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Optimization of PCR Protocols for ITS rDNA Amplification of Yeasts Isolated from *Apis mellifera* Honeycomb

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ABSTRACT

Yeast is a microorganism that can be found in honeycomb. Yeast identification is a process to find and identify new species. One of which is molecular identification of yeast with rDNA sequences in the ITS (Internal Transcribed Spacer) region. Before carrying out molecular identification, it is necessary to optimize yeast DNA amplification methods to obtain good DNA sequences that ease the yeast identification process. The purpose of this study was to discover the optimum PCR (Polymerase Chain Reaction) protocols for the identification of yeasts isolated from Apis mellifera honeycomb based on the ITS rDNA. This study used 3 PCR (Polymerase Chain Reaction) protocols, i.e., from M-K18; M-E08; and M-M11. *This results study shows that the optimum PCR protocol was from M-M11 which produced clear and whole DNA fragment luminescences.*

Introduction

Yeast is a eukaryotic unicellular microorganism of the fungi kingdom that reproduces asexually by budding and sexually without being covered by the fruit body (Kurtzman *et al.*, 2011). The presence of yeast in nature is abundant covering water/aquatic (sea), atmosphere (air), and land/terrestrial (land) (Ashliha & Nur, 2014). Yeast can be found in honey comb (Thacker, 2012). The presence of yeast in honeycomb plays a vital role in the bee's life, giving rise to a symbiotic mutualism. Bee act as a vector for yeast from one habitat to another, while yeast plays a role in the conversion and maturation process of nectar into honey on honeycomb carried out enzymatically (Savitri *et al.*, 2017).

Flower availability as a nectar source is the cause of honey diversity. This diversity causes yeast diversity that helps the honey production process. Honey that has been produced will be stored in cells made of bee wax, commonly called honeycomb. Honey can only be produced by a honey bee (Prasetyo *et al.*, 2014). One of the honey bees types in Indonesia is *Apis mellifera*. Studies on yeast presence in *Apis mellifera* honeycomb are still limited, and therefore, exploration and identification are needed. Identification is a method to discover yeast diversity in nature (Maulana, 2011).

In 1951, Wickerman became a pioneer in the identification and development of a taxonomy of yeast. This research was marked by 100 genus and 700 yeast species

identified (Kanti & H.J.D., 2011). In 2011, there were 1500 identified yeast species. However, this development was still far from representing the whole yeast species because yeast species are estimated to be 150.000 in number in practice. Hence, there are still 99% of yeast species unidentified (Candra, 2015).

Molecular yeast identification is based on the rDNA gene sequence data (Maulana, 2011). This gene consists of coding and non-coding areas. The non-coding area has an Internal Transcribed Spacer (ITS) with high sequence diversity with a size of ±700 bp, which has many copies in the genome, is easy to analyze, this gene is often used in many studies so that its gene sequence is already available in the NCBI database, and is in accordance with for the identification of yeast molecules (Anggraini *et al.*, 2019).

In the identification of groups of fungi including yeasts, several specific primers have been set to support the success of DNA amplification. One of the forward and reverse primers that are often used is ITS5 and ITS 4 with sizes between 450 to 750 bp (Fajarningsih, 2016).

Method variation in molecular yeast isolate identification is closely related to the work steps to collect desired data, adjusted to the ease of method and financial aspect availability. The financial aspect is related to the utilization of several chemicals, which are relatively expensive with the procurement of materials that sometimes take a long time so that research breakthroughs are needed to overcome these problems (Syafaruddin & Tri, 2011).

This breakthrough is in the form of optimization of existing methods, in terms of chemical use, selection of primer pairs, and setting the annealing temperature, so the most optimal method is found for molecular identification of yeast isolates (Joko *et al.*, 2011). The optimal method can be seen based on the success of ITS rDNA amplification which can help at the DNA sequencing stage, making it easier to identify yeast species (Nugroho *et al.*, 2013).

Based on this explanation, it is known that research on optimization PCR (Polymerase Chain Reaction) protocol for the identification of yeasts isolated from *Apis mellifera* honeycomb based on the ITS rDNA is the first step that will be used as a reference for further study that is expected to provide benefits. Therefore, this research needs to do considering the role and benefits of yeast for the needs of other living things.

Materials and Methods

This research is experimental research by presenting qualitative descriptive data. The materials used in this study consisted of yeast isolates collected from *Apis mellifera* honeycomb located at beekeeping Kembang Joyo Sriwijaya, Kab. Malang. The mediums used in this study consists of YMA and PDA.

While other materials consist of agarose gel, blue loading dye, buffer TAE, chloramphenicol 500 mg, Diamond™ nucleic acid dye-Ethidium Bromide (Et-Br), DNA ladder 1 kb, Kit PCR GoTaq® Green Master Mix, Nuclease-Free Water (NFW), and custom primers from Integrated DNA Technologies (IDT) is Forward Primer ITS5 (5'-TCCTCCGCTTATTGATATGC-3') and Reverse Primer ITS4 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (Maulana, 2011).

Sample Preparation

Collected samples were temporarily stored in a cooler box, which was transferred to a cool and dry place, 1 g of honeycomb was taken to be dissolved in a sterile aquadest of 90 ml, vortexed, poured into the sterile YMA medium for 1 ml, and incubated for 48-72 hours.

Yeast Isolation & Morphology Observation

Furthermore, microscopic observation of yeast morphology was carried out based on several parameters, i.e., length, width, vegetative cell shape, and budding type (Kreger, 1987; dalam Suryaningsih *et al.*, 2018). Yeast isolate purification was conducted in order with the yeast working culture production. Isolate inoculation to the

sterile YMA medium for ±3 oses and incubated for 48-72 hours to obtain a pure and single colony. This purification was conducted at least two times. The yeast working culture production was then carried out by transferring representative single colony yeast culture to the PDA media to be incubated for 3-7 days.

Yeast DNA Extraction

DNA extraction was carried out on pure and single yeast cultures of 1 week of age. This study, using the extraction method that comes from the boiling method. As for the extraction method used, namely a pure culture was taken using a sterile inoculation needle for ±3 oses, put into the microcentrifuge tube, added with NFW 50 µl, vortexed to be dissolved, heated for 10 minutes in 98 ^{o}C, homogenized using spin down to create two layers, i.e., the bottom layer as the pellet and the upper layer as the DNA template, and transferred the DNA template into the microcentrifuge tube. It was then continued with the measurement of DNA extraction product concentration and purity values using a spectrophotometer (Kanti *et al.*, 2018).

Yeast DNA Amplification

The yeast DNA amplification was carried out with the PCR technique. This study used 3 PCR (Polymerase Chain Reaction) protocols, i.e., M-K18 from Kanti *et al.* (2018); M-E08 from Ediningsari (2008); and M-M11 from Maulana (2011). PCR component cocktail used was referring to the Promega GoTaq® Green Master Mix protocol with a reaction volume of 25 µl, consists of GoTaq® Green Master Mix 12.5 µl, NFW 5.5 µl, Forward Primer ITS5 1 µl, Reverse Primer ITS4 1 µl, and DNA template 5 µl, put into the PCR mini tubes, homogenized using spin down for 3-5 seconds, put into PCR machine blocks, and executed the PCR running process. Before that, the PCR protocol program utilized should be regulated. This study used three PCR protocol variations for the optimization that are presented in Table 1.

Table 1. PCR (*Polymerase Chain Reaction*) Protocol Variations

No	Protocol	Stage	Temperature (ºC)	Time	Cycle
1.	M-K18	*Pre-denaturation*	95	1’30”	35
		Denaturation	95	30”	
		Annealing	55	30”	
		Extension	72	1’	
		Post-extension	72	15’	
2.	M-E08	*Pre-denaturation*	94	2’	40
		Denaturation	94	15”	
		Annealing	56	30”	
		Extension	68	1’	
		Post-extension	68	10’40”	
3.	M-M11	*Pre-denaturation*	95	2’	40
		Denaturation	95	15”	
		Annealing	58	30”	
		Extension	68	1’	
		Post-extension	68	10’40’’	

Note: ‘: minute “ : second

The DNA amplification result was analyzed by electrophoresis agarose gel 2% in 1xTAE buffer, colored with Et-Br. The product of DNA amplification was poured into the well for 3 µl with a loading dye of 1 µl. Electrophoresis was conducted at a speed of 100 volts for 30 minutes. Luminous reading of the band length and DNA thickness between 300-900 bp will be compared to the DNA ladder 1kb and visualized with the gel documentation instrument under UV light.

Results and Discussion

Yeast Isolation

This study obtained four isolates with codes of IKS-1, IKS-2, IKS-3, and IKS-4. Isolates that managed to be isolated had various growing phases, i.e., fast-growing and slow-growing. It is due to differences in adaptation ability to the growth medium environment (Maulana, 2011). The results of yeast microscopic observation are presented in Table 2 and Figure 1.

Table 2. Observation Result Yeasts Microscopic

Isolate Code	Microscopic Parameter			
	Size		Shape	Budding Type
	Length (µm)	Width (µm)		
IKS-1	2,6-5,7	2,3-5,3	Round	Multilateral
IKS-2	5,2-12,2	2,2-4,5	Round-Oval-Cylinder	Multilateral
IKS-3	3,7-9,6	2,7-4,1	Pseudomiselium	Multilateral
IKS-4	2,8-4,6	1,4-3,7	Oval-Cylinder	Multilateral

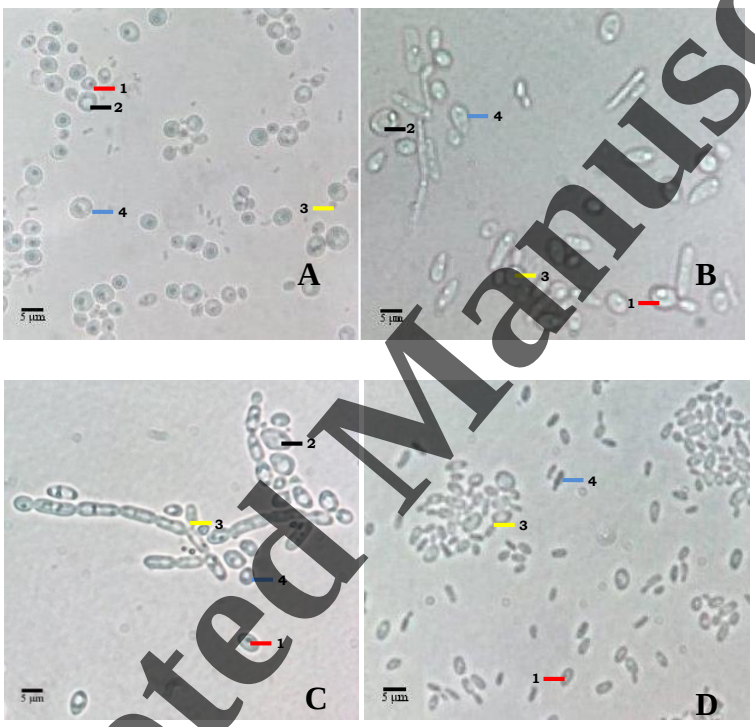

Figure 1. Yeasts Microscopic. (A) IKS-1, (B) IKS-2, (C) IKS-3, and (D) IKS-4
Note: 1) Nucleus, 2) Vacuole, 3) Budding, 4) Cell Wall

Based on (Table 2) and (Figure 1), the characteristics of isolates that managed to be isolated were following yeast characteristics with the multilateral-budding types (Kurtzman *et al.*, 2011). The multilateral-budding type is the type of shoot is commonly found in the Genus *Candida*, Genus *Saccharomyces*, Genus *Pichia*, Genus *Hansenula*, Genus *Debaryomyces*, and Genus *Torulaspora* (Sulastri, 2019). In addition, it also has round, oval, oval, pseudomycellium, and cylindrical shapes.

The isolates that were successfully isolated had various sizes. This size depends on the growth and type of species. Yeasts are 2-50 m long and 1-10 m wide. In addition, no characteristics such as the presence of hyphae and flagella were found, so the isolates that were successfully isolated were not fungi and bacteria. It follows Walker's (2009) statement that yeast is a microorganism that cannot form hyphae and move due to the absence of flagellum.

Yeast DNA Extraction

The methods were from the boiling method used to aims to extract DNA from yeast cells. The results of the quantitative analysis of yeast DNA extraction products are presented in Table 3.

Table 3. Quantitative Analysis Result Yeasts DNA Extraction Products

Method Name	Isolate Code	Spectrophotometry Result	
		Concentration (µg/ml)	Purity (Å260/ Å280)
Boiling Method	IKS-1	20,06	2,034
	IKS-2	12,22	2,118
	IKS-3	21,55	1,759
	IKS-4	10,32	1,870

Based on (Table 3), there was a significant difference in DNA concentration and purity values. It is related to several factors, such as temperature, time, steps, and reagents used in extracting DNA. Quantitative analysis results of DNA extraction, indicate that the concentration and purity values of the resulting DNA are good enough to be forwarded to the DNA amplification stage. Good DNA concentrations for the DNA amplification are 10-100 µg/ml, while good purity values for the DNA amplification are 1.8-2.0. If the value obtained is lower than the above values, it shows contamination from phenols, polysaccharides, or other proteins, while a ratio over the above values shows contamination of RNA (Nur'utami, 2011).

Meanwhile, the incubation temperature was appropriate to extract DNA and generate relatively high concentration and purity values on isolates of IKS-1 and IKS-4, which were within the range of 1.8-2.0. The heating with an incubation temperature of 98 ºC for 10 minutes was perceived to accelerate the yeast wall's lysis, and therefore, DNA can be extracted easily and denaturation inhibitor proteins. Besides, may increase yeast cell wall permeability that allowed inflows of liquid and other materials surrounding the cell and outflows of materials from the cell (Maknunah, 2017). The high temperature is also beneficial to inactivate enzymes, especially DNAse that may damage DNA (Nugroho *et al.,* 2013).

While the other two isolates possessed a relatively low DNA quality. It is demonstrated by the presence of IKS-2 and IKS-3 with absorbance ratios of more and less than the 1.8-2.0 range. The poor purity value might be caused by an incomplete DNA extraction which allowed encapsulated DNA within the cell. Besides, there was also an incomplete other particle elimination that was potential to be inhibitors that hindered the DNA amplification process (Sunarno *et al.*, 2014). The next influencing factor to DNA extraction success was the yeast cell waste thickness which required another reagent for the cell wall lysis. Using NFW as the solvent is perceived as sufficient to extract DNA because NFW could help avoid DNA degradation by nuclease and dissolve reactor concentration to the appropriate final concentration.

Optimization PCR Protocol

The PCR protocols used for yeast DNA amplification in this study were based on three references, i.e., PCR protocols from M-K18; M-E08; and M-M11. The results of visualization of yeast DNA amplification products with PCR protocols from M-K18; M-E08; and M-M11 are presented in (Figure 2) below.

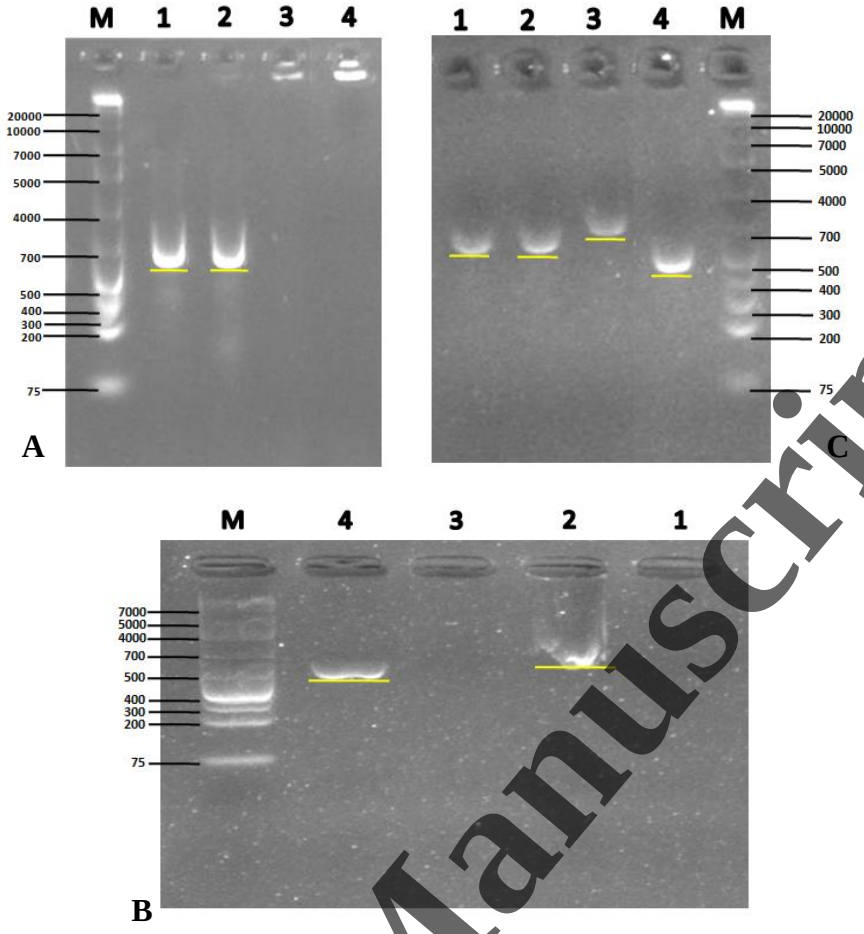

Figure 2. Visualization Yeasts DNA with (A) M-K18; (B) M-E08; and (C) M-M11 Protocol. Information: (M)=Marker, (1)=IKS-1, (2)=IKS-2, (3)=IKS-3, and (4)= IKS-4

Based on the results of visualization of yeast DNA amplification products from several PCR protocols above, it was shown that there was a significant difference in the visualization of DNA bands. This is closely related to several factors such as the number of cycles; temperature and time at each stage, especially at the denaturation and annealing stages; components in PCR cocktails; quantity of DNA extracted as template DNA; and the primer pairs used. When viewed from the results of visualization of yeast DNA amplification products with the first PCR protocol, 2 isolates showed DNA band fragments in the wells that did not migrate properly and completely to the positive pole.

This is because the number of cycles required for all stages of DNA amplification is still lacking. The number of cycles used in the first PCR protocol is only 35 cycles and this is the least number of cycles when compared to the number of cycles in the other 2 PCR protocols, which is 40 cycles. It is also supported by Amalia's (2013), statement stating that yeast DNA amplification with 35 cycles, the amplification reaction was not optimal and perfect.

Besides, the DNA amplification product visualization result in the second PCR protocol also showed two isolates that did not amplificated well and perfectly. It might be caused by a non-optimal denaturation temperature. The second PCR protocol was carried out at a denaturation temperature of 94 ^{o}C for 15 seconds. According to Deekshit *et al.,* (2013), The denaturation stage in the amplification process caused DNA structure separation from double helixes to be a single helix, and therefore, an optimal temperature is

required, i.e., 95 ^{o}C for 30 seconds. The not optimal temperature may cause a renaturation process, while overtime may affect DNA Taq-polymerase enzyme works. Therefore, it may affect the PCR process success (Joko *et al.,* 2011).

Furthermore, the pre-denaturation stage also plays a role in the PCR process's success. This stage plays a role in ensuring the number of DNA template molecules to be doubled to denaturation them all. The temperature used in this stage is adjusted to the denaturation stage, with a long time of 2-5 minutes (Muladno, 2010). The annealing stage is the next crucial factor in the primer adhering process so that it becomes the key to DNA amplification. This stage should be adjusted to the properties of the primer used. The annealing temperature applied is a temperature with a 5 ^{o}C difference under the melting temperature (Tm) of the primer (Cindy, 2017). This study used ITS5 and ITS4 as the primer pair in which each primer had a Tm of 58 ^{o}C and 63 ^{o}C. Hence, linked with Tm temperatures of primers, the annealing temperature should be in the range from 53 ^{o}C to 58 ^{o}C.

From the annealing temperature on several PCR protocols used in this study, the annealing temperature that met the standard was on the third PCR protocol of 58 ^{o}C, showing that all isolates were well-amplificated. Meanwhile, visualization results from the other two protocols showed isolates that did not well-amplificated. A not optimal annealing temperature may cause mispriming (Hikmatyar *et al.,* 2015). Besides, a poor PCR component cocktail is also a factor reducing the annealing stage success, where specific primer targets were also affected by enzyme activity, $MgCl_2$, dNTPs component availability, and DNA template concentration and purity values (Rakhmana *et al.,* 2015).

When connected to the concentration and purity values of yeast DNA extraction results from the second DNA extraction method, there are isolates that were absent or did not amplificated well and perfectly on the first and second PCR protocols, i.e., IKS-1, IKS-3, and IKS-4. Meanwhile, these three isolates had relatively high concentrations values of 20.06, 21.55, and 10.32 µg/ml and their purity values were within the range of 1.8-2.0 with 2.034, 1.759, and 1.870. The failure of the three isolates on the DNA amplification stage might be caused by several factors, such as contaminants that affected the DNA quality.

DNA with good quality is pure DNA with DNA fragment luminescence on an electrophoresis gel. Impure DNA might still contain proteins, polysaccharides, RNA, and others. If these contaminants are present in a substantial number, they may affect primary adherence to DNA template during the amplification process (Afshan *et al.,* 2017). Also, the number of DNA templates used may be a factor influencing DNA amplification success. Rahman *et al.,* (2013) stated that a DNA template of 1 µl had produced the best amplification product, marked by thick, complete, and non-smear DNA bands.

Too low of a DNA template will cause DNA target to be not amplificated well because primers are not adhering to the DNA target, while too much of a DNA template also results in a poor PCR product. It is because primer cannot adhere to the specific target gene, and therefore, amplification of other genes resembling the target gene is possible. The DNA template number determination should be adjusted to the number of forward and reverse primer numbers used in the yeast DNA amplification.

Table 4. Yeasts DNA Band Length Comparison

Nama Protokol PCR	Panjang Band DNA (bp)			
	IKS-1	IKS-2	IKS-3	IKS-4
M-K18	563	563	-	-
M-E08	-	594	-	475
M-M11	504	504	588	444

If you look at the comparison of the length of the yeast DNA band with the three PCR protocols presented in the form (Table 4) above, it shows that there are differences

in the length of the DNA band in all yeast isolates with a size of 444-563 bp. The variation in DNA band length was caused by differences in PCR conditions in amplifying yeast DNA. However, this difference in DNA band length is still understandable because it is still in the range of 300-900 bp, where this size is a measure of the length of the ITS area in yeast (Citra, 2019). According to Katsu *et al.* (2003), the ITS area of yeast rDNA varies greatly between species with a size of 300-900 bp and this is also supported by the statement of White *et al.* (1990); in Rahayu *et al.* (2015), that the combination of ITS5 and ITS4 primers in the ITS-1 and ITS-2 regions has a size range of 563-602 bp.

The average ITS rDNA region can be amplified with ITS5 and ITS4 primer pairs. This is also proven in the research of Rakhmana *et al.* (2015), which showed that amplification of yeast rDNA with a combination of ITS5 and ITS4 primers had a DNA band length of 583 bp. In the research of Rahayu *et al*, (2015), also showed that amplification of yeast rDNA with a combination of ITS5 and ITS4 primers had a DNA band length of 537 bp. In the research of Maulana (2011), it was shown that amplification of yeast rDNA with a combination of ITS5 and ITS4 primers had DNA band lengths ranging from 350-600 bp.

According to Nugroho *et al.*, (2013), the ITS5 and ITS4 primer pair is the universal primer pair for the fungi group, including yeast, to be used to amplify the whole ITS rDNA region. This statement is proved in this study where yeast DNA amplification visualization results using the PCR protocol from M-M11 produced whole and clear DNA bands.

Conclusion

Based on the study, conclusions are made, the optimum PCR (Polymerase Chain Reaction) for identification of yeasts isolated from *Apis mellifera* honeycomb on ITS rDNA target gene was the PCR protocol from M-M11 that generated visualization of whole and clear DNA fragment respectively.

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