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Isolation, Characterization and Identification of Yeast in Pindang Eggs as a Starter for Fermented Chicken Eggs

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ABSTRACT

Background: The yeast in pindang eggs is indigenous. Isolation, characterization and identification of yeast in boiled eggs were carried out to determine the genus of yeast. The yeast has the potential to be a starter for fermented chicken eggs. This study aims to determine the characteristics of the yeast genus that has been isolated from boiled eggs and to determine the genus and species of yeast that have the best ability as a starter based on pH, total yeast count and reducing sugar content in fermented chicken eggs.

Methods: This study was carried out by isolating, identifying, characterizing the yeast in pindang eggs and then carrying out chicken eggs fermentation by adding yeast with the ability to ferment sugar in chicken eggs.

Result: The results show that pindang eggs are thought to contain the genera Candida, Hyphopichia and Pichia. These three yeasts have the ability to ferment sugar, making them suitable as starters for fermented chicken eggs. *Pichia kudriavzevii* is the best starter for fermented chicken eggs, producing the lowest pH (5.830) and the lowest reducing sugar content (0.058%) with low total yeast count (4.7×10^4 CFU/mL).

Key words: Chicken eggs, Fermented, Pindang eggs, Starter, Yeast.

INTRODUCTION

Pindang eggs are a method of preserving eggs that combines the addition of tannins, salting and boiling. The water content in pindang eggs ranges from 71.1-79.8%, with a pH value of 6.7 (Nusi *et al*., 2020). The boiling process with a tanning agent for pindang eggs cannot completely eliminate the microbes in the eggs, especially yeast. The presence of carbohydrates, neutral pH and high water content in pindang eggs make them conducive to the appearance of yeast. To determine the presence of yeast in pindang eggs, it is necessary to isolate and identify them.

Yeast found in chicken eggs includes *Candida albicans*, *Candida catenulate*, *Candida famata*, *Candida guilliermondii*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida peliculosa*, *Candida zeylanoides*, *Hyphopichia burtonii*, *Rhodotorula rubra*, *Trichosporon asteroides* and *Trichosporon coremiiforme* (Cafarchia *et al*., 2019). Some yeasts are tolerant to tannins. The genus of yeast identified from the ingredients used in making pindang eggs can be considered indigenous. Yeasts with known characteristics and identified as functional yeast have the potential as starters.

Fermented eggs are egg products that have undergone a fermentation process. It can improve the functional properties of emulsification and stabilization in egg products and can be applied to other advanced food preparations (UNECE, 2010). The use of yeast in egg fermentation is a method of eliminating glucose in chicken eggs (Asaithambi *et al*., 2022). The success of yeast in fermenting sugar in fermented eggs is evaluated using total yeast count, pH and reducing sugar content tests. The total number of microbes in chicken eggs is 10^2 to 10^5 CFU/mL (Chousalkar *et al*., 2021). During the fermentation process, the yeast metabolizes and divides, causing the total number of yeast to increase as the starter is added (Rosita *et al*., 2021). pH is an important indicator in the fermentation process. Yeast can only grow in mildly acidic pH range of 4-6. A decrease in pH after fermentation indicates that the starter is growing optimally. The reduction in sugar content occurs due to the utilization of glucose by yeast for metabolism. Yeast can metabolize various sugars, including glucose (Pratama *et al*., 2019).

Therefore, some of the indigenous yeasts in pindang eggs have the potential to act as fermentation starters in fermented eggs. Based on this description, this study was

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carried out to isolate, characterize and identify yeast in pindang eggs as a starter for fermented chicken eggs.

MATERIALS AND METHODS

Research period

This study was conducted during the year 2022-2024 at Laboratory of Livestock Product Processing Technology, Faculty of Animals Husbandry, Universitas Padjadjaran, Indonesia.

Macroscopic and microscopic observation

Macroscopic observation included examining the characteristics recorded include colony shape, texture, color, surface, elevation and edges (Naiman *et al*., 2014). Microscopic observation included examining yeast cells, including cell shape, budding and the presence or absence of hyphae or pseudohyphae and the presence or absence of capsules (Bitew *et al*., 2023).

Capsule staining test

Capsules appear as pale blue circles surrounding purple cells (Kurtzman *et al*., 2011).

Physiological and biochemical properties

a. pH: The pH test was carried out using a pH meter.

b. Sugar Fermentation Test (Prescott and Klein, 2002): The media used in the sugar fermentation test included glucose, sucrose, lactose, maltose and galactose.

c. Urease Test (Kurtzman *et al*., 2011): The media used in the urease test was Agar Base Urea media.

Enzymatic test

a. Proteolytic test: The medium used for the proteolytic test was skim milk agar.

Proteolytic index =

$$\frac{\text{Diameter of clear zone (mm) - Diameter of colony (mm)}}{\text{Diameter of colony (mm)}}$$

b. Amylolytic test: The medium used for the amylolytic test is rice starch agar, prepared by mixing 0.2% soluble starch in MEA media.

c. Amylolytic index =

$$\frac{\text{Diameter of clear zone (mm) - Diameter of colony (mm)}}{\text{Diameter of colony (mm)}}$$

Test yeast's ability as a fermentation starter

a. Total yeast: Yeast was cultured on MEA media up to 10^{-5} using the spread plate method. The plates were incubated for 48 hours at 30°C.

b. Reducing sugar content: The reducing sugar content in fermented chicken eggs was measured using the Nelson-Somogyi method.

Molecular identification of amylolytic yeast

Identification of yeast was carried out by identifying the 18S rRNA gene, with stages including DNA isolation of pure yeast isolates, DNA amplification using Polymerase Chain Reaction (PCR), agarose gel electrophoresis of PCR results, sequencing of the 18S rRNA gene and construction of a phylogenetic tree. In this study, DNA isolation and amplification were carried out simultaneously using a direct PCR kit (KOD FX Neo, Toyobo) following the company's protocol. The PCR machine used was a personal mastercycler from the Eppendorf brand using Universal Primer F: ITS-1 (F) with the sequence TCC GTA GGT GAA CCT GCG and Primer R: ITS-4 with the sequence TCC TCC GCT TAT TGA TAT GC. The PCR product was sent to PT Genetika Science Jakarta for sequencing. The sequencing data was then edited using the BIOedit program and analyzed using the Basic Local Alignment Search Tool (BLASTN).

RESULTS AND DISCUSSION

Macroscopic and microscopic observation

Table 1 shows that yeast isolates tpi 01, tpi 02, tpi 03 and tpi 04 share the same colony morphology characteristics: round shape, butyrous texture, shiny and smooth surface, convex elevation and entire edges. Isolate tpi 05 exhibits irregular shape, friable texture, white color, dull and rough surface, umbonate elevation and filamentous edges. Meanwhile, isolate tpi 06 exhibits irregular shape, membranous texture, cream color, dull surface, flat elevation and undulate edges. Meanwhile, morphology characterization showed that tpi 02, tpi 03 and tpi 04 share the same cell morphology characteristics: round, oval and elliptical shapes with no hyphae or pseudohyphae. Isolates tpi 01 and tpi 05 presence of pseudohyphae and round shape. Isolate tpi 06 exhibiting elliptical and oval shapes with no hyphae or pseudohyphae. The six yeast isolates exhibited multilateral budding and did not form capsules.

Physiological and biochemical properties

Yeast isolate pH

Table 2 shows that the six isolates exhibit varying pH levels, ranging from 4.39 to 5.16. Isolates tpi 02 and tpi 04 exhibited the lowest pH among the isolates, indicating that these isolates produce more acid, leading to the lowest pH compared to other isolates.

Sugar fermentation test

Isolates tpi 02, tpi 03, tpi 04 and tpi 05 showed a strong positive reaction to glucose, sucrose, maltose and galactose but negative reaction to lactose. Isolate tpi 06 only showed a strong positive reaction to glucose (Table 2).

Urease test

The six isolates showed positive results on urea test (Table 2). This suggests that these yeasts can hydrolyze urea in the media.

Enzymatic

Proteolytic assay

Table 3 shows isolate tpi 06 exhibited proteolytic activity with a clear zone of 36 mm. According to (Sabaria *et al*., 2024), an isolate with a PI <1 is considered to have a low PI.

Table 1: Macroscopic and microscopic characteristics of yeast.

Isolate code	Macroscopic characterization							Yeast isolate cell	Shape	Pseudohifa/ Hifa	Budding	Capsule test cell	Capsule
	Colony	Shape	Texture	Color	Surface	Elevation	Margin						
tpi 01		Cocci	*Butyrous*	White	Glistening, smooth	*Convex*	*Entire*		Cocci	Pseudohifa	Multilateral		-
tpi 02		Cocci	*Butyrous*	White	Glistening, smooth	*Convex*	*Entire*		Cocci, oval, elips	-	Multilateral		-
tpi 03		Cocci	*Butyrous*	White	Glistening, smooth	*Convex*	*Entire*		Cocci, oval, elips	-	Multilateral		-
tpi 04		Cocci	*Butyrous*	White	Glistening, smooth	*Convex*	*Entire*		Cocci, oval, elips	-	Multilateral		-
tpi 05		*Irregular*	*Friable*	White	Dull	*Umbonate*	*Fillamentous*		Cocci, oval	Pseudohifa	Multilateral		-
tpi 06		*Irregular*	*Membranous*	Cream	Dull	*Flat*	*Undulate*		Elips	-	Multilateral		-

Amylolytic test

Isolates tpi 02, tpi 03, tpi 04 and tpi 05 exhibited amylolytic activity with amylolytic index (AI) values ranging from 0.22 to 0.96 (Table 4). This clear zone formation occurs through a process where starch reacts with iodine, forming a complex compound that appears dark blue (Sukmawati *et al*., 2021).

Fermented chicken eggs

The selected yeast isolates used as a starter for fermented chicken eggs, tpi 04, tpi 05 and tpi 06, represent three different genera: Candida, Hyphopichia and Pichia. In this study, the activity of yeast as a starter for fermented chicken eggs was observed through the pH of fermented chicken eggs, total yeast count and reducing sugar content.

pH of fermented chicken eggs

The pH value of TA 1 has an average of 5.84 ± 0.08^{a} and a CV value of 1% (Fig 1). TA 2 has an average pH of 6.20 ± 0.35^{b} and a CV of 6%. TA 3 has an average pH of 5.83 ± 0.09^{b} and a CV of 2%.

Total yeast

Fig 2 shows that the total yeast count for TA 1 has an average value of 1.5×10^{5a} CFU/mL with and a CV of 20%. TA 2 has an average value of 1.6×10^{5a} CFU/mL and a CV of 4%. TA 3 has an average value of 4.7×10^{4b} CFU/mL and a CV of 25%.

Reducing sugar content

The reducing sugar content in fermented chicken eggs for TA 1 has an average value of $0.060\%\pm0.0082^{b}$ and a CV of

Table 2: Physiological properties and biochemical activities of yeast.

Isolate code	pH yeast	Sugar fermentation test					Urease test
		Glucose	Sucrose	Maltose	Lactose	Galactose	
tpi 01	5.00	++	+	+	-	++	*Delayed positive*
tpi 02	4.39	++	++	++	-	++	*Delayed positive*
tpi 03	4.45	++	++	++	-	++	*Delayed positive*
tpi 04	4.39	++	++	++	-	++	*Delayed positive*
tpi 05	4.69	++	++	++	-	++	*Delayed positive*
tpi 06	5.16	++	-	-	-	-	*Delayed positive*

Description:+: Positive test result, no gas formation.

++: Positive test result and gas formation.

-: Negative test result.

Table 3: Yeast proteolytic test.

	Proteolytic test			
Isolate code	Clear zone	Diameter of colony (mm)	Diameter of clear zone (mm)	Proteolytic index (IP)
tpi 06		35.6	36	0.01

Table 4: Yeast amylolytic test.

	Amylolytic test			
Isolate code	Clear zone	Diameter of colony (mm)	Diameter Clear zone (mm)	Amylolytic index (IA)
tpi 02		8	15.65	0.96
tpi 03		8	14.00	0.75
tpi 04		9	13.05	0.45
tpi 05		9	11.00	0.22

14% (Fig 3). TA 2 has an average value of 0.068%±0.005[a] and a CV of 7%. TA 3 has an average value of 0.058%±0.005[b] and a CV of 9%.

The results of identification to the yeast genus level in this study are referenced from "The Yeast: A Taxonomic Study" by Kurtzman *et al*. (2011).

Genus candida

Based on the results, the yeast isolates identified as potentially belonging to the genus Candida included tpi 01, tpi 02, tpi 03 and tpi 04. Candida typically exhibit macroscopic colony morphology characterized by white to cream color, round shape, convex elevation and smooth surface. According to Sachivkina *et al*. (2021), Candida cells are generally round to elongated, with or without pseudohyphae and reproduce asexually through multilateral budding. Research conducted by Saikia *et al.* (2024), Candida produced white to creamy, smooth and butyrous colonies and Genus Candida does not have capsules. Yeasts of this genus can typically grow in a pH range of 3-7 (Sherrington *et al*., 2017). They are known to ferment glucose, sucrose, maltose and galactose and have the capability to hydrolyze urea (Nwaguma *et al*., 2019). According to Modrzewska *et al*. (2016), several Candida species do not have proteolytic activity, such as *Candida guilliermondii*, *Candida krusei*, *Candida rugosa* and *Candida lusitaniae*. Furthermore, de Souza *et al*. (2021) also found a Candida species that does not have proteolytic activity. Based on these characteristics, it can be concluded that isolates tpi 01, tpi 02, tpi 03 and tpi 04 exhibit traits consistent with the genus Candida (Balia, 2014).

Genus hyphopichia

Based on the identification results, the yeast isolate tpi 05 exhibited characteristics similar to the genus Hyphopichia. Hyphopichia is identified having a white to pale white color, irregular shape, wrinkled and fibrous surface with raised elevations and filamentous edges. According to Zhang *et al*. (2021), cell morphology of Hyphopichia is described as ovoid with pseudohyphal shapes and these yeasts reproduce asexually through multilateral budding. Genus Hyphopichia can ferment glucose, sucrose, maltose and galactose. Ma *et al*. (2022) carried out the urease and pH tests of yeast in the genus Hyphopichia. The urease test produces a positive result, which is indicated by a color change to pink in the media and the optimal pH for growth is in weak acids, pH 4-7. Genus Hyphopichia can hydrolyze starch, indicating amylolytic activity. Hyphopichia does not exhibit proteolytic activity. Based on the characteristics that have been described, it can be concluded that isolate tpi 05 has similarities to the genus Hyphopichia, a fermentative yeast (Wang *et al*., 2020).

Genus pichia

Based on the identification results, the yeast isolate tpi 06 is likely to belong to the genus Pichia. According to (Abbas *et al*., 2019), Pichia have white to cream color, round to irregular shape, dull surface and flat elevation. Microscopically, the cells are ovoid, elliptical and elongated. Pichia reproduces asexually through multilateral budding and while some species may form pseudohyphae, true hyphae are rare (Shrivastava and Sharma, 2023). A study found that the genus Pichia does not have capsules in its cells. Yeast pH, sugar fermentation tests and urease tests have varying results for each genus. The genus Pichia can grow at pH 2.5-7.5. Only glucose can be fermented by the genus Pichia and shown positif result on urea test

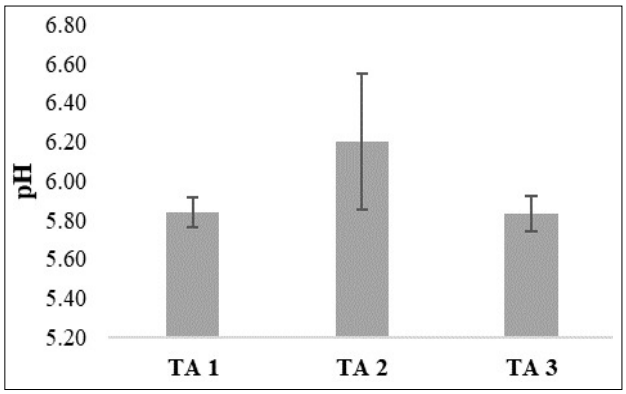

Fig 1: pH value of fermented chicken eggs.

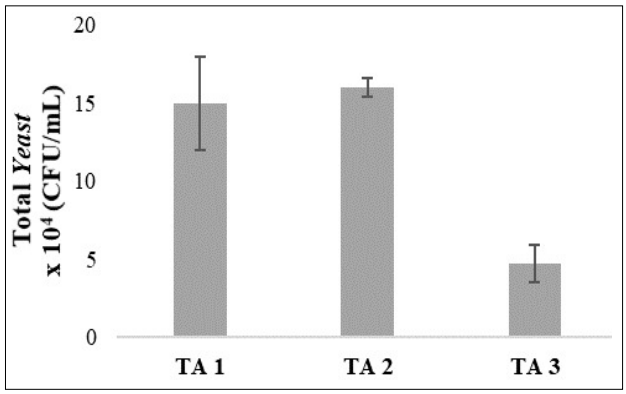

Fig 2: Total yeast count of fermented chicken eggs.

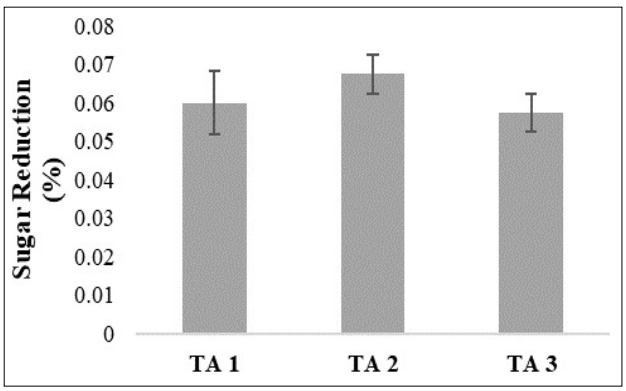

Fig 3: Reducing sugar content of fermented chicken eggs.

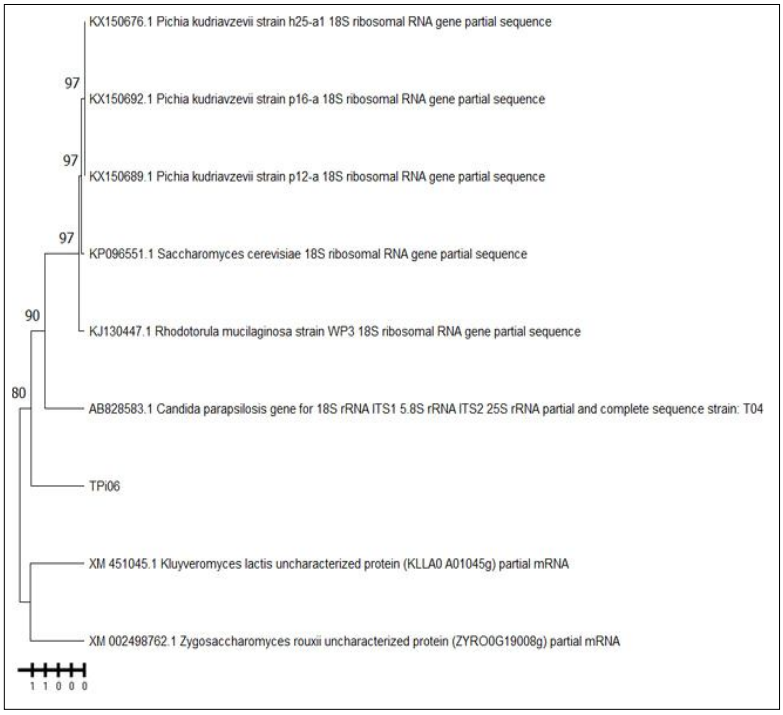

Fig 4: Phylogenic tree.

(Muñoz *et al*., 2022). Based on the characteristics that have been described, it can be concluded that isolate tpi 06 has similarities to the genus Pichia, an oxidative yeast.

pH, sugar reduction and total yeast of fermented chicken eggs

Fig 1 show that the pH values differ among the yeast genera used. For fermented chicken eggs with the genus Candida (TA 1), the average pH is 5.84; with the genus Hyphopichia (TA 2), it is 6.20 and with the genus Pichia (TA 3), it is 5.83. The decrease in pH is caused by yeast fermentation activity. As research conducted by (Jagadeesh *et al.,* 2022) shows, in yeast metabolism there is an increase in pH at the beginning, but along with the fermentation process, the pH value will decrease. The pH of fermented chicken eggs with the addition of the genus Pichia has the lowest value among the other genera. According to Chu *et al*. (2023), Pichia can produce higher levels of organic acids compared to Candida due to its tolerance to low pH and organic acids. Fermented chicken eggs with the addition of the genus Candida have a slightly lower pH than those with the genus Pichia. A study by (Bressani *et al*., 2020) indicates that the genus Candida produces oxalic acid and other organic acids.

Total yeast counts in fermented chicken eggs show that adding yeast starters of different genera results in varying average total yeast levels Fig 2. Candida genus produced an average total yeast count of 1.5×10^5 CFU/mL, Hyphopichia genus produced an average total yeast count of 1.6×10^5 CFU/mL, while Pichia genus produced an average total yeast count of 4.7×10^4 CFU/mL. The genus Pichia has the lowest total yeast count among the genera tested. According to (Vicente *et al*., 2021), this is because Pichia can only ferment glucose, providing limited nutrition for growth. In contrast, the genera Candida and Hyphopichia produced higher total yeast counts compared to Pichia. According to (Bao *et al*., 2021), the genus Pichia can rapidly consume sugar, leading to low reducing sugar content. Research conducted by Nidhilangelo and Antony (2024) on cocoa beans shows that fermentation by yeast can reduce sugar levels up to 4 times the normal value. The coefficient of variation for each genus varies. The coefficient of variation for fermented chicken eggs with the addition of the genus Candida is 14%, for the genus Hyphopichia is 7% and for the genus Pichia is 9% (Fig 3).

Molecular identification of amylolytic yeast

In this study, the yeast genus that has the best ability as a starter in fermented chicken eggs is the genus Pichia. The resulting pH was 5.83, total yeast was 4.7×10^4 and the reducing sugar content was 0.058%. To see the type of species of this genus, molecular identification was conducted. BLAST results with genome data at the National Center for Biotechnology Information (NCBI) show that molecularly the yeast isolate tpi 06 is identified as *Pichia kudriavzevii* with 99.5% homology. The construction of a phylogenetic tree for *Pichia kudriavzevii* is detailed in Fig 4.

CONCLUSION

Yeast that has been isolated from pindang eggs is thought to have similarities with the genus *Candida, Hyphopichia* and *Pichia*. Yeast *Pichia kudriavzevii* has the best ability

as a starter in fermented chicken eggs with the resulting pH of 5.83, total yeast of 4.7×10^4 and reducing sugar content of 0.058%.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

There is no animal used in this study.

Conflict of interest

All authors declare no conflict of interest.

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