final product. However, detailed analysis of volatile compounds and further sensory evaluations are necessary to fully understand the impact of different yeast strains on flavor development.

4. Conclusion

This study successfully isolated and characterized yeast strains from fermented tofu whey used in the traditional Mơ tofu production process in Vietnam. Three yeast strains, Y12.1, TA18, and TA32, were investigated for their fermentation activities. Molecular identification confirmed that strains TA18, TA32 and Y12.1 were *Pichia kudriavzevii Candida tropicalis* and *Kluyveromyces marxianus* respectively. The fermentation experiments revealed that higher temperatures promoted greater yeast diversity, with the highest yeast counts and diversity observed at 30-34°C. The carbohydrate utilization profiles indicated that TA18 and TA32 had extensive capabilities in fermenting various sugars, which could potentially enhance the flavor of tofu whey by metabolizing oligosaccharides like raffinose. In

addition, strain Y12.1 could increase lactic acid concentrations during fermentation. The findings of this study provide a foundation for future research aimed at developing microbial preparations for tofu production, contributing to the improvement of traditional tofu-making practices in Vietnam. This is acknowledged that further safety assessment for potential strains used as starter culture in tofu whey fermentation needs to be conducted.

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