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Optimization of Various ITS rDNA Amplification Protocol of Yeast Isolated from Giant Honey Beehives (*Apis dorsata*)

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ABSTRACT

Indonesia is a country with high variability of microorganisms, including bacteria, yeast, and fungi. Yeast isolates could be isolated from the honeycomb of *Apis dorsata*. Molecular approaches were used to identify yeast using ribosomal DNA gene sequences, called the ITS gene. The optimum condition for DNA extractions and amplifications are needed for the successfully of molecular identification. Therefore, it is necessary to optimize the DNA extraction and amplification of several protocols to obtain good identification results. This study aimed to compare the effects of DNA extraction with various temperatures and different amplification protocols. LIPI reference DNA extraction protocol with the boiling method and variations in incubation time of 10, 15, and 20 minutes at a temperature of 98° C. Meanwhile, for the amplification of yeast DNA using a variety of different amplification protocols. The results showed the optimal time of incubation was 10 minutes in K1 isolates with DNA purity of 1.896. meanwhile, for isolates K2, K3, and K4 each with a purity of 2.246, 2.335, and 1.748. optimal DNA amplification results were indicated by the presense of DNA bands for each sample K1, K2, K3, and K4, namely 503, 542, 492, and 526 bp. In this study, it can be concluded that the optimal incubation time for the extraction process is 10 minutes. In addition, the optimal amplification protocol was shown in the DNA bands in all sample.

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INTRODUCTION

Yeast is a eukaryotic unicellular microorganism from the fungi kingdom that lives as a parasite or saprophyte. In Indonesia, yeasts are common microorganism in tropical rain forests. Yeast isolates that have been identified from the estimated number of yeast diversity in the world are still around 1% of the total 89 yeast genera listed in the yeast monograph list. There are 37 genera of yeast or about 42% can be found in Indonesia, hence the potential for exploring yeast diversity is very large. In addition, the climate and landscape in Indonesia are very suitable for the life of microorganisms, including yeast (Jumiyati et al., 2012).

Identification of yeast needs to be done to find new genera or species by exploring various ecosystems in Indonesia and believing that there are still many yeasts in nature that have not been found and their numbers are higher than known yeasts (Asliha et al., 2014). Yeast has been long utilized in various fermented food and beverage productions, such as beer, *tapai*, and wine. Yeast in the fermentation process acts to degrade substrates to formulate the structure, aroma, and texture in food and beverages (Sumerta & Kanti, 2017). Along with

technological advances, yeast utilization expands from the industrial sector to the energy sector that develops renewable energy such as bioethanol and biofuel as alternatives to fossil fuel. Yeast isolates can be extracted from beehives. Beehives are hexagonal cells from wax mixed with propolis sourced from the plants. Beehives are used to contain bees, pollen, and larva (Rospita et al., 2014).

Commonly, the conventional identification based on morphology, i.e., macroscopic and microscopic observations were used for identification. Conventional identification can also be completed by biochemical and physiological tests, including sugar fermentation ability, vitamin requirements, growth temperature, urease test, carbon and nitrogen element assimilation ability, and cycloheximide resistance (Rukmana, 2015; Suryaningsih et al., 2018). However, conventional identification has several disadvantages, e.g., the process takes a long time, and it is susceptible to identification mistakes, particularly in closely related species. In addition, it is due to the limited yeast morphological characters for the identification and the lack of references. Thus, it is crucial to have a method to overcome these drawbacks, i.e., identification using molecular markers to improve the accuracy of yeast identification (Maulana, 2011).

Yeast identification using the molecular approach is currently performed using genetic sequence data. Generally, specific genes used in accurate and rapid yeast identification are ribosomal DNA (rDNA). It includes the internal transcribed spacer (ITS), a non-coding area in the ribosomal DNA. This study employed this area because the ITS has high nucleotide sequence variations between species, identifying closely related yeasts species (Hartati et al., 2021; Maulana, 2011).

Yeast DNA amplification was carried out using the Thermo Cycler equipment with a pair of forward and reverse primers. The PCR process results were visualized using gel documentary to observe DNA band from the PCR product. This successfully process was influenced by yeast DNA extraction and amplification that should be carried out optimally. Hence, it is necessary to optimize a PCR for yeast ITS rDNA amplification isolated from giant honey beehives (*Apis dorsata*). This optimization process expected to acquire an appropriate protocol for yeast ITS rDNA amplification and be beneficial for studies regarding identification using molecular methods. This study proposed to compare the effects of DNA extraction with various temperatures and different amplification protocols.

MATERIALS AND METHODS

This research was conducted from January 2020 - July 2020. The tools used in this study included: Erlenmeyer flask, petri dish, ose needle, electric stove, micropipette, micropipette tips, Microtube, laminar air flow (LAF) cabinet, autoclave, vortex mixer, microsentrifuge, DNA *documentation / transiluminator*, spectrophotometer DNA quantitave analysis [BioDrop], light microscope, thermo cycler [Multigene optimax], electrophoresis.

The materials used in this study included: yeast isolate that was isolated from *Apis dorsata* honey beehives. Samples of beehives were obtained around the Darungan Lumajang Resort, East Java (8°12'27"S 112°56'05"E). The Potato dextrose agar (PDA) was used as a medium to which the antibiotic *chloramphenicol* was added. Yeast DNA extraction using nuclease-free water. Kit PCR GoTaq® Green Master Mix (Promega). Amplification of yeast DNA using ITS 4 (5'-TCCTCCGCTTATTGATGC 3'), and ITS 5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') primer pairs (Maulana, 2011). Buffer TAE, 1 kb DNA Marker, Diamond dye, Agarose gel 2%, Loading dye.

Yeast Isolation

After obtaining the *Apis dorsata* honey beehives sample, the initial stage was carried out by isolating a sample of 10 grams then dissolving it with 90 ml of sterile distilled water and then vortexing. The sample suspension was stored in the electric deep freezer at -80° C. The PDA medium was prepared according to the packaging instructions and sterilized in an autoclave for 30 minutes at 121° C, 1 atm pressure. After being sterile, the medium was poured as much as 20 ml on Petri dish and added the antibiotic *chloramphenicol* concentration of 1 mg/ml. Meanwhile, for the isolation of yeast i.e, 10 µL of the suspension of the nested sample was poured on the PDA medium using the spreader. Cultures then incubated at room temperature for three days until yeast colonies were appeared. Each appared colony was purified and transferred to a new PDA medium.

Yeast Morphology Observation

Yeast morphology was observed using macroscopic observations consists of several parameters such as shape, color, size, texture,

margins, elevation. Microscopic observation was observed using binocular microscope (Nikon ECLIPSE E100), such as cell shape, budding pattern, and the presence or absence of pseudo hyphae.

Yeast DNA Extraction

The yeast DNA extraction was extracted using the protocol from LIPI with the boiling method (Kanti *et al*., 2018). Yeast isolates were taken with sterile ose, put in a tube containing 50 µl of NFW (*nuclease free water*), and vortexed until homogeneous. The samples were then heated at a temperature of 98° C with three variations of the incubation time, namely 10 minutes, 15 minutes, and 20 minutes. After completion of incubation, spin down until there are two layers and the top layer is transferred to a new tube. The concentration and purity of DNA were measured using BioDrop Touch Duo Version 7144 V1.0.5 at a wavelength of 260 and 280 nm.

Optimization of Yeast DNA Amplification of Various Protocols

The obtained DNA was amplified using Go Taq® Green Master Mix [Promega] protocol. PCR cocktail composition that has been made with a total reaction volume of 25 µl consisting of GoTaq® *Green Master Mix* 12,5 µl; NFW 5,5 µl; ITS5 *Forward* Primer 1 µl; ITS4 *Reverse* Primer 1 µl; and DNA *template* 5 µl. The amplification used three temperature and time control protocols aimed at optimization. The temperature and times during the PCR process were shown in the Table 1.

The amplification results were performed electrophoresis using 2% agarose gel containing 1 µl of *Diamond dye Nucleic Acid* (Promega), DNA ladder 1 kb (Thermo Scientific), and amplified samples were inserted into each well for 1 hour 15 minutes, a voltage of 50 V. The results were visualized with DNA *documentation/transiluminator* EnduroTM GDS Labinet to observed the amplified DNA bands.

RESULTS AND DISCUSSION

Yeast isolated from the *Apis dorsata* beehives were grown on a PDA medium and obtained four isolates coded by K1, K2, K3, and K4. Then, macroscopic and microscopic observations were carried out. Both observations estimated that four isolates are different genus. The results of macroscopic and microscopic observations of each yeast isolate were presented in Table 2.

Macroscopically, four yeast isolates were round-shaped. The four isolates were creamy-white in color, thick and slimy in texture, and having different margins for each isolate. It was serrated, wavy for K1 and K2, respectively, and uniformed for K3 and K4. All isolates were raised in elevation. Meanwhile, microscopic observation showed that the shape of isolate K1 was oval, isolate K2 and K3 were round-oval, and isolate K4 was ovoid. The yeast isolates obtained varied in size with a length between 2-7 µm and a width between 1-7 µm. The germination pattern of all isolates was multilateral, except for isolate K1 that was monopolar. The appearance of four yeast isolates microscopically under the microscope with 1000x magnification is illustrated in Figure 1.

Table 1. Third Protocols of Temperature and Time of Yeast DNA Amplification

No.	Protocols	PCR Steps	Temperature (°C)	Time	Cycle
1.	**Maulana**	*Pre-denaturation*	95	2'	
		Denaturation	95	15"	
	(2011)	*Annealing*	58	30"	40
		Extension	68	1'	
		Post-extension	68	10'40"	
2.	**Ediningsari**	*Pre-denaturation*	94	2'	
	(2008)	*Denaturation*	94	15"	
		Annealing	56	30"	40
		Extension	68	1'	
		Post-extension	68	10'40"	
3.	**Kanti et al, (2018)**	*Pre-denaturation*	95	1'30"	
		Denaturation	95	30"	
		Annealing	55	30"	40
		Extension	72	1'	
		Post-extension	72	15'	

Note: ' minute, "second

Table 2. Observation results of macroscopic and microscopic yeast isolates

No	Characteristics	Observation of yeast isolates			
		K1	K2	K3	K4
1.	**Macroscopic observation**				
	a. Shape	Round	Round	Round	Round
	b. Color	Creamy	Creamy	Creamy-white	White
	c. Texture	Thick	Thick	Thick	Thick
	d. Margin	Serrated	Wavy	Uniformed	Uniformed
	e. Elevation	Raised	Raised	Raised	Raised
2.	**Microscopic observation**				
	a. Size	(4,5-7x1-4) µm	(2-4x3-5) µm	(2-6x3-7) µm	(3,5-7x2,5-6) µm
	b. Cell Shape	Oval	Round-Oval	Round-Oval	Ovoid
	c. Germination	Monopolar	Multilateral	Multilateral	Multilateral
	d. Pseudohypae (+/-)	+	-	-	-

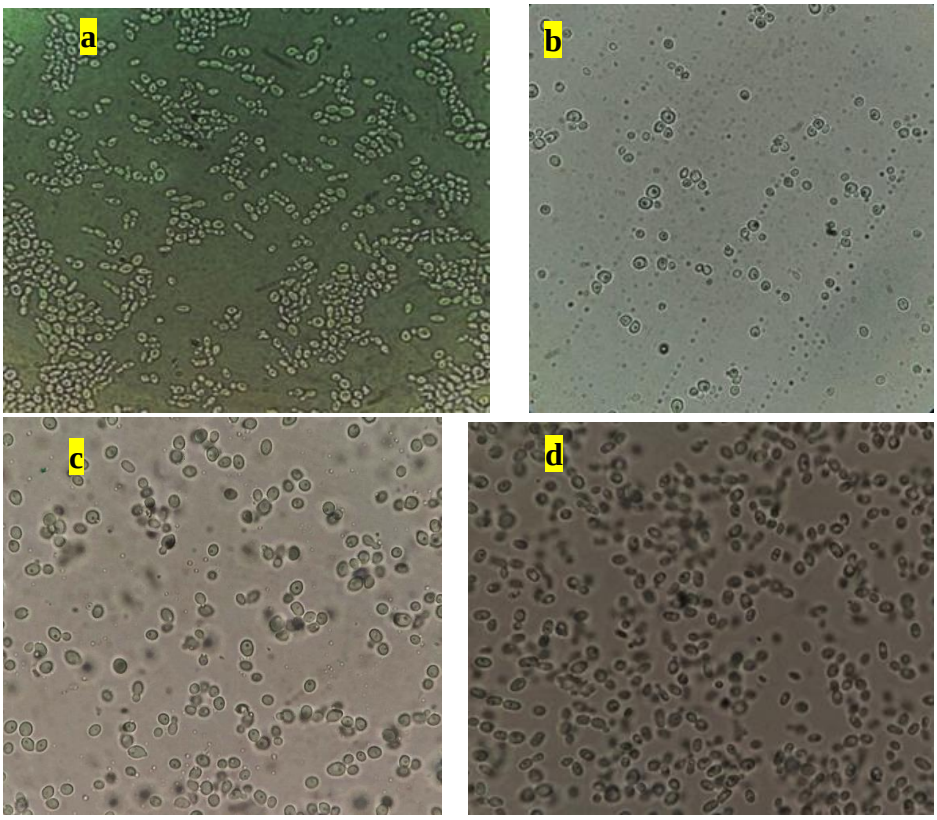

Figure 1. (a) Isolate K1, (b) Isolate K2, (c) Isolate K3, (d) Isolate K4 Under a microscope with a magnification of 1000x

Comparing macroscopic and microscopic observation data of four yeast isolates from the *Apis dorsata* beehive with literature from Cletus Kurtzman et al., (2011) "The Yeast: A Taxonomy Study" show that isolate K1, K2, K3, and K4 had a similar characteristic with *Pichia*, *Candida*, *Torulospora*, and *Wickerhamomyces* genus yeast, respectively. However, it requires further analysis to obtain an accurate and appropriate result.

The molecular analysis was initiated by DNA extraction using the boiling method reference protocol from LIPI with incubation time variations of 10, 15, and 20 minutes in 98ºC. This protocol was chosen in this study because it has several advantages, such as minimal materials, fast, and affordable. DNA extractions aim to generate DNA isolates. The DNA extraction results determine the success of subsequent DNA analysis processes. Therefore, the DNA extraction process should be

carried out carefully and aseptically to avoid contamination. Yeast DNA extraction results were measured for its concentration and purity. The concentration results of each measurement are presented in Table 3.

The DNA isolate with the highest concentration was K2 during the 10-minute incubation time, i.e., 14.15 µl/mg with a purity of 2.246 (Table 3). Meanwhile, the lowest concentration was the isolate K4 during the 15-minute incubation time, i.e., 0.663 µl/mg with a purity of 2.119. According to Sambrook et al., (1989), pure DNA isolate has a purity value between 1.75 – 2.0. If the purity value is less than 1.75 then it indicates the presence of protein contamination, while the purity value more than 2.0 indicates the presence of RNA contamination. In this study, DNA isolates that showed pure DNA wthout any protein or RNA contamination were isolates coded K1 and K4 with a purity of 1.896 and 1.746, respectively. The pure DNA results were obtained from the DNA extraction process with an incubation time of 10 minutes.

Yeast DNA isolates showed pure on each isolate was then subjected to amplification. The amplification protocol for the cocktail recipe on the PCR reaction was Go Taq® Green Master Mix [Promega]. Primers used on the ITS rDNA target area were primer ITS4 (reverse) and primer ITS5 (forward). These primers were universal primers to amplify all ITS rDNA areas in fungi (Maulana, 2011).

Three different temperatures and times during the PCR process was used for optimization based on Ediningsari, (2008), Maulana, (2011) and Kanti et al., (2018) protocols. It was necessary to acquire excellent yeast DNA and optimal PCR products for the subsequent analysis. The success in proliferating DNA using PCR depends on sufficient template DNA, annealing temperature, and appropriate primer selection (Nugroho et al., 2013). DNA from the PCR product amplification was subjected to electrophoresis and DNA bands visualization using gel documentation. The results of yeast DNA bands visualization from the three protocols are presented Figure 2.

Based on the three yeast DNA amplification protocols showed that the protocol by Kanti et al. (2018) generated a DNA band for all yeast isolates. It contrasts, Maulana (2011) and Ediningsari (2008) protocols did not generated DNA bands for all yeast isolates, it means that the optimal protocol for amplification of yeast DNA is the protocol of Kanti, et.al. However, the DNA quality from protocol Kanti et al., (2018) was less good due to the presence of a smear. Smear is DNA that is cut into pieces and small size (Ekasari et al., 2012). Smear's presence is possibly due to the polysaccharides in yeast isolates that were extracted during the DNA extraction. Besides polysaccharides, a smear may present due to RNA contamination during the DNA extraction process (Rahayu et al., 2015).

Table 3. Spectrophotometric Results Data with Incubation Times of 10, 15, and 20 Minutes at the Time of Yeast DNA Extraction

Sample Code	Incubation Time	Spectrophotometric Result	
		Concentration (µl/mg)	λ 260/ λ 280
K1	10'	2.646	1.896
	15'	4.922	1.656
	20'	6.127	1.644
K2	10'	14.15	2.246
	15'	8.501	2.698
	20'	8.558	1.413
K3	10'	11.98	2.335
	15'	8.436	2.491
	20'	12.35	1.387
K4	10'	1.052	1.748
	15'	0.663	2.119
	20'	1.749	8.721

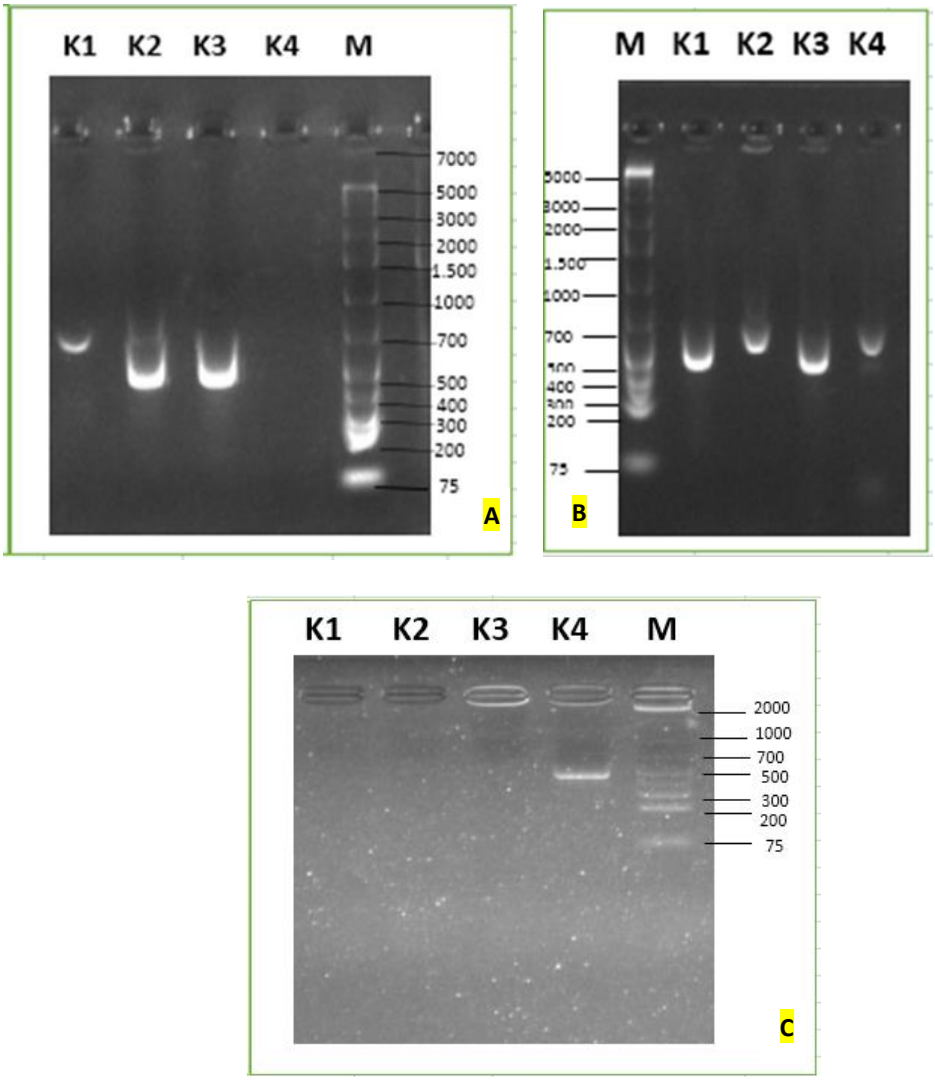

Figure 2. A. DNA band protocol Ediningsari (2008), B. DNA band protocol Atit Kanti, et al. (2018), C. DNA band protocol Maulana (2011).

Table 4. Comparison of DNA Band(s) Length Results in The Three Amplification Protocol

No	PCR Temperature and Time Setting Protocol	Length Band DNA (bp)			
		K1	K2	K3	K4
1	Ediningsari (2008)	719 bp	579 bp	588 bp	-
2	Maulana (2011)	-	-	-	457 bp
3	Kanti, *et al* (2018)	503 bp	542 bp	492 bp	526 bp

In this study, the DNA band of K4 isolate appeared as united, non-smearing using the protocol by Maulana, (2011). Although the result of DNA extraction processes was qualified, however, this protocol did not generate any DNA band in the isolates K1, K2, and K3. It might be due to several factors, e.g., a non-adhering primer on target DNAs, non-denatured DNAs, or unsuitable annealing temperature. Annealing temperature that is too high result in low PCR products, while annealing that is too low results in non-specific DNA amplification results (Prakoso et al., 2016). Annealing temperature in the PCR process highly affected the primer adherence process, and hence, a transformation of one degree causes the failure of primer adherence (Gusmiaty et al., 2012; Nurjayadi et al., 2020). It also applied to the protocol of Ediningsari (2008) on the isolate of K4. The length comparison of DNA band generated from the three protocols is presented in Table 4.

Based on table 4, comparing yeast DNA band(s) length using three protocols of PCR temperature and time assignment demonstrate different DNA band(s) lengths. However, the length of ITS area ranges from 300-900 bp (Citra, 2019; Wardani et al., 2020). The difference in DNA fragment size (DNA fragment polymorphism) of yeast DNA amplification results was caused by nucleotide base location distribution in genomes, which became the site of primer adherence. DNA band from these different amplifications showed that DNA band(s) size influences the population diversity (Gusmiaty et al., 2012).

CONCLUSIONS

Four yeast isolates were successfully isolated from giant honey beehives (*Apis dorsata*) which showed different characteristics observed from macroscopic and microscopic morphology. Based on the results of the study, the yeast DNA extraction process used the boiling method with temperature variations from LIPI has shown the most optimal DNA purity with a time of 10 minutes.

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