Genotypic Characterization of Rhizopus species from Tempeh and Usar: Traditional Inoculum of Tempeh in Indonesia

TATI BARUS1*, JASON WIRANATA SANJAYA2, DAVID TANDJUNG2, ANASTASIA TATIK HARTANTI1, ADI YULANDI1, AND VIVITRI DEWI PRASASTY2

1Master of Biotechnology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jakarta 12930, Indonesia; 2Department of Biology, Faculty of Biotechnology, Universitas Katolik Atma Jaya, Jakarta 12930, Indonesia;

Soybeans tempeh (tempeh) is processed by fermentation using Rhizopus spp. Tempeh is an important source of protein in Indonesia. The traditional inoculum in tempeh fermentation locally is known as Usar, which is made from the leaves of Hibiscus tiliaceus. However, Rhizopus information from Usar is still limited. Therefore, this study aims to identify and investigate the genetic diversity of Rhizopus species from Usar and tempeh based on the Internal Transcribed Spacer (ITS) sequences and the Random Amplified Polymorphic DNA (RAPD) markers. Twenty-three Rhizopus strains were isolated from Usar and ten Rhizopus strains were isolated from tempeh. Based on ITS sequences, the isolates were similar to Rhizopus microsporus (30 isolates) and Rhizopus delemar (3 isolates) with 98-99% similarity. The genetics of R. microsporus and R. delemar are varied and different from the genetics of R. microsporus from tempeh. The growth temperature of R. microsporus varies from 33°C to 48°C and R. delemar can grow to a maximum at 33°C. This research needs to be continued to obtain information about the role of Rhizopus from this study in determining the quality of tempeh.

Key words: diversity, ITS, RAPD, Rhizopus, tempeh

Tempe kedelai (tempe) diolah melalui fermentasi menggunakan Rhizopus spp. Tempeh adalah salah satu sumber protein penting di Indonesia. Inokulum tradisional dalam fermentasi tempe dikenal sebagai Usar yang terbuat dari daun Hibiscus tiliaceus. Namun, informasi Rhizopus dari Usar masih terbatas. Oleh karena itu, penelitian ini bertujuan untuk mengidentifikasi dan mengkaji keragaman genetik spesies Rhizopus dari Usar dan tempeh berdasarkan urutan sekuen Internal Transcribed Spacer (ITS) dan penanda Random Amplified Polymorphic DNA (RAPD). Dua puluh tiga Rhizopus strains were isolated from Usar dan sepuluh strain Rhizopus dari tempeh. Berdasarkan ITS sequences, isolates were similar to Rhizopus microsporus (30 isolates) dan Rhizopus delemar (3 isolates) with 98-99% similarity. Genetik R. microsporus dan R. delemar bervariasi dan berbeda dari genetic R. microsporus dari tempeh. Suhu pertumbuhan R. microsporus bervariasi dari 33°C hingga 48°C dan R. delemar dapat tumbuh hingga maksimum pada 33°C. Penelitian ini perlu dilanjutkan untuk mendapatkan informasi tentang peran R. microsporus dan R. delemar dari penelitian ini dalam menentukan kualitas tempeh.

Kata kunci: ITS, keragaman, RAPD, Rhizopus, tempeh

Soybeans tempeh (tempeh) is a traditional fermented food from Indonesia. It has been consumed as main source protein by Indonesian for years. It contains essential compounds such as vitamin B12 (Keuth and Bisping 1994), isoflavon and essential fatty acids. It has also been reported that tempeh have many health benefits such as in preventing free radicals (Esaki et al. 1996). Tempeh can prevent diarrhea (Sudigbia 1999) and anemia (Astuti 1999) because of increased iron availability during fermentation. Tempeh can also stimulate the formation of good bacteria populations in the intestines (Stephanie et al. 2019).

Tempeh is made through the fermentation of soybean, mainly by Rhizopus spp. Therefore, Rhizopus spp. is known as an economically important mold in Indonesia. Many species of Rhizopus spp. such as R. oligosporus, R. oryzae, R. arrhizus, and R. stolonifer were previously identified from Indonesian tempeh (Dwidjoeputro and Wolf 1970). In the current taxonomic system, R. arrhizus is considered as a synonym of R. oryzae (Abe et al. 2010), R. oligosporus as synonym of R. microspores (Dolatabadi et al. 2014), and R. oryzae as synonym of R. delemar (Abe et al. 2007). At present, tempeh producers rarely use Usar (traditional inoculum) (Fig 1) because they generally use commercial inoculum. Usar is made by growing Rhizopus spp. on Hibiscus tiliaceus leaves for 24 hours and then dried. After that,
it is ready to be used as an inoculum in tempeh fermentation. However, information about *Rhizopus* spp. from Usar was not yet available.

Many molecular techniques are available to study genotypic characterization of *Rhizopus* (Abe et al. 2007). To identify *Rhizopus* spp. often done based on ITS sequences (Iwen et al. 2002; Lott et al. 1998). Abe et al. (2007) reported the classification of three *Rhizopus* species, i.e. *R. microsporus*, *R. stolonifer*, and *R. oryzae*. Therefore, in this study ITS sequences were used to assess the genetic diversity of *Rhizopus* isolates from Usar (Fig 1) and tempeh.

Beside ITS sequences, various molecular methods have been developed to get more accurate data on the genetics of an organism. One of them is random amplified polymorphic DNA (RAPD) marker using polymerase chain reaction (PCR). RAPD markers have been successfully used in assessing differentiating fungal genetic diversity, such as the genetic diversity of *Colletotrichum* spp. (Mahmodi et al. 2014) and *Rhizopus stolonifera* (Vágvölgyi et al. 2004). Therefore, this study aims to identify and investigate the genetic diversity of *Rhizopus* species from Usar and tempeh based on the Internal Transcribed Spacer (ITS) sequence and the Random Amplified Polymorphic DNA (RAPD) markers.

**MATERIALS AND METHODS**

*Rhizopus Isolation.* *Rhizopus* isolates have been isolated from Usar were taken from Yogyakarta, Central Java-Indonesia. Usar sampling was carried out from Yogyakarta because to our knowledge that only in this area Usar was still used as an inoculum in tempeh production. A total 10 tempeh is collected from Yogyakarta and Solo, Central Java-Indonesia. All these tempeh are produced using commercial inoculums. A total of 23 pieces of Usar that grow *Rhizopus* has been cut off and homogenized in sterile 0.85% w/v NaCl by the use of a Stomacherlab-blender 400 (Seward Medical, London, UK) for 1 minute. All isolates were grown on potato dextrose agar (PDA) and incubated at 28°C. All suspected *Rhizopus* isolates were stored at 4°C for further analysis.

**DNA Extraction.** Genomic DNAs of *Rhizopus* isolates were extracted from four days-old mycelia grown on PDA using the Phytopure™ DNA Extraction Kit (GE Healthcare, UK) according to the manufacturer's protocol. DNAs were visualized on 1% electrophoresis agarose gel (Promega, Madison, USA) then stained with ethidium bromide (Sigma-Aldrich, USA).

**Amplification of Its Region and Sequencing.** Amplification of ITS region was performed using the GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) and the primer pair ITS4 (5′–TCCTCCGCTTATTGATATGC–3′) and ITS5 (5′–GGAAGTAAAAGTCGTAACAAGG–3′) (White et al. 1990). A total 50 µL of reaction mixtures were used, containing 1 µL DNA template, 10 µL 5X KAPA Taq EXtra Buffer, 2.5 µL of each primer, 1.5 µL 10 mM dNTPmix, 3.5 µL MgCl2, 28.5 µL nuclease-free water (NFW), and 0.5 µL KAPA Taq EXtra HotStart DNA Polymerase. PCR conditions were set as follow: initial denaturation at 94 °C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing at a temperature of 55°C for 30 seconds, and extension at 72°C for 1 minute. Final elongation was set at 72°C for 5 minutes. PCR products were visualized on 1% agarose gel and stained with ethidium bromide. PCR products were then partially sequenced at Macrogen Inc., Republic of Korea. The DNA sequencing results were compared to the GenBank database using BLASTN (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree was constructed using MEGA7. The branch support was analyzed by 1000x bootstrap analysis.

**Growth of Rhizopus Isolates on Various Temperature.** All isolates were grown on potato dextrose agar (PDA). To determine the optimal growth temperature, all *Rhizopus* isolates obtained in this study were grown on various temperature, i.e. 33°C,
Amplification of Random Amplification of Polymorphic DNA. Amplifications of RAPD markers were conducted using single six primers (Table 1) through GeneAmp® PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA). Amplification of RAPD was carried out with a total of 25 μL of the reaction mixture with the same composition carried out with Barus et al. (2019). Amplifications of RAPD markers were performed with the same condition also with Barus et al. (2019). Annealing conditions were done based on the melting temperature of each primer (Table 1). Products of amplification were separated by electrophoresis in agarose gel (1% w/v). The agarose gel was stained with ethidium bromide and UV transilluminator was used to visualize the PCR products in agarose gel. The 1 kb ladder (Fermentas) was used as weight marker. Clearly resolved each band was manually scored for the presence (1) or absence (0) to make binary data. Dendrogram analysis among all the Rhizopus was computed using Roderic D.M. Page software. The unweighted pair group method analysis (UPGMA) was used for clustering and Tree View software was used for interactive visualization of the dendrogram.

RESULTS

A total of twenty-three Rhizopus isolates had been isolated from Usar (TB23-TB45) and ten Rhizopus isolates had been isolated from tempeh (TB46-TB55) (Fig 2). ITS sequences were successfully amplified and each PCR amplification showed DNA fragments with single band at 700 bp (Fig 2). BLASTN results of ITS sequence (± 600 nucleotides) showed that 30 isolates (TB23-TB25, TB27, TB29-TB36, TB38-TB55) were Rhizopus microsporus with similarity about 98-100%. Only three isolates (TB26, TB28, TB37) were Rhizopus delemar with similarity about 98-100%. All the ITS sequences have been submitted to GenBank with accession numbers MF445258 - MF445290.

The phylogenetic tree based on the ITS sequences showed that the thirty three of Rhizopus isolates were divided into two clusters (Fig 3). The first cluster consisted of R. microsporus (30 isolates) and the second cluster consisted of R. delemar (3 isolates).

All Rhizopus spp. isolates were grown at 33°C, 42°C, 45°C, and 48°C. The growth temperature for all isolates R. microsporus varied. Eight strains (Table 2) of R. microspores could grow up to 48°C, thirteen strains could grow up to 45°C, seven strains could grow up to 42°C, and two strains could grow up to 33°C. Conversely, three R. delemar isolates could only grow up to 33°C.

Genomic DNAs isolated from 33 Rhizopus isolates were subjected to obtain RAPD-PCR markers using six primers (Table 1), but only 9 out of 33 Rhizopus isolates produced distinct and reproducible band RAPD marker using these primers. The dendogram (Fig 4) describes the genetic similarity R. microsporus and R. delemar was successfully created. UPGMA dendrogram based on RAPD – PCR separated the R. microsporus and R. Delemar in two main clusters. Among all R microsporus, the smallest genetic similarity (GS) (35%) was found between TB34 and TB35 and the largest GS (63%) was found between TB32 and TB33. R. microsporus from tempeh (TB49) is most similar to TB32 with GC 54% and most different with TB34 with GC 38%. R. delemar TB26 and R. delemar TB37 have genetic similarity 64%.

DISCUSSION

A study by Bressa et al. (2017) showed that lifestyle enhanced health-promoting bacteria. A previous
reported that gut microbiota in obese subjects and/or with Type-2 Diabetes were different from lean and non-diabetic subjects (Patterson et al. 2016). To get beneficial gut microbiota population, probiotics consumption and dietary fibers are strongly recommended. Holscher (2017) reported that low fiber intake is associated with increased chronic diseases, such as obesity, cardiovascular disease, type 2 diabetes, and colon cancer. Tempeh, a popular fermented food in Indonesia, is one source of fiber-rich food.

The main microorganism in fermentation of tempeh is Rhizopus spp. At present, many molecular techniques are available for identification of Rhizopus spp. However, internal transcribed spacer (ITS) is often used (Abe et al. 2003. Based on ITS sequence showed that 30 isolates (TB23-TB25, TB27, TB29-TB36, TB38-TB55) were Rhizopus microsporus with similarity about 98-100% and three isolates (TB26, TB28, TB37) were Rhizopus delemar with similarity about 98-100%. The ITS regions have become an important molecular target for fungal taxonomy and identification (Iwen et al. 2002). Due to greater sequence variations, the ITS domains are more suitable for species identification (Iwen et al. 2002; Lott et al. 1998). Therefore ITS sequences are widely used to identify and assess fungal genetic diversity.

**Table 2 Growth on various temperatures of thirty-three Rhizopus isolates isolated from Usar and tempeh.**

| Source | Isolate code | Species          | Growth temperature (°C) |
|--------|--------------|------------------|-------------------------|
|        |              |                  | 33 | 42 | 45 | 48 |
| Usar   | TB24         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB25         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB31         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB32         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB33         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB39         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| tempeh | TB48         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| tempeh | TB54         | R. microsporus   | ✔  | ✔  | ✔  | ✔  |
| Usar   | TB23         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB27         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB30         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB35         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB36         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB38         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB40         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB43         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB45         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| tempeh | TB46         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| tempeh | TB52         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| tempeh | TB53         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| tempeh | TB49         | R. microsporus   | ✔  | ✔  | ✔  | -  |
| Usar   | TB29         | R. microsporus   | ✔  | ✔  | -  | -  |
| Usar   | TB34         | R. microsporus   | ✔  | ✔  | -  | -  |
| Usar   | TB41         | R. microsporus   | ✔  | ✔  | -  | -  |
| Usar   | TB42         | R. microsporus   | ✔  | ✔  | -  | -  |
| Usar   | TB44         | R. microsporus   | ✔  | ✔  | -  | -  |
| tempeh | TB47         | R. microsporus   | ✔  | ✔  | -  | -  |
| tempeh | TB51         | R. microsporus   | ✔  | ✔  | -  | -  |
| tempeh | TB50         | R. microsporus   | ✔  | ✔  | -  | -  |
| tempeh | TB55         | R. microsporus   | ✔  | ✔  | -  | -  |
| Usar   | TB26         | R. delemar      | ✔  | -  | -  | -  |
| Usar   | TB28         | R. delemar      | ✔  | -  | -  | -  |
| Usar   | TB37         | R. delemar      | ✔  | -  | -  | -  |
Fig 2 Results of PCR amplification sequences of internal transcribed spacer (ITS) sequence of Rhizopus isolates. M: Marker 1-kb lambda ladder. TB23-TB45: Rhizopus isolates from Usar. TB46-TB55: Rhizopus isolates from tempeh.

Fig 3 Phylogenetic tree generated from the internal transcribed spacer (ITS) sequences of 33 isolates of Rhizopus species isolated from Usar and tempeh.
In the past, it was reported that various *Rhizopus* species were used to make tempeh (Dwidjoseputro and Wolf 1970). In this study it was shown that only *R. microspores* were found in tempeh samples. This finding is similar to the report by Hartanti *et al.* (2015), where tempeh collected from 28 locations throughout Indonesia only contained *R. microspores*. This is caused by the use of commercial inoculums that only contain the *R. microsporus*.

Surprisingly, in this study found three isolates of *R. delemar* from Usar. Based on the RAPD marker (Fig 4), TB26 and TB27 are in the same cluster, but they are different types represented by different RAPD markers. Information on *R. delemar* in tempeh is still limited. Therefore, the role of *R. delemar* in determining the quality of tempeh needs to be further investigated. The species of *Rhizopus* may have an important contribution to the variety of tempeh flavor and nutritional value. The different species of *Rhizopus* have different metabolic activities. Moreover, it has been reported that *R. delemar* produced fumaric acid and malic acid (Abe *et al.* 2007).

Figure 3 showed that ITS sequences were not sufficient to distinguish *R. microsporus* up to the variety level. This can be seen from the phylogenetic tree which *R. microsporus* var. *azygosporus*, *R. microsporus* var. *chinensis*, *R. microsporus* var. *oligosporus*, *R. microsporus* var. *rhizopodiformis*, and *R. microsporus* var. *tuberosus* were all grouped as one cluster (Cluster 1). This indicated that the ITS sequences were not sufficient to distinguish *R. microsporus* up to the variety level. This report is in line with Hartanti *et al.* (2015) which ITS sequences were not sufficient to distinguish *R. microsporus* species from tempeh up to the variety.

*Rhizopus* is the main microorganism in making tempeh. Information about the growth of *Rhizopus* isolates on various temperature is important as a basis for selecting isolates to be used as inoculums in tempeh fermentation. The growth temperature for all isolates *R. microsporus* strains varied (Table 2). This was found that *Rhizopus microsporus* (TB32) can grow up to 48°C and *Rhizopus microsporus* (TB55) can grow up to 32°C (Table 2). Barus *et al.* (2019) reported that *Rhizopus microsporus* (TB32) produced tempeh with higher antioxidant activity compared to *Rhizopus microsporus* (Tb55).

Figure 4 showed that RAPD markers can show genetic variation in nine *Rhizopus*. Previously it has been reported that RAPD marker can be used as an important technique to investigate for the genetic variations of fungal (Dwivedi *et al.* 2018). These reported are in line with our result, where RAPD marker can also distinguish the genetic variations of nine *R. microsporus* and two *R. delemar* well.

Genetic of all *R. microsporus* isolate from Usar (TB26, TB30, TB32-TB37) were different from the genetic *R. microsporus* from tempeh (TB49). Furthermore, the genetics of *R. microsporus* and *R. delemar* derived from Usar also varied. RAPD markers of 24 isolates (TB 23-TB25, TB27-TB29, TB31, TB38, TB39-TB48, TB50-TB55) have not been successfully amplified using several primers (Table 1) even though they have been repeated several times. This might be accessible using another primer.

**ACKNOWLEDGEMENTS**

This study was funded by the Competitive Grant Program of Atma Jaya Catholic University of Indonesia.
REFERENCES

Abe A, Asano K, Sone T. 2010. A molecular phylogeny-based taxonomy of the genus *Rhizopus*. Biosci Biotechnol Biochem 74(7): 1325-1331. doi: 10.1271/bbb.90718.

Abe A, Oda Y, Asano K, Sone T. 2007. *Rhizopus delemar* is the proper name for *Rhizopus oryzae* fumaric-malic acid producers. Mycologia 99(5): 714-722. doi: 10.3852/mycologia.99.5.714.

Astuti M. 1999. Iron availability of tempe and uses in iron deficiency anemia. The Complete Handbook of Tempe: The Unique Fermented Soyfood of Indonesia 41-45.

Barus T, Halim R, Hartanti AT, Saputra PK. 2019. Genetic diversity of *Rhizopus microsporus* from traditional inoculum of tempeh in Indonesia based on ITS sequences and RAPD marker. Biodiversitas Journal of Biological Diversity 20(3): 847-852. doi: 10.13057/biodiv/d200331.

Bressa C, Bailén-Andrino M, Pérez-Santiago J, González-Soltero R, Pérez M, Montalvo-Lominchar MG, Maté-Muñoz JL, Domínguez R, Moreno D, Larrosa M. 2017. Differences in gut microbiota profile between women with active lifestyle and sedentary women. PLoS One 12(2): 1-20. doi: 10.1371/journal.pone.0171352.

Dolatabadi S, Walther G, Van Den Ende AG, De Hoog G. 2014. Diversity and delimitation of *Rhizopus microsporus* from traditional inoculum of tempeh in Indonesia based on ITS sequences and RAPD marker. Biodiversitas Journal of Biological Diversity 20(3): 847-852. doi: 10.13057/biodiv/d200331.

Hartanti AT, Rahayu G, Hidayat I. 2015. *Rhizopus* species from fresh tempeh collected from several regions in Indonesia. Hayati Journal of Biosciences 22(3):136-142. doi: 10.4308/hjb.22.3.136.

Holscher HD. 2017. Dietary fiber and prebiotics and the gastrointestinal microbiota. Gut microbes 8(2):172-184. doi: 10.1080/19490976.2017.1290756.

Iwen PC, Hinrichs SH, Rupp ME. 2002. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Medical mycol. 40(1): 87-109. doi: 10.1080/mmny.40.1.87.109.

Keuth S, Bisping B. 1994. Vitamin B12 production by *Citrobacter freundii* or *Klebsiella pneumoniae* during tempeh fermentation and proof of enterotoxin absence by PCR. Appl Environ Microbiol. 60(5): 1495-1499. doi: 10.1128/AEM.60.5.1495-1499.1994.

Lott TJ, Burns BM, Zancopé-Oliveira R, Elie CM, Reiss E. 1998. Sequence analysis of the internal transcribed spacer 2 (ITS2) from yeast species within the genus Candida. Current microbiology 36(2): 63-69. doi: 10.1007/s002849900280.

Mahmodi F, Kadir J, Puteh A, Pourdad S, Naseh A, Soleimani N. 2014. Genetic diversity and differentiation of *Colletotrichum* spp. isolates associated with Leguminosae using multigene loci, RAPD and ISSR. The Plant Pathology Journal 30(1): 10-24. doi:10.5423/PPJ.OA.05.2013.0054.

Patterson E, Ryan PM, Cryan JF, Dinan TG, Ross RP, Fitzgerald GF, Stanton C. 2016. Gut microbiota, obesity and diabetes. Postgraduate Medical Journal 92(1087):286-300. doi: 10.1136/postgradmedj-2015-133285.

Stephanie, Kartawidjajaputra F, Silo W, Yogiara, Suwanto, A. 2018. Tempeh consumption enhanced beneficial bacteria in the human gut. Food Res. 3(1):57-63. doi: 10.26656/fr/3.1.2018.230.

Sudigbia I. 1999. Tempe in the management of infant diarrhea in Indonesia. The Complete Handbook of Tempeh: the Unique Fermented Soyfood of Indonesia 23-40.

Vágvölgyi C, Heinrich H, Ács K, Papp T. 2004. Genetic variability in the species *Rhizopus stolonifer*, assessed by random amplified polymorphic DNA analysis. Antonie van Leeuwenhoek 86(2): 181-188. doi: 10.1023/B:ANTO.0000036123.48215.fc.