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Research Article

First Report of *Pediococcus acidilactici*: Bacterium Harbored in *Lysurus periphragmoides* Slimy Spore Mass

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ABSTRACT
Bacterium is a cosmopolitan microbe. It can be live everywhere and anywhere. They only need a simple nutrition from environment. Some of them also do a symbiosis for completing the nutrients. Lysurus periphragmoides was found in Arboretum in IPB University. The mushroom has a special structure for the head part of the fruiting body. On the head, the slimy spore mass was covering the part. The slimy was wet, sticky, and stinky. From the slime, bacterium was isolated using Luria Bertani Agar medium and incubated for 35C. Bacterium coded as Lyz222 was successfully isolated from the slimy spore mass. The colony looks like the Lactic Acid Bacteria with the specific odor smelled as milk odor. The colony was circular shape, milky white, concave surface, and mucoid density. The cell was coccus and as Gram-positive bacterium. Molecular identification was done for 16S rRNA. Then, the phylogenetic was constructed using RAxML analysis in CIPRES website. The bacterium strain Lyz222 was identified as Pediococcus acidilactici. The bacterial presence on the head part with slimy spore mass is because the insect or other animal that have touched the L. periphragmoides part. The animal or insect was harboring the bacterium from other source previously, before attaching the L. periphragmoides slimy spore mass. The Pediococcus was commonly found on the milk product, or recently report as on the fruits and vegetables. The result of this study is the first report that Pediococcus as P. acidilactici was found on the slimy spore mass.

Key words: *Cosmopolitan microbe, insect attaching, milk odor, slimy spore mass*

Introduction

Bacterium associated a mushroom has been reported mostly as a helper bacterium, such as ectomycorrhizal helper bacterium (Fitter & Garbaye, 1994; Frey-Klett et al., 2007). Generally, mycorrhizal helper bacteria (both for ectomycorrhiza and arbuscular mycorrhiza) strains classify from bacterial Gram-negative (Frey-Klett et al., 2005) and bacterial Gram-positive (Poole et al., 2001; Schrey et al., 2005). The bacteria enhance mycorrhizal function symbioses for plant and the nutrient uptake to the fungus and plant, and they help promote a defense

mechanism from other pathogen or unbeneficial microbes (Frey-Klett et al., 2007). One kind mushroom as Truffle fruiting body saved many bacteria as a dominant microbiome in all phases of truffle fruiting body (Liu et al., 2021). Then, some reports about a bacterium as a phyllosphere microbe on mushroom are not popular to be discussed.

Lysurus periphragmoides is known as stinky and horn mushroom and belongs to Phallaceae order (Caffot et al., 2018). Recently, *L. periphragmoides* was found in Indonesia (Hermawan et al., 2021). *L. periphragmoides* head contains a special liquid or slime that harbours many spores (Hermawan et al., 2021). The *Lysurus* fruiting body is very sterile of other microorganism when it was egg phase. During the development, this egg structure transforms as a real fruiting body that opens the sterile parts. One microorganism that very possible attach or live in this part is the bacterium. There are not any reports yet about the bacterium attached or harbored on the *Lysurus* slime. In this study, one species of bacterium was successfully isolated from the slime of *L. periphragmoides*. The bacterium identified as *Pediococcus acidilactici* through molecular study. This information is being a new and first report that the bacterium as *Pediococcus* was found in *L. periphragmoides* slime spore mass. Normally, the bacterium as *Pediococcus acidilactici* is found as prebiotic microorganism.

Materials and Methods

1. Bacterial Isolation

The slimy spore mass was collected from a fresh *Lysurus periphragmoides* fruiting body. The fruiting body was found by Hermawan et al. (2021). The slimy spore mass was mixed with sterile natrium chloride (NaCl) solution 0.85%. The suspension was centrifugated at 5.000 rpm for 45 minutes (Partida-Martinez et al., 2007). Then, the suspension was poured into Luria Bertani Agar (LA) medium by pour plate method (Sanders, 2012). The sample was incubated in 35 °C for three days. One type of colony was appeared, then it was picked and transferred to the new LA medium. The single cell purification was conducted by quadrant streak plate. The colony was only one type based on the morphological colony appearance and cell shape. The bacterium strain was coded as Lyz222.

2. Slime Spore Mass pH Checking

The slime was checked about the pH using Litmus paper test. The slime was diluted by distilled water (pH 7). The indicator paper would be changed according to the result test.

3. Morphological Observation

The bacterial strain Lyz222 was cultured in LA medium for morphological observation. The morphological observation was conducted for 2-days-incubation bacterial strain. The colony morphologies were observed as color, density, elevation, and shape. Then, the cell morphologies for shape, size and stained cell were observed too. The bacterium was stained using Gram Staining (Crystal Violet 1%, Iodine Solution 0.5%, Ethanol 95%, and Pfeiffer's Solution). The morphological observation was done, but not to clear distinguish and decide the genus or species. Then, the molecular identification was needed to ensure the genus till species.

4. Effect of Temperature Treatment on Growth

The pure bacterium was cultivated into many Luria Bertani Broth. The bacterium was inoculated onto tube containing LB medium. The various temperatures (30 °C, 33 °C, 35 °C, 37 °C, 40 °C, 43 °C, and 45 °C) were used in this study to check the temperature growth type. The incubation was conducted for 3 days. The indicator was the turbid colour changing. The LB medium without bacterial inoculation became a negative control.

5. Molecular Identification

The bacterium strain Lyz222 was cultured in Luria Bertani Broth (LB) medium. It was incubated in 35 °C for one day. Next, the bacterial suspension was centrifugated at 5.000 rpm for 20 minutes. The supernatant was removed from the tube. The bacterial cell as a pellet was mixed with CTAB solution. Then, the

next step followed the protocol of DNA extraction from Hermawan et al. (2020). The DNA concentration was used for 100 ng/µl for the amplification process. The DNA was amplified using bacterial primer, as 27F as a forward and 1492R as a reverse primer. The amplification was conducted with 40 µL of total reaction. The PCR mixture was included 20 µL PCR mix of 2X Kappa Fast 2G, 2 µL of 10 pmol of each primer, 4 µL 100 ng template DNA, and 12 µL ddH2O. the amplification was conducted using a Thermoline PCR.

The bacterial PCR program was following the condition by Mora et al. (1998), as follows: pre-denaturation at 94 °C for 5 min, denaturation at 94 °C for 30 seconds, annealing at 57 °C for 45 seconds, then extension at 72 °C for 90 seconds, and finally post-extension at 72 °C for 10 minutes. The cycles from denaturation to the extension were set for 30 cycles. The amplicon was estimated on 1 % agarose gels and visualized by the Gel DocTM XR system. PCR products were sent to the 1st Base Malaysia company for sequencing using Sanger dideoxy method.

6. Phylogenetic Tree Construction

The sequence results were assembled using the ChromasPro software. The assembled sequence was deposited into GenBank (https://www.ncbi.nlm.nih.gov/) to get the GenBank accession number. Then, The Basic Local Alignment Search Tool (BLAST) was conducted in the NCBI website. The genera would be shown as the BLAST result. The BLAST result was showing the genus as *Pediococcus*. The current publication that reported about *Pediococcus* was chosen as the basic reference sequences for phylogenetic tree. Sequences of *Pediococcus* species from Doi et al. (2009) were downloaded and *Lactobacillus casei* as an outgroup (Table 1). Single gene analysis was conducted to construct the phylogenetic tree. All reference sequences, Lyz222 sequence, and out group were aligned using Clustal X software and transferred as the Phyllip format file (Hermawan & Khairillah, 2021). The phylogenetic tree was built using Randomized Axelerated Maximum Likelihood (RAxML) Black Box that was generated on CIPRES (Stamatakis, 2014). The Bootstrap analyses as 1000 replications were used in this phylogenetic tree reconstruction. The Bootstrap (BS) value ≥ 60 was shown on the branch.

Table 1. Species, Isolate Code and GenBank Acc. Number in this study

Species	Isolate Code	GenBank Acc. Number16S rRNA
Pediococcus acidilactici	DSM 20284^T	AJ305320
Pediococcus acidilactici	Lyz222	OM400519
Pediococcus argentinicus	CRL 776	NR_042623
Pediococcus argentinicus	KGWM1-1	MN176360
Pediococcus claussenii	DSM 14800^T	AJ621555
Pediococcus damnosus	DSM 20331^T	AJ318414
Pediococcus ethanolidurans	Z-9T	AY956789
Pediococcus ethanolidurans	CUPV1441	MH298647
Pediococcus inopinatus	DSM 20285^T	AJ271383
Pediococcus lolii	NGRI 0510Q^T	AB362985
Pediococcus parvulus	JCM 5889^T	D88528
Pediococcus pentosaceus	DSM 20336^T	AJ305321
Pediococcus stilesii	FAIR-E 180	NR_042401
Pediococcus stilesii	LMG 23082^T	AJ973157
Lactobacillus casei	ATCC 334	AF404708

Results and Discussion

The *Lysurus periphragmoides* fruiting body has many parts that can be explored for the microbiome diversity, especially for the slime on the head part. The slime as known as slimy spore mass contains a dark liquid and spore. The slime is really wet and sticky. This criterion makes the possibility of bacteria to live in the slime. This part is also opened to the environment. Many organisms or microorganisms can touch and drift into this part. One possibility is from bacteria. One kind colony species was appeared on the isolation medium. Then, the colony was purified using quadrant streak for single cell colony (Figure 1A). The colony has creamy color and odor on the LA medium. The bacterium was coded as Lyz222. The colony morphology showed circular shape, milky white color, concave surface, and mucoid density. The cells were coccus, Gram-positive bacterium, and 2.8–3.5 µm in diameter (Figure 1B). The Lyz222 strain produced strong odor like a milk or fermented milk smell. We assumed that the Lyz222 strain was categorized as Lactic Acid Bacteria group. As the environmental in the slime spore mass, the slime had the pH as around 6. This pH was available for the Lactic Acid Bacteria group growing.

The 16S rRNA was very useful gene to identify various bacteria (Woese & Fox, 1977). The bacterial strain Lyz222 assembled sequence was submitted into GenBank as OM400519 (GenBank Accession Number). Then, Homology nucleotide BLAST of the Lyz222 16S rRNA was classified as *Pediococcus* genus specifically as *Pediococcus acidilactici* (Table 2). The top four of the identification results are *P. acidilactici* with 99.52% as the percent identity. But the second rank of the BLAST result was for *P. pentosaceus* Strain LAB2 with 99.52% as the percent identity too. The phylogenetic tree supported the identification as *P. acidilactici* with 100% BS value (Figure 1). The comparison between *P. acidilactici* and *P. pentosaceus* in the phylogenetic was related as sister clade. Based on the homological analysis, our bacterium can be identified as *P. pentosaceus*. Among of *P. acidilactici* and *P. pentosaceus* was difficult to distinguish by phenotypic properties (Holland et al., 2011). It should be continued for molecular identification

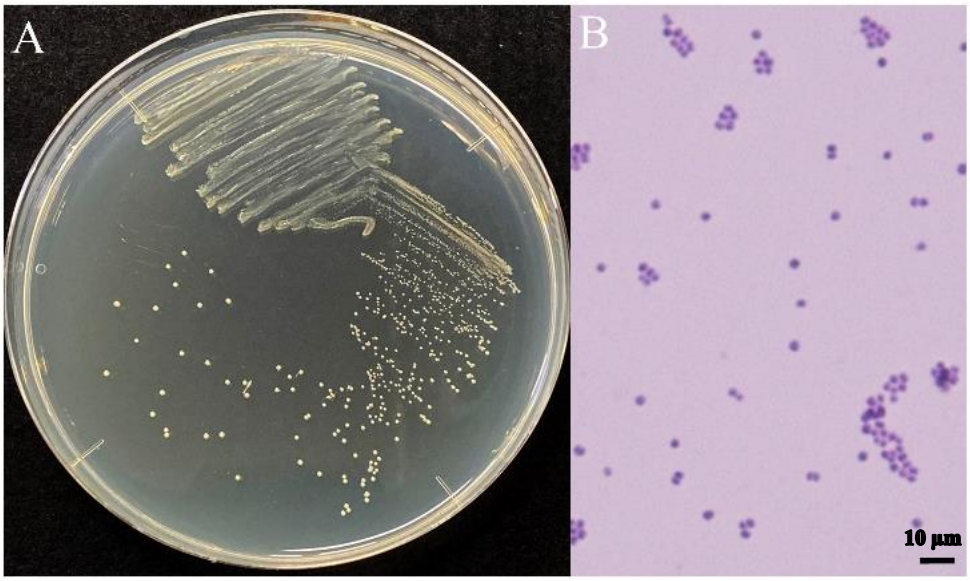

Figure 1. Bacterial Lyz222 morphology on LA medium for 2 days incubation at 35°C. (A) colony on LA medium; (B) cell morphology. Scale bar on B is 10 µm.

Table 2. Species, Isolate Code and GenBank Acc. Number in this study

Species	Strain Code	Max Score	Total Score	Query Cover	E. Value	Percent Identity
Pediococcus acidilactici	R8	2654	2654	99%	0.0	99.52%
Pediococcus pentosaceus	LAB2	2654	2654	99%	0.0	99.52%
Pediococcus acidilactici	PMC65	2649	13229	99%	0.0	99.45%
Pediococcus acidilactici	PMC48	2649	13229	99%	0.0	99.45%
Pediococcus acidilactici	HBUAS 56082	2649	2649	99%	0.0	99.45%

Based on the table 2 of phenotypic differentiation of *Pediococcus* by Holland et al. (2011), the differentiation between *P. acidilactici* and *P. pentosaceus* can be showed by the various growth temperatures. The *P. acidilactici* can grow at 45 °C, while *P. pentosaceus* cannot grow. As our result, the Pediococcus strain Lyz222 could grow at 30 till 45 °C. It proofed that *Pediococcus* strain Lyz222 was not *P. pentosaceus*. The *P. pentosaceus* strain LAB2 with the GenBank accession number JQ716921 should be reconfirmed as the correct name in *Pediococcus acidilactici* species. The GenBank description for the *P. pentosaceus* Strain LAB2 was not published yet. The homology BLAST nucleotide in GenBank cannot be assumed strongly to identify the species level. The advance analysis was needed to conform the species level name, i.e. phylogenetic tree analysis, Figure 2.

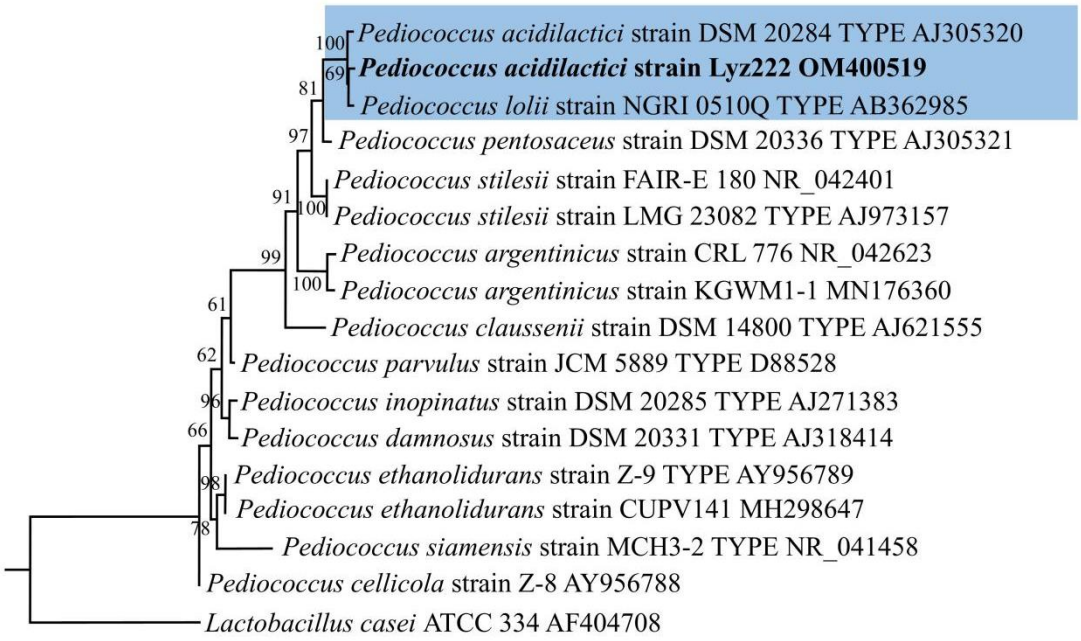

Figure 2. *Pediococcus* phylogenetic tree based on 16S rRNA using RAxML Black Box. Bootstrap (BS)≥60 was shown on the branch. *Pediococcus* strain Lyz222 must be in bold.

Current studies report that the group of *Pediococcus* spp. could be isolated directly from fresh fruit and vegetables. Todorov & Dicks (2009) found the presence of *P. pentosaceous* in marula fruit. Subsequently, this species also found in Maize leaf, Euphoria longana fresh fruit, *Telfairia occidentalis*, *Amaranthus spinosus*, Lychee fruit, grapefruit, and cantaloupe

melon (Dalie et al., 2010; Emerenini et al., 2013; Panthavee et al., 2017; Taroub et al., 2019; Zhao et al., 2012). Holland et al. (2011) stated that *P. pentosaceous* was closely related to *P. acidilactici*. They have similarities in phenotypic but differences in rRNA sequences. In contrast, there was no report about the presence of this group in mushrooms. This is the first report of the presence of the *Pediococcus* group identified as *Pediococcus acidilactici* (strain Lyz222) in a slimy spore mass of *L. periphragmoides*.

The possibility of the presence of *P. acidilactici* isolated from *L. periphragmoides* might originate from spores (spore bearing) or from external environment. Although there were report of *Lysurus* spore could be spread by mycophagous insect/saphropagous flies in their fesses (Chen et al., 2014), however the latter presumption is more likely due to the condition during bacterial isolation that the fungus was not in the egg phase but mature phase where at this stage the head and stem are not wrapped by the volva membrane which allows contamination from the environment. It is also noted that in the surrounding of the mushroom there are several trees whose fruits have fallen to the ground and flies that land on mushroom head because they are attracted by odors originated from mushroom slimy spore mass. To date, there have been no reports of bacteria associated with *L. periphragmoides*, hence this is the first report on bacterium, specifically lactic acid bacterium *Pediococcus acidilactici*, isolated from slimy spore mass of *L. periphragmoides*.

Conclusion

Lysurus periphragmoides slimy spore is appeared from mature fruiting body. One bacterium was isolated from the slimy spore mass, identified as *Pediococcus acidilactici* strain Lyz222. This is the first record and information that there is bacterium colonised the *Lysurus periphragmoides* slimy spore mass. The possibility of *P. acidilactici* living on *L. periphragmoides* slimy spore mass might be sourced from spores (spore bearing) or from external environment.

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