

Early Detection of Endobacteria in *Rhizopus* spp.

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Abstract: *Rhizopus* spp. are commonly used in bio-industrial processes such as manufacturing traditional fermented foods. However, one species, *R. microsporus* has been reported to contain toxin-producing endobacteria. Therefore, it is essential to evaluate the *Rhizopus* strains' safety before application as tempe inoculum. The study aimed to detect the presence of endobacteria from seven strains of *Rhizopus* spp. isolated from Tempe and four strains from other substrates using molecular and staining techniques. Molecular analyses were conducted using a PCR approach of 16S rDNA with primer sets 10F and 1541R. The presence of endobacteria was confirmed with the LIVE /DEAD® bacterial viability kit. The bacteria were identified molecularly by a phylogenetic approach using the Neighbour-Joining method and p-distance model in MEGA 6 with species of endo hyphal bacteria of *Mortierella elongata* as an outgroup. Molecularly, only *R. microsporus* IPBCC 13.1131 isolated from tempe in Cilacap, Central Java, was found to contain endobacteria. The presence of endobacteria was confirmed by the appearance of the red and green luminescence in hyphae, not in the spores. These endobacteria are identified as *Curtobacterium* sp. The study is the first report of described endo hyphal *Curtobacterium* from *R. microsporus* tempe starter.

Keywords: Endobacteria, *Rhizopus* spp., Tempe, *Curtobacterium*

Introduction

Rhizopus spp. such as *Rhizopus oligosporus*, *R. oryzae*, *R. arhizus*, *R. stolonifer* and *R. microsporus* (Zygomycota, Mucorales, Mucoraceae) (Schipper and Stalpers, 1984) were abundant in various substrates in nature, often used as inoculants in the soybean tempe industry (Nout and Rombouts, 1990; Astuti *et al.*, 2000; Tamam *et al.*, 2019). In addition, those species also play an essential role in other bio-industries, such as steroids (Donova, 2021), amylase (Ait Kaki El-Hadef El-Okki *et al.* (2017), protease (Negi *et al.*, 2020), lipase (Satomura *et al.*, 2015) and various organic acids production (Ghosh and Ray, 2011).

Intriguingly, some strains of *R. microsporus* are associated with bacterial endosymbionts. These bacteria have been demonstrated to modify host morphology, sporulation, metabolite synthesis, and other symbiotic relationship-related characteristics. According to Partida-Martinez *et al.* (2007a), the symbiotic bacterium from the genus *Burkholderia* is in charge of the production of rhizoxin and the toxic cyclopeptide rhizonin. The toxins

harm humans, animals, and plants (Dolatabadi *et al.*, 2016). Lackner and Hertweck (2011) demonstrated that *R. microsporus* contained symbionts will attacks rice plants and illicit rice seedling blight, while non toxinogenic strains did not. Recently Itabangi *et al.* (2022) reported that a bacterial endosymbiont of the fungus *Rhizopus microsporus* drives phagocyte evasion and opportunistic virulence. The order of Mucorales members is sometimes colonized or influenced by endosymbiotic bacteria. For example, *Mortierella elongata* were also reported to harbor endobacteria similar to *Candidatus glomeribacter* which produces endotoxins

Lackner *et al.* (2009) suggested that fungi with industrial potential should be endobacteria-free. Therefore, for tempe production, the starters should include only food-grade microbes and not produce harmful metabolite. The presence of endobacteria must be detected and the protocol for detecting endobacteria on *Rhizopus* spp. must be developed.

Currently, methods for detecting endobacteria include molecular analysis and staining as performed by Partida-Martinez and Hertweck (2005); Sato *et al.* (2010);

Hoffman and Arnold (2010). In addition, molecular analysis can be performed using Polymerase Chain Reaction (PCR) techniques and Fluorescent in Situ Hybridization (FISH) techniques (Hoffman and Arnold, 2010). Molecular analysis can detect endobacteria symbiosis by amplifying the 16S rDNA sequence. This approach takes advantage of the gene's unique variable regions, which are genus-or species-specific for all bacteria (Loong *et al.*, 2016) and differs from the rDNA sequence in eukaryotes. In addition, 16S rDNA sequences can facilitate the identification of bacteria that are difficult to identify conventionally (Woo *et al.*, 2008). Among the three methods, the PCR technique with universal bacterial primers and general staining is used to detect endobacteria of unknown species.

Despite the existence of identification techniques, there is no available information about the presence of endobacteria other than *B. rhizoxinica* in *R. microsporus* CBS 339.62 from Indonesian tempe (Dolatabadi *et al.*, 2016). Poisoning due to the consumption of tempe has never been reported. Therefore, it is suspected that *Rhizopus* spp. used as a starter was endobacteria-free. However, research on the presence of endobacteria in *Rhizopus* spp. needs to be carried out to guarantee the quality of tempe inoculants.

The present study aimed to detect the presence of endobacteria in *Rhizopus* spp. IPB-CC collections derived from tempe and other substrates are the basis for developing methods for detecting endobacteria on *Rhizopus* spp. and developing a quality control technique for a starter used in tempe production.

Materials and Methods

Fungal Strains

About eleven strains of *Rhizopus* spp. and one strain of *Rhizopodopsis javensis* from accession number IPB-CC (Table 1) were used in this study. Those seven strains are isolated from tempe from two main islands in Indonesia: Sumatra and Java, while others are isolated from fruit and plant. The fungal

strains were grown in potato dextrose agar to prepare the inoculum. The loosely-associated bacteria (potentially contaminating bacteria and exobacteria) from the fungal culture strains were removed using the modified van Tieghem method (Harrower, 1989). Mycelium was free of exobacteria and was grown on PDA media covered with a cellophane membrane.

Endobacterial Detection Through Staining Methods

Mycelium was free of exobacteria and was grown on PDA media covered with a cellophane membrane. First, the mycelium was harvested and mounted on a polycarbonate membrane filter (black filter with 0.2 µm pore size, Advantec, Tokyo, Japan) and stained for 15 min with a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA) (Boulos *et al.*, 1999). Next, the filter was rinsed with filter-sterilized (0.2 µm pore size, Advantec) distilled water and placed on a microscope slide. Preparations were observed under blue and green light with a fluorescence microscope (B ×51, Olympus, Tokyo, Japan) equipped with a Charge-Coupled Device (CCD) camera (E-620, Olympus).

Endobacterial Detection Through Molecular Methods

The fungal strain's DNA was extracted using CTAB (Cetyltrimethyl Ammonium Bromide according to Hidayat and Pinatih (2017). The DNA extract was then examined for quality DNA purity using a Nanodrop Spectrophotometer. DNA extracts were stored at -20°C before use. DNA extracts were stored at -20°C before use. PCR amplified the 16S RNA with the primer 10F (5'-AGTTTGATATCCTGGTCCAG-3') and 1541R (5'-AAGGAGGTGATCCAGCCG-3') (Sato *et al.*, 2009). The PCR mixture (10 µL) was prepared by combining 1× MyTaq HS Red Mix, 0.5 pmol primer, dNTPs, and buffer in a PCR cycler. The amplification conditions used were: Initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 15 sec each, attachment at 55°C for 15 sec and elongation at 72°C for 10 sec, followed by final elongation.

Table 1: Name, accession number, and substrate locality, of strains used in this study

No	Isolate name	No. accession	Substrate/host	Location
1	<i>Rhizopus</i> sp.	BN	Dragon fruit	Unknown
2	<i>Rhizopus</i> sp.	BN-B	Dragon fruit	Unknown
3	<i>R. microsporus</i>	IPBCC 13.1127	Tempe	Medan, North Sumatera Utara
4	<i>R. microsporus</i>	IPBCC 13.1128	Tempe	Labuhan Batu, North Sumatera Utara
5	<i>R. microsporus</i>	IPBCC 13.1131	Tempe	Cilacap, Central Java
6	<i>R. microsporus</i>	IPBCC 13.1137	Tempe	Jambi, Sumatra
7	<i>Rhizopus</i> sp.	R1	Tempe	Sukabumi, West Java
8	<i>Rhizopus</i> sp.	R2	Tempe	Malang, East Java
9	<i>Rhizopus</i> sp.	R3	Tempe	DI Yogyakarta
10	<i>R. stolonifer</i>	IPBCC 88.010	Unknown	CBS 110.17
11	<i>Rhizopus</i> sp.	R14	Ginger 1a-2-1	Cirebon, West Java
12	<i>Rhizopodopsis javensis</i>	R20	<i>Ficus</i> kecil 13	Bogor, West Java

At 72°C for 5 min. The PCR results were then electrophoresed on 1% agarose gel with 1× TAE buffer. Electrophoresis was carried out at 100 V for 25 min. Finally, the gel was immersed in EtBr (Ethidium Bromide) solution for 30 min and visualized using Syngene gel imaging. Endobacterial PCR products with staining results were sent to 1st base, Malaysia, for sequencing.

Identification and Phylogenetic Analysis of Endobacteria

Endobacterial sequences were edited with Seqtrace-0.9.0 software (Stucky, 2012), then endobacteria were identified with the BLAST (basic local alignment search tool) program on GenBank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

The identification is carried out by reconstructing the phylogenetic tree. The phylogenetic tree is built based on the sequences of endobacteria found and the sequences of bacteria from the genus within the same family. Endobacterial sequences that have been found in Mucoromycotina were selected as the outgroup. Phylogenetic tree reconstruction was carried out using the Neighbor-Joining model p-distance method in MEGA v 6 software with 1000× replications. Bootstrap values >60% are displayed in the tree.

Results

Endobacterial Detection Through Staining Methods

From ten strains of *Rhizopus* and one strain of *Rhizopodopsis* used in the study, the presence of endobacteria was observed only in *Rhizopus microsporus* IPBCC 13.1131. Microscopic observations using the LIVE/DEAD BacLight bacterial viability kit, which is specific for bacteria and can distinguish between live and dead cells, showed that bacteria colonized the fungal hyphae. Green fluorescent endobacteria move inside the hyphae.

Figure 1A represent on the observations result, showed that endobacteria were only present in hyphae. Endobacteria were not detected in the spores. Sporulation of *R. microsporus* IPBCC 13.1131 did not differ from other strains.

In addition to the presence of endobacteria, the LIVE/DEAD® Bacterial Viability Kit can be used to determine the viability of endobacteria. The green color luminesce indicated the endobacteria in *R. microsporus* were alive and the red glow indicated that the endobacteria were dead.

Even though the mycelium has been stained, the glow does not appear when the fluorescence microscopy is in an inactive state (Fig. 1B). In the negative control, luminescence was not seen on the hyphae when the fluorescence condition was active and inactive (Fig. 1C-D).

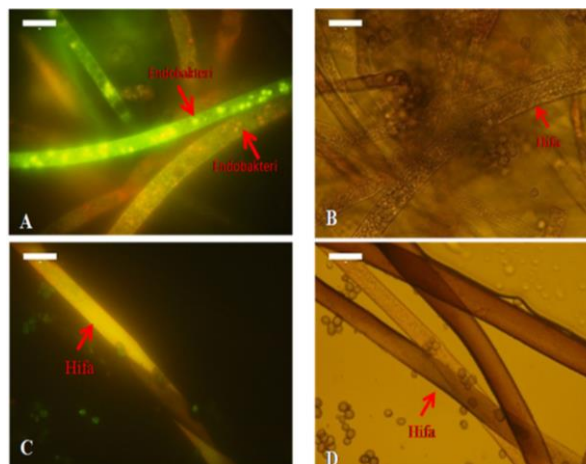


Fig. 1: Fluorescence microscope (B ×51, Olympus, Tokyo, Japan) observation. (A) Endobacteria on *R. microsporus* IPBCC 13.1131 fluorescence mode (B) Fluorescence mode off, (C) R20 mycelium visualization with fluorescence mode, (D) R20 mycelium visualization under full light observation (fluorescence mode off). Scale = 20 µm

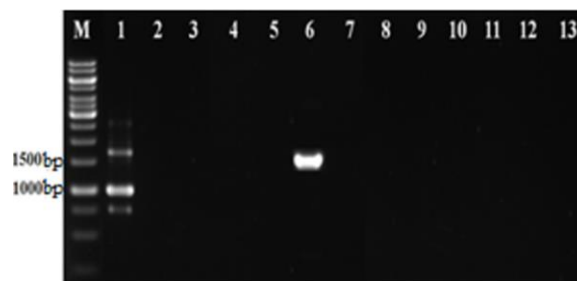


Fig. 2: Endobacterial 16S rDNA electropherogram of *Rhizopus* spp. and *Rhizopodopsis javensis* using 1% Agarose gel electrophoresis. Caption: M = marker 1 kb, numbers 1-13 respectively: Positive control: *E. coli*, endobacteria of *Rhizopus* spp. BN, BN-B, IPBCC 13.1127, IPBCC 13.1128, IPBCC 13.1131, IPBCC 13.1137, R1, R2, R3, IPBCC 88.010, R14 and *Rhizopodopsis javensis* R20

Endobacterial Detection Through Molecular Methods

In order to confirm endobacteria in *Rhizopus* spp., PCR amplification of the bacterial 16S rRNA gene was conducted using 4-10 ng of template DNA extracted from all fungal strains used (Fig. 2). The template DNA used in this study is a mixture of fungal and bacterial DNA, so the quality of the bacterial DNA is unknown. However, according to Sato *et al.* (2010) usually 10 ng of bacterial DNA templates is sufficient for use in PCR. A clear single band of endobacteria in this study was found with a template DNA concentration of 10 ng.

The positive control used during PCR was *Escherichia coli* DNA. The sticking temperature at the time of detection is 55°C. The *E. coli* DNA band in this study was seen as a double band.

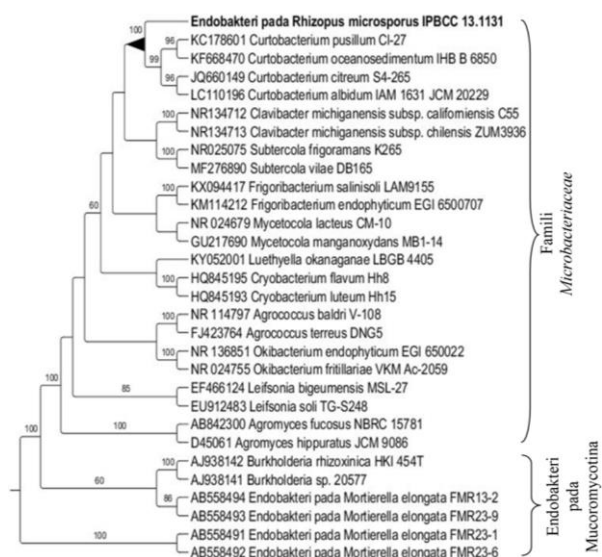


Fig. 3: Phylogenetic tree of the 16S rDNA gene of endobacteria in the hyphae of *Rhizopus microsporus* IPBCC 13.1131, a member of the Microbacteriaceae family and an endobacterium of Mucoromycotina. Bootstrap values >60% are displayed on branches

This might have occurred because the attachment temperature was too low or the concentration of DNA was too high. According to Ishikawa *et al.* (2000), the optimum attachment temperature for primers 9F and 1541R was 65°C with a DNA concentration of 0.1 ng. The electrophoresis result showed that the 16S. rRNA gene was well separated and aligned to the 1,500 bp in lane 6 for endobacteria in *R. microsporus* IPBCC 13.1131.

Based on their genomic sequences, the tree depicts the evolutionary connections between several bacterial taxa or lineages. The phylogenetic analysis (Fig. 3) revealed that the endobacteria in *R. microsporus* IPBCC 13.1131 existed as a single lineage in a clade containing *Curtobacterium* species. The bootstrap value of the clade branch containing endobacteria in *R. microsporus* IPBCC 13.1131 and *Curtobacterium* was 100%. However, the species of this endobacteria cannot be determined.

The endobacteria may have a more recent common ancestor than the other bacteria in the clade, according to this evidence. These endobacteria are closely related to *Burkholderia*, endobacteria found in Mucoromycotina and the endobacteria in *Mortierella elongata*. Higher bootstrap values which is >60% indicate stronger support for the branching pattern observed.

Discussion

A bacterial viability staining kit revealing the green illumination, which showed that cells still-alive and having an undamaged membrane observed in the

hyphae of *Rhizopus microsporus* IPBCC 13.1131. The bacteria reside in the cytoplasm of the hyphae within the fungal mycelium; there isn't a specific hyphal structure present. Different from this study, Lackner and Hertweck (2011) supported by Moebius *et al.* (2014) reported that endobacteria not only living inside hyphae, but can also be found in sporangium. They also stated that the presence of *B. rhizoxinica* in *R. microsporus* would affect spore formation. According to Partida-Martinez *et al.* (2007b), *R. microsporus* cultures that have endobacteria will sporulate while those that do not contain endobacteria do not form spores.

Strains with slight sporulation characteristics are preferred to be used as tempe starters. Therefore *R. microsporus* containing endobacteria must be freed from endobacteria before use. Even at low quantities, the production of toxins can have harmful effects, such as chronic or acute liver and kidney damage (Alshannaq and Yu, 2017). In addition *R. microsporus* is also associated with human infection (Kerr *et al.*, 1988; Chen *et al.*, (2021). Hyphae can be freed from exobacteria by growing the culture in 2% MEA media added with the antibiotic ciprofloxacin as much as 40 µg/mL (Hoffman and Arnold, 2010), Therefore only endobacteria left inside the hyphae.

To separate real fluorescence signals from general background fluorescence, the negative control is a crucial reference point. It helps confirm the viability evaluation based on the observed staining patterns and gives confidence in the correctness of the results from the experimental samples. This finding is in accordance with the results of Partida-Martinez and Hertweck (2005).

More thorough and reliable evidence of endobacteria within fungal hyphae can be acquired by combining fluorescent microscopy staining with PCR analysis. Fluorescence identifying can give a visual indication of where the bacteria might be located inside the hyphae, whereas PCR can verify their existence by specifically amplifying and sequencing their DNA. According to Hoffman and Arnold (2010), the LIVE/DEAD® Bacterial Viability Kit also colors the mitochondria and nucleus of the fungus. Therefore the staining method cannot be used as a standard method for detecting endobacteria but must be confirmed further by a PCR method.

In the PCR analysis, the size of the amplicon can also be an indication that what is being amplified from the mixed template DNA is endobacterial DNA. Endobacteria in *R. microsporus* IPBCC 13.1131 are 1557 bp in size, similar to the size of *Burkholderia* sp., endobacteria in *R. microsporus* found by Partida-Martinez and Hertweck (2005). Partida-Martinez *et al.* (2007a) found two species of *Burkholderia rhizoxinica*, endobacteria on *R. microsporus* var. chinensis isolated from rice seeds from Japan and *B. endofungorum*

isolated from *R. microsporus* obtained from peanuts from Mozambique. Both of these species have a 16S rDNA region of around 1525-1527 bp. *Candidatus glomeribacter endobacteria* discovered by Sato *et al.* (2010) on *M. elongata* also has an amplicon close to 1500 bp, which ranges from 1422-1440 bp. In order to confirm further, determination of bacterial species requires several analyzes, such as hybridization and fatty acid analysis (Spierings *et al.*, 1992).

Curtobacterium as endobacteria has been reported by Baltrus *et al.* (2017). However, no information about the host mold was mentioned. According to Bulgari *et al.* (2014), the genus *Curtobacterium* (family Microbacteriaceae) includes a wide range of bacteria isolated from different environments, such as soil, cheese vat, residential carpet, and plants. Most *Curtobacterium* is reported to live as endophytic symbionts in plants which are useful as decomposers and affect plant growth, except for *C. flaccumfaciens* which is reported as a plant pathogen (Chase *et al.*, 2016).

As many as 21% of 33 strains of *R. microsporus* (8 clinical strains, 20 strains of food origin, 4 strains from nature, and 1 strain of unknown origin) studied by Dolatabadi *et al.* (2016) contained endobacteria and among the strains examined, there were 9 strains from Indonesian tempe. *Curtobacterium* is a new record for the endohyphae bacteria *R. microsporus* from Indonesian tempe.

Endobacteria can produce toxins that harm human health, damaging the liver and kidneys, causing cancer, and even damaging nerves (Kabak *et al.*, 2006; Partida-Martinez *et al.* 2007a). Rohm *et al.* (2010) reported that toxins are produced when conditions in the tempe fermentation process are below standard. The standard conditions for tempe fermentation are around 30°C for 24 to 96 h. The toxin was identified after five days of the incubation process.

Conclusion

In conclusion tempe starter, as an inoculant containing *Rhizopus*, must be guaranteed free of harmful endobacteria. Therefore, detection from the results of this study can be proposed as a standard method for detecting endobacteria in *Rhizopus*, a bio-industrial agent as a tempe starter in Indonesia. The new genus of endobacteria, *Curtobacterium* associated with *R. microsporus* from Indonesian tempe in the world has been identified in this study. The function of these bacterial-fungal interactions and their intricate connection to the fungal-plant interaction may be the subject of further research.

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Author's Contributions

Ratna Noviyanti: Wrote the first draft of the manuscript and executed the research in the laboratory.

Gayuh Rahayu: Designed the research and approved the final manuscript.

Rida Oktorida Khastini: Managed the analyses of the study, literature searches, read and approved the final manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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