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FULL PAPER

Mold characterization in "RAPRIMA" tempeh yeast from LIPI and over-fermented Koro Pedang (Jack Beans) tempeh

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Abstract

Tempeh mold is tempeh starter mixed with legumes as a substrate. Generally, tempeh molds are *Rhizopus* sp. but they are not specific to a particular genus. This study aimed to identify and characterize the molds in "RAPRIMA" tempeh of LIPI Indonesia and over-fermented (60 hours) koro pedang tempeh. The characterization method in this research utilized media-specific molds, analyzed by most chamber and microscopic, biochemical analysis with Analytical Profile Index (API-test), and molecular with ITS sequencing by PCR Product. The results showed that "RAPRIMA" tempeh yeast had total molds of 4.94 log CFU/mL on YMA media and 4.85 log CFU/mL on MEA media, while over-fermented tempeh had total molds of 4.84 log CFU/mL on YMA media and 4.60 log CFU/mL on YMA media. The characteristics of molds were black, white, and oval mycelia. The API-test analysis revealed that "RAPRIMA" tempeh and over-fermented tempeh yeast was *Rhizomucor*. The ITS sequencing by PCR products showed that it was *Rhizopus microsporus*. The genus mold characterization of RAPRIMA and over-fermented koro pedang tempeh was *Rhizopus* sp. and gives good physical characteristics.

1. Introduction

Tempeh is a fermented food ingredient from legumes using various Rhizopus molds, such as *Rhizopus oligosporus*, *Rhizopus oryzae*, *Rhizopus stolonifer* (Nout and Kiers, 2005; Maryati *et al*., 2019). Based on data from the Statistics Indonesia (2010), soybean consumption in Indonesia reaches 2.2 million tons/year, of which 50% of soybean processing consumption is in the form of tempeh, 40% of tofu, and 10% in the form of other products (such as tauco, oncom, soy sauce, miso, and natto) (David Owens, 2014; Djajasoepena *et al*., 2014). Therefore, making tempeh has become an industry of Indonesian society.

Tempeh requires some characteristics quality tempeh, such as tempeh odor, white color- slightly grayish, and tempeh taste (Yarlina and Astuti, 2021). The white color is caused by the presence of mycelia in molds growing on the surface of soybean seeds. The degradation of components causes the taste of tempeh in soybean seeds, which is triggered by the enzymes produced by molds after the fermentation process occurs (Barus *et al*., 2008; Maryati *et al*., 2019).

Tempeh inoculum is a collection of spores that are used for breeding in the process of tempeh. The growing mold will decompose the protein in soybeans and produce a mycelium that binds together to make compact cakes of tempeh (Yarlina and Astuti, 2021). Tempeh mold will grow optimally if it already has adequate environmental conditions, including the temperature used of 30-37^{o}C, the optimum relative humidity (RH) at 70-85%, and the optimum pH of 3.5-5.5 (Radita *et al*., 2017).

The type of mold and the time of fermentation determine the production and activity of the enzyme's amylase, lipase, and protease, those will work to break down carbohydrates, fats, proteins contained in the beans and determine the nutritional quality of the tempeh

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produced (Weng and Chen, 2011; Gonçalves *et al*., 2016). The type of mold in tempeh is dominantly still not known with certainty. It is only known that the ones that are often used in the producing of tempeh are *R. oligosporus* and *R. oryzae* (Ikasari and Mitchell, 1994; Tahir *et al*., 2018; Pangastuti *et al*., 2019).

Rhizopus oryzae is longer than *R. oligosporus*, resulting in denser tempeh, while *R. stolonifer* has irregular and polygonal spores. Each type has different enzyme activity capabilities. Besides that, they have different abilities in producing antibiotics and biosynthetic vitamins to make other physical and chemical characteristics in tempeh (Thanh and Nout, 2004; Nguyen and Ranamukhaarachchi, 2020).

Rhizopus oligosporus is a mold generally playing a significant role in producing fermented tempeh. *Rhizopus oligosporus* mold has the characteristics of other colonies appearing brownish gray, solitary sporangiophores and reach 1 μm and 10-18 μm in diameter. The sporangiophores of *R. oligosporus* are transparent (sub hyaline) and gradually becomes brownish; rhizoids are short branched and grow opposite the sporangiophores, such as towards the substrate with smooth or slightly rough cell walls. The ripe sporangium is round brown to black, with a diameter of 100-180 μm, and the sporangium forms spores as a means of reproduction (Jennessen *et al*., 2008). *Rhizopus oligosporus* can survive in temperatures 25°C – 45°C with an optimum temperature of 37°C and can grow with 0.88 of water activity (a_w) (Sugai-Guérios *et al.*, 2019; Rosni *et al*, 2020)

The quality of tempeh is strongly influenced by the quality of the starter used for inoculation. Generally, the laru or tempeh starter used is RAPRIMA LIPI molds. However, there has been no specific determination of what mold is dominant in the laru. Therefore, this study identified and characterized the molds in the RAPRIMA tempeh, where over-fermented was conducted by Analytical Profile Index (API-Test) analysis and molecular biology identification was carried out by PCR test.

2. Materials and methods

2.1 Materials

The material used was RAPRIMA molds from LIPI Indonesia as a starter for Tempeh and Koro Pedang Beans as legumes and substrate. Tempeh was made by soaking, peeling, steaming, inoculating RAPRIMA molds with koro pedang beans. They were fermented for 60 hrs using a temperature of 25°C and 60% of relative humidity. The analytical media used were Yeast Mold Agar (YMA), Malt Extract Agar (MEA), Potatoes Dextrose Agar (PDA), and Sodium Chloride. The analytical tools used were a Moist chamber by a petri dish, prepared glass and cover glass, API CH 20C AUX well, and PCR tools kit.

2.2 Propagation, isolation and purification media

A total of 1 g of sample of RAPRIMA molds was diluted with 9 mL of sterile Sodium chloride up to the third dilution. Isolation of the molds was carried out by pouring 1 mL of the sample from the 10^{-4}, 10^{-5}, 10^{-6} dilution into a petri dish that already contained a selective medium. The selective media were Yeast and Mold Agar (YMA) and Malt Extract Agar (MEA) medium (Nguyen and Ranamukhaarachchi, 2020).

The molds isolate purified using Potato Dextrose Agar (PDA) medium. Molds colonies growing in Petri dishes were isolated for each different colony. The colonies were purified until they produced a single colony and were ready for analysis.

2.3 Molds characterization by moist chamber and microscopic

First, sub-culturing and purification of isolated microorganisms were conducted, then continued with a moist chamber (Tahir *et al*., 2018). This method was carried out aseptically in a petri dish. PDA was dripped onto the slide. After freezing, cut it in half, and then one of the cut parts was discarded. The remaining side was left on the slide. Aseptically, the spores of pure molds were taken and attached to the side of the remaining agar on the slide. After that, Vaseline was put at each corner of the cover glass, then the cover glass was closed on top. The petri dish with pure molds was incubated for 72 hrs. Furthermore, the results were observed under a microscope and a reference book to observe the morphology (de la Maza *et al*., 2020).

2.4 Molds identification by biochemical analysis

RAPRIMA molds and over-fermented tempeh were identified for 60 hrs using the API Kit test with a 20C AUX API card (Laboratory of Biofarma, Indonesia). API test was done by analyzing biochemical characterization and morphological analysis to determine the types of microorganisms and species of molds isolate (Alakeji *et al*., 2015).

2.5 Molds identification by PCR test

2.5.1 DNA extraction

DNA extraction was carried out using Promega Crop DNA. As much as 0.3 g of mycelium was scraped from a petri dish using a sterile spatula and then put into a microtube, then 293 μL of 50 mM EDTA (pH 8) and 7.5 μL lyticase enzyme (20 mg/mL) were added. Incubated

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mixed culture at 37°C for 60 mins, then centrifuged at 13,000×*g* for 2 mins. The precipitate was dissolved in Nuclei Lysis Solution and homogenized again (Dannaoui, 2009).

The added 100 µL DNA of protein precipitation solution, then shaken for 20 s and allowed to stand on ice for 5 mins and centrifuged at 12,000×*g* for 3 mins. The supernatant containing DNA pallet was added to 300 µL of isopropanol (Tonge *et al.*, 2014).

The DNA pellet was purified by adding 70% ethanol and then centrifuged at 13,000×*g* for 5 mins. The pellet was added with 25 µL of DNA dehydration solution and 1 µL of RNase solution and mixed using vortex for 5 s. The DNA isolate was incubated at 37°C for 15 mins, and then the DNA isolate was stored in the refrigerator.

2.5.2 PCR Amplification

PCR amplification in the ITS-1 and ITS-4 rDNA regions was carried out using ITS1 and ITS4 primers with an annealing temperature of 45°C. ITS-1 primer (5' (TCC GTA GGT GAA CCT GCG G) 3') was used as the reverse primer, and ITS4 primer (ITS4 5' (TCC TCC GCT TAT TGA TAT GC) 3') was used as the forward primer (Hartanti *et al*., 2015).

The amplification reaction is in 3 steps. The first stage was the denaturation process at a temperature of 94°C for 1.5 mins. The second stage was the annealing process at a temperature of 45°C for 1 min. The third stage was extending the DNA chain at a temperature of 72°C for 3 mins. In this study, PCR amplification lasted for 35 cycles. PCR amplification was analyzed to obtain Data and Information sequences using NCBI data (Abe *et al*., 2006).

3. Results and discussion

3.1 Total plate count of RAPRIMA molds and over-fermented tempeh

Tempeh was a fermented product gained from mixing two raw materials, legumes, and a starter in the form of *Rhizopus* sp. The quality of a tempeh product is strongly influenced by the quality of the starter used in the inoculation process. Tempe inoculum can also be referred to as starter tempeh and is known as molds (Wipradnyadewi *et al*., 2011). The production of this tempeh relies on the type of mold *Rhizopus* sp., especially from the species *R. oligosporus* (Redi, 2020). In Indonesia, forty *Rhizopus* strains were isolated from Tempeh and there is difficulty in tracing the genetic diversity of the strains used for tempeh production. The best tempeh molds for tempeh production were *R. oligosporus*, a significant microorganism that has an essential role during the fermentation process of making tempeh products (Nurdini *et al.,* 2015; Sjamsuridzal *et al.*, 2021).

The results of mold propagation on RAPRIMA molds inoculum and over-fermented tempeh can be seen in Table 1. Mold growth on the selection medium dominated the plate used. The growth of blackish-white mold colonies is shown on the plate, and on the growth medium, some filaments have a structure consisting of fine threads called hyphae (Ajdari *et al*., 2011; Hartanti *et al*., 2015). The results of the total plate count showed that the mold could be isolated and purified for microscopic characteristic analysis.

The good characteristic of molds will produced Tempeh with good quality, such as a compact solid cake, grayish white in color, and a distinctive tempeh aroma (Barus *et al*., 2019). A total 0.3% RAPRIMA molds inoculum from LIPI concentration was mixed with 100-gram soybeans to produce tempeh with a very compact texture and density (Triyono *et al*., 2017). The results of the total plate count in Table 1 showed that the dominant mold growing on the selection medium was responsible for being the inoculum in the tempeh-making process. The mold is due to the viability of the spores produced being uniform and having high spores, so that it allowed the spores to be quickly dispersed and germinated in a short time (Barus *et al*., 2019).

3.2 Isolation and identification of molds in moist chamber

Growing spores and mycelium were conducted in the moist chamber whereas characteristics identification of molds was done on the agar media. In the moist chamber

Table 1. The result of molds propagation on YMA and MEA media by total plate count

Sample	Medium	Dilutions			SPC (CFU/g)	Log (CFU/g)
		10^{-4}	10^{-5}	10^{-6}		
RAPRIMA LIPI Indonesia	YMA	9	1	1	8.5×10^5	4.93
		8	-	-		
	MEA	8	1	1	7×10^5	4.85
		6	2	-		
Over-fermented Tempeh 60 hrs	YMA	10	1	-	7×10^5	4.85
		4	-	1		
	MEA	6	1	1	4×10^5	4.60
		2	-	-		

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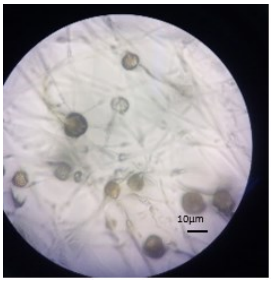
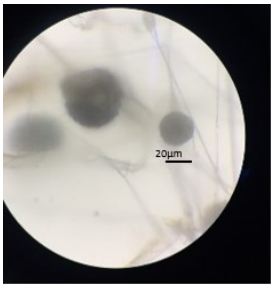
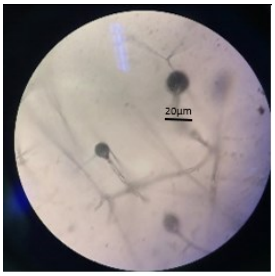
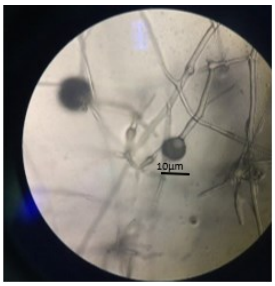
Table 2. Identification and characteristic morphology of molds isolate by moist chamber method

Sample	Media	Morphology			Microscopic
		Spongiospora Color	Miselium Color	Conidia type	
Raprima "LIPI" Indonesia	YMA	Yellowish-black	White	Oval	
	MEA	Grayish black	White	Oval	
Over-fermented Tempeh 60 hrs	YMA	Black	White	Oval	
	MEA	Grayish black	White	Oval	

method, the characteristics were observed under a microscope to see the morphology of the spores and mycelium produced from the mold and the type of mold growth can be observed (Tahir *et al*., 2018). The isolation of RAPRIMA molds and over-fermented tempeh revealed that isolates of molds could grow on YMA and MEA selection media. They further analyzed them with a moist chamber to identify their types and morphology. The isolation and identification of molds in the moist chamber can be seen in Table 2.

The results obtained from the isolation and identification of molds in the moist chamber showed physical characteristics of spongiospora, sporangium, mycelium, and rhizoids (Thanh *et al*., 2007; Wipradnyadewi *et al*., 2011). The microscopic characteristics observed included the color of the conidia, the color of the mycelium, and the shape of the resulting conidia. On the color of the conidia, the results showed a yellowish black color and a grayish-black color. The resulting color revealed the dominant color characteristics produced by *Rhizopus* sp. molds (Jennessen *et al*., 2008). The color of the conidia in *Rhizopus* sp was brownish gray. These molds are commonly found in the tempeh and inoculum used (Wipradnyadewi *et al*., 2011). The results obtained in this method indicate that the molds growing were specific YMA and MEA selection media. In this case, the media were a selective medium for the growth of microorganisms of the *Rhizopus* sp.

The color of the mycelium molds produced was white, and the mycelium made in the isolate had a non-septate hyphae structure with smooth hyphae. The mycelium characteristics of this isolate gave the physical characteristics of the tempeh produced (Handoyo and Morita, 2006). The mycelium isolate can form cotton-like look, so the Tempeh characteristics were compact cakes and white due to the color of the mycelium produced (Barus *et al*., 2019).

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The morphological involvement of length and diameter sporangiophore's by slide culture techniques (Hartanti *et al.*, 2020). The shape of the columella produced in this isolate was round, tending to oval, and large at 50 - 40 µm. *Rhizopus* sp. has dark-colored stolons and rhizoids, large and black sporangia, slightly rounded columella, and cup-shaped apophysis with vegetative hyphae penetrating the substrate (Fardiaz, 1992). The sporangiophore *Rhizopus* sp. measures mostly within 1,000 µm and 1,700 µm in length (Hartanti *et al.*, 2020).

Based on microscopic morphological characteristics, the results obtained on isolates indicated white-gray colonies with a height of about 1 mm. In addition, sporangiophores could be single or in groups with a sub headline of a brownish color. The rhizoids in these molds were very short, smooth, or slightly rough-walled, with up to 1000 m length and 10-18 m in diameter. Sporangia were spherical, brownish-black when ripe, and 100-180 m in diameter. The columella was spherical to semi-spherical in shape with an oval shape. Sporangiaspores were round, elliptical, or irregular, 7-10 mm in length, forming a brownish mass (Jennessen *et al*., 2008; Febriani *et al*., 2018). The microscopic morphology indicated *Rhizopus* sp, but the specific species in these molds had not been confirmed.

3.3 Molds isolate identification by application programming interface test

The identification of mold isolates with the API test 20C AUX showed that the two isolates were included in *Rhizomucor* species. It showed some changes in the biochemical characteristics in the well of API test, which was positive results (bioMérieux SA, 2010). Positive (+) results have occurred in the active ingredients of API 20 AUX in *Rhizomucor* species, such as Galactose, N-Acetyl-D-Glucosamine, Cellobiose, maltose, trehalose, sorbitol, xylose, glycerol, and glucose (Moreira *et al.*, 2020).

Rhizomucor is one of the genera of zygomycetes, including *R. oligosporus*, *R. oryzae* and *R. stolonifer*. These fungi are primarily saprophytic fungi and capable of assimilating several different sugars such as glucose, mannose, galactose, xylose, arabinose, cellobiose, and even some polymers these sugars (Millati *et al*., 2005; Sivakanthan *et al*., 2019). The genera *Rhizopus*, *Mucor*, and *Rhizomucor*, belong to the order Mucorales. The three genera can produce molds that are not septum, have asexual spore structures called sporangiospores, and are found in sporangium (Osuntokun *et al*., 2018; Walther *et al*., 2019).

Rhizomucor can produce enzymatic compounds that inhibit aflatoxins and play an essential role in fermentation. *Mucor* sp. and *Rhizopus* sp. can produce hydraulic enzymes, including protease, amylase, and lipase. In the fermentation process, the enzyme produced by this type of mold is rennin, a crucial compound in cheese making. The lipase enzymes produced by molds can be used to produce chitosan (Purwijantiningsih *et al*., 2005; Sivakanthan *et al*., 2019).

In the tempeh fermentation process, *Rhizomucor* sp. is one of the molds playing a role in shaping the physical characteristics of tempeh. Other molds found in tempeh include *R. oryzae*, *R. oligosporus*, *Mucor indicus*, *Mucor circinelloides*, *Geotrichum candidum*, *Aureobasidium pullulans*, *Alternaria alternate* and *Cladosporium oxysporum* (Nout and Kiers, 2005; Sanjukta and Rai, 2016; Pangastuti *et al*., 2019). However, the mold *Rhizomucor* sp. is not included in the dominant type of molds in tempeh. The molds *R. oryzae* and *R. oligosporus* were the most dominant molds in tempeh (Wipradnyadewi *et al*., 2011).

3.4 Identification molds isolate by polymerase chain reaction test

Two isolates were successfully isolated, followed by molecular analysis using PCR test. The results of PCR test based on homology equations showed that two isolates, such as KP1 (RAPRIMA molds) and KP2 (over -fermented tempeh), were 100% similar with *R. microsporus* (CBS 631.82). The result indicated similarities between the isolates and *R. microsporus* were identical, so the isolates identified were *R. microsporus* molds. The phylogenetic tree produced on the isolate of Raprima mold from LIPI Indonesia and the over-fermented tempeh isolate can be seen in Figure 1.

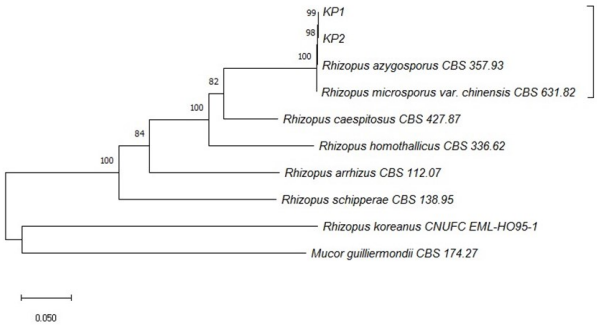

Figure 1. Phylogenetic tree of isolate molds Raprima "LIPI" Indonesia (code sample: KP1) dan Over-fermented tempeh 60 hrs (code sample: KP2) with ITS-1 dan ITS-4 rDNA Primers

Rhizopus microsporus is a significant microorganism in the tempeh fermentation process. The molds found in Indonesian tempeh from various regions generally contain *R. microsporus* (Hartanti *et al*., 2015). The type of molds or inoculum in tempeh fermentation is referred to as laru tempeh. Besides *R. microsporus*, other types of

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molds, including *R. arrhizus*, *R. foormosaensis*, *Rh. oryzae*, *Rhizomucor*, and *R. stolonifer*, have a role in the fermentation of beans into tempeh (Barus *et al*., 2019).

Rhizopus microsporus can produce enzymes, including lipase, protease, and amylase. This type of mold will degrade protein, fat, and carbohydrates in legumes into simple components, so that the nutrients produced are easier to digest (Starzyńska-Janiszewska *et al*., 2015; Barus *et al*., 2019). Lipase enzymes in molds have a role in lipid degradation to produce fatty acids, in which fatty acids in the fermentation process will affect the color and texture of tempeh. Protease enzymes have a role in degrading proteins or polypeptides into free amino acids, in which amino acids will affect the taste and aroma of tempeh. Amylase enzyme acts as a starch degrader, reducing sugars that will be used as a carbon and energy source (Benabda *et al*., 2019).

The type of mold used in the process of fermenting is very decisive due to the characteristics and quality of tempeh (Barus *et al*., 2019). The types of molds have their respective roles in the fermentation process. In addition, the type of mold will also affect the nutritional value providing benefits to the body.

4. Conclusion

Identification and characterization of molds in tempeh fermentation affect the characteristics and the quality of tempeh. The mold identification method in RAPRIMA molds and over-fermented tempeh had three steps such as propagation, isolation, and testing of physiological properties by microscopic. The two mold isolates characterized by biochemical API 20C AUX were *Rhizomucor* and indicated by molecular PCR Test were *Rhizopus microsporus*. *Rhizomucor* and *Rhizopus microporous* isolates gave characteristics quality of tempeh, such as nutrition and physical characteristics.

Conflict of interest

The authors declare no conflict of interest.

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