Identification of Contaminant Fungi on *Pedetan*, an Dry Fish Product of Lemuru (*Sardinella lemuru*)

Ni Made Ayu Suardani Singapurwa¹, *Dewa Ngurah Suprapta¹, Ida Bagus Wayan Gunam², I Gusti Ngurah Alit Susanta Wirya³, Khamdan Khalimi³

¹Laboratory of Biopesticide, Faculty of Agriculture Udayana University, Jl. PB. Sudirman Denpasar Bali Indonesia.
²Laboratory of Food Science, Faculty of Agricultural Technology Udayana University, Jl. PB. Sudirman Denpasar Bali Indonesia.
³Laboratory of Biotechnology, Faculty of Agriculture Udayana University, Jl. PB. Sudirman Denpasar Bali Indonesia.

*Corresponding author email: biop@dps.centrin.net.id*

**Abstract**

Dry fish products of Lemuru (*Sardinella lemuru*) known in Bali under the name "pedetan" is one kind of dried seasoned processed products that is quite popular among the people of Bali, especially in Jembrana Regency. In general the process is done through drying lemuru fish in the sun so it is possible for them to grow and develop the contaminant fungi that may affect the health of consumers. This study aims to isolate and identify fungi that contaminate pedetan. Pedetan samples were taken from 10 villages which are the pedetan production centers in Jembrana Regency. The identification was done macroscopically, microscopically, and molecularly through analysis of 18S rRNA gene. The results of this study indicate that there are four types of fungi that are found as contaminants of pedetan, namely *Aspergillus flavus*, *Aspergillus aculeatus*, *Aspergillus niger*, and *Aspergillus tubingensis*. Efforts are needed to improve the composition of spices in order to maximally reduce the population of contaminant fungi in pedetan.

**Keywords:** *Sardinella lemuru*, pedetan, contaminant fungi

**1. Introduction**

Lemuru fish (*Sardinella lemuru*) is widely used as raw material for making fishery products, especially in a fish canning industry, partly processed into salted fish, fish meal and pedetan. Pedetan is a spicy dried fish food product, which is processed by people in Jembrana Regency of Bali Province. The communities processing lemuru fish into pedetan starts when the raw materials of lemuru fish are received, then its scales are cleaned, the stomach contents and the spine are discarded, and split into a butterfly shape and washed. The clean lemuru fish is mixed with traditional Balinese spices and dried in the sun for 2-3 days (Singapurwa *et al*., 2014). Different pedetan processing from the process of receiving raw materials to the distribution process (Singapurwa *et al*., 2017a; Singapurwa *et al*., 2017b) and storage of lemuru fish with different packing materials affect the quality and safety of the resulting pedetan (Singapurwa *et al*., 2017c).

The presence of microbes that can contaminate pedetan is able to reduce the quality of the product because of decay. Implementation of the feasibility of basic food processing with Good Manufacturing Practice (GMP) and Sanitation Standard Operating Procedures (SSOP) is able to reduce microbial contaminants that can contaminate lemuru fish pedetan (Singapurwa *et al*., 2016; Singapurwa *et al*., 2017b; Singapurwa *et al*., 2017d). Total microbials in lemuru fish before and after applying GMP and SSOP are respectively 1.56 x 10⁵ CFU/g (Singapurwa *et al*., 2017b) and 5.5 x 10⁴ CFU/g (Singapurwa *et al*., 2017d), and after packaging of 15.22 x 10⁴ CFU/g (Singapurwa *et al*., 2017c). Pedetan damage occurs because of mold on the surface of fish and the mold which often contaminates dried fish are *Aspergillus, Penicilium, Rhizopus,* and *Fusarium* (Olajuyigbe *et al*., 2014; Jimoh *et al*., 2014; Olajuyigbe *et al*., 2017).

Mold *Aspergillus* sp. is a multicellular type of mold that is opportunistic and often contaminates food. Several species of *Aspergillus* sp. which often contaminate food are *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus terreus*, *Aspergillus fumigates*, *Aspergillus tamari*, *Aspergillus sydowii*, and *Aspergillus versicolor* (Putra, 2016; Kamil, 2016). Putra (2016) reports that *Aspergillus* sp. which contaminates salted fish in Beringharjo Yogyakarta market, Indonesia from 11 samples tested, 45% species are *A. flavus* and 45% species are *A. niger* while the rest are *A. tamari* and *A. sydowii*. While the research done by Kamil (2016) shows that the type of shrimp *Aspergillus* sp. which contaminates the salted fish in the Kenjeran Surabaya market, Indonesia from 9 samples tested are 100% of *A. tamari*, 89% of *A. flavus*, 67% of *A. sydowii*, 56% of *A. niger*, and 56% of A. versicolor. This shell has a spore that is light and easy to spread in the air, thus easily contaminates open food items such as pedetan.
Based on the above background, the identification of contaminant fungi in lemuru fish pedetan is made with the aim to know the species of contaminant fungi and predict the possible effects on human health.

2. Research Methods

2.1. Sampling and Isolation of Contaminant Fungi from pedetan

Sampling was conducted in 10 villages in pedetan production center in Jembrana Regency, Bali, Indonesia namely Perancak Village, Pengambengan, Baler Bale Agung, Air Kunung, Baluk, Pangkung Gayung, Melaya, Yeh Sumbul, Banyu Biru, and Berangbang. The number of samples tested for each village was 10 pedetan samples so that there were 100 samples of pedetan. Subsequently each sample of the pedetan was weighed 1 g and dilution series were carried out from $10^{-1}$ to $10^{-6}$. Furthermore, at dilution of $10^{-6}$ in each sample was taken 100 μl of suspension and inserted into 10 ml of PDA medium in a Petri dish and incubated at room temperature for 2 days.

![Figure 1. Pedetan, an dry product of Lemuru Fish](image)

2.2. Macroscopic and Microscopic Observations

A macroscopic observation was done by looking at the morphology of a fungal colony that grew in a Petri dish at each dilution. Morphological characteristics were seen from the colony, colony color, colony size, texture and growth of colonies and conidia.

A microscopic observation was performed by taking samples aseptically with sterile Ose needles. Mold is placed above the object glass which had been given one drop of Aquadest. Cover glass was placed on top of object glass that already contained molds, and observations were conducted with a microscope with 400 times magnification. Computer readings were done with Olympus Microscope CX23 which was connected with OptiLab Microscop Camera. All microscopic and microscopic observation processes were performed aseptically.

2.3. Identification of Aspergillus sp. based on 18S rRNA sequencing

Four Aspergillus sp. namely Aspergillus sp. isolate AyS-A, Aspergillus sp. isolate AyS-B, Aspergillus sp. isolate AyS-C, and Aspergillus sp. isolate of AyS-D were isolated from pedetan then maintained at Biopesticide Laboratory, Faculty of Agriculture Udayana University. DNA extraction of the mycelia was performed using Genomic DNA Purification Kit Thermo. Miselia grown in Potato Dextrose Broth (PDB) medium was placed in a mortal and added with 180 μl digestion solution then crushed until soft, then added with 20 μl proteinase K solution, 20 μl RNase A solution, 200 μl lysis solution, 400 μl ethanol 50% and then was applied for vortex. It was then transferred into Genomic DNA Genetic Purification Column. The obtained DNA was then used as a template for PCR. The primer used was the primer pair of ITS1 (5’TCCCTCCGCTTATTGATATGC3’) and ITS4 (5’ TCCGTAGGTGAAACCTGCGG 3’). Nucleotide sequences were determined by using ABI-Prism 3100-Avant Genetic Analyzer. The sequenced DNA sequences were then trimmed and assembled using the ChromasPro Version 1.5 program. The data that had been assembled was further BLASTed with data that had been registered in NCBI (National Center for Biotechnology Information) through the site [http://www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST); then the data were analyzed again by aligning the sequence by using MEGA version 6.0 program. Then, the data were analyzed using PAUP 4.0b program with Maximum Parsimony method with bootstrap 1,000 replications. Then the phylogeny tree was designed using TreeGraph 2.0.
3. Results and Discussion

3.1 Contaminant fungi on pedetan

The results of observation on each sample in each village of pedetan production center showed the presence of mold contamination on lemuru fish product as shown in Table 1. Singapurwa et al. (2016) report that on lemuru fish stocks there is no contamination caused by Salmonella, Vibrio cholera, Staphylococcus aureus and Escherichia coli in fresh lemuru fish. Sulieman et al. (2014) point out that microbial contamination of Kejeik dried fish in Sudan is caused by Aspergillus niger, Alternaria, Penicillium sp., Halophilic bacteria, Rhodotorula and Cryptococcus laureate yeasts, but no E. coli, Salmonella, V. cholera, S. aureus, Listeria monocytogenes, and Vibrio parahaemolyticus.

Table 1

<table>
<thead>
<tr>
<th>Sampling Location</th>
<th>Number of Sampel</th>
<th>Population of Aspergillus sp. in pedetan of lemuru fish (CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perancak</td>
<td>10</td>
<td>Isolate AyS-A: 6.14 x 10^5, Isolate AyS-B: 5.27 x 10^6, Isolate AyS-C: 3.47 x 10^6, Isolate AyS-D: -</td>
</tr>
<tr>
<td>Pengambengan</td>
<td>10</td>
<td>3.28 x 10^6, - 4.72 x 10^6, -</td>
</tr>
<tr>
<td>Baler Bale Agung</td>
<td>10</td>
<td>6.34 x 10^6, 3.26 x 10^6, 5.64 x 10^6, -</td>
</tr>
<tr>
<td>Air Kuning</td>
<td>10</td>
<td>3.46 x 10^6, - 4.87 x 10^6, -</td>
</tr>
<tr>
<td>Baluk</td>
<td>10</td>
<td>3.59 x 10^6, 8.16 x 10^6, - 8.28 x 10^6</td>
</tr>
<tr>
<td>Pangkung Gayung</td>
<td>10</td>
<td>6.82 x 10^6, 3.24 x 10^6, 2.58 x 10^6, -</td>
</tr>
<tr>
<td>Melaya</td>
<td>10</td>
<td>5.25 x 10^6, 6.71 x 10^6, -</td>
</tr>
<tr>
<td>YehSumbul</td>
<td>10</td>
<td>2.55 x 10^6, 1.87 x 10^6, 8.75 x 10^5, -</td>
</tr>
<tr>
<td>Banyubiru</td>
<td>10</td>
<td>7.92 x 10^5, - 3.82 x 10^6, 3.82 x 10^6</td>
</tr>
<tr>
<td>Beranghang</td>
<td>10</td>
<td>5.62 x 10^5, - 3.82 x 10^6, -</td>
</tr>
</tbody>
</table>

- : not detected

Dry fish products produced by sun drying or by fumigation process can create contamination by mold. Mold that often contaminates dried fish products is Aspergillus sp. (Akinyemi et al., 2011; Olajuyigbe et al., 2014) in addition to other types of mold such as Penicillium, Trichoderma, Fusarium, Rhizopus, Mucor, Neurospora (Junaid et al., 2010; Jimoh et al., 2014; Olajuyigbe et al., 2017). Sam et al. (2015) report that there are 23 different species of mold that contaminate Tuticorin dried fish products in India, and dominant fungi are A. flavus and A. niger. In the dried fish smoke in Nigeria found 9 genus molds that contaminate fish with a range of 2.00 x 10^7 - 3.09 x 10^8 CFU/g, and of all the genus is only strain Aspergillus sp. which potentially produce aflatoxin.

Aspergillus sp. can produce aflatoxin which if consumed will be accumulated in the body, so it can cause chronic health disorders, such as hepatitis B and hepatocellular cancer (Handayani and Setyaningsih, 2006). As many as 50% of A. flavus population can produce aflatoxin. Aflatoxin produced by A. flavus is influenced by fish species and environmental conditions. A. flavus can produce 0.001 - 5.492 μg/kg aflatoxin B1 and A. niger can produce 0.01 - 2.960 μg/kg aflatoxin G1 in Tuticorin fish products (Sam et al., 2015). Akinyeti et al. (2011) also reported that A. flavus and A. parasiticus isolated from dried fish by fuming process can produce aflatoxin of 0.030 ppb – 1.150 ppb, while Olajuyigbe et al. (2014) said that A. flavus and A. tamari isolated from dried fish can produce aflatoxin of 1.05 - 25.00 μg/kg.

Efforts are needed to improve the composition of herbs in order to maximally reduce the population of contaminant fungi in pedetan. Restu (2014) and Syifa et al. (2013) point out that coriander and garlic herbs are effective to inhibit fish damage during storage because they contain anti-microbial ingredients. Sitepu et al. (2012) have observed that galangal spice can inhibit the growth of A. flavus, and turmeric may inhibit the Curvularia lunata fungus.

3.2. Macroscopic and Microscopic Observation Results

The macroscopic observation results show that A. flavus isolate AyS-A has the following characteristics: light green colonies with granular and compact colonies (Fig. 2). Microscopic observation shows that the size of conidia A. flavus isolate AyS-A was 6.12 μm x 3.19 μm, vesicle diameter 32.08 μm, and conidiofor length 175.39 μm. Nyongesa et al. (2015) report A. flavus isolated from maize with Malt Extract agar medium having a yellowish green colony, with a vesicle diameter of 18-36 μm and a 3.5 - 5μm conidial length. The mold A. flavus has conidiofor, rounded vesicles, and has a rounded and smooth to slightly coarse conidia, cannot produce exudate and can dissolve pigment.
The macroscopic observation results show that *A. aculeatus* isolate AyS-B has the following characteristics: round and black colonies. However, microscopic observation shows that the size of conidia *A. aculeatus* isolate AyS-B is 3.00 μm x 3.29 μm, vesicle diameter 37.98 μm, and conidiofor length of 180.25 μm (Figure 3).

Nyongesa et al. (2015) report *A. aculeatus* isolated from corn with Potato Dextrosat Agar medium has a dark brown to blackish colony, with a vesicle diameter of 48-74 μm and a conidia length of 4-5μm. Mold *A. aculeatus* has conidiofor, round-shaped vesicles, and has rounded and rough conidia, cannot produce exudate but can produce solvent yellow lemon pigment. Mold *A. aculeatus* has the synonym *A. japonicas* var. *aculeatus* (lizuka) Al-Musallam and the mold are included in the familia Trichocomaceae (Baba et al., 2015) and these species can be isolated from decomposed soil and fruit.

The macroscopic observation results show that *A. niger* isolate AyS-C has a characteristic black colony. Microscopic observation shows that the size of conidia *A. niger* isolate AyS-C is 3.91 μm x 3.38 μm, vesicle diameter 40.99 μm, and conidiofor length 210.32 μm (Figure 4). Nyongesa et al. (2015) report *A. niger* isolated from corn with Potato Dextrosat Agar medium has a black colony, with a vesicle diameter of 37-52 μm and a conidia length of 4-6 μm. *Kapang A. aculeatus* has conidiofor, round-shaped vesicles, and has a rounded and rough conidia, unable to produce exudates and pigment solvents.
Figure 4
Colony and microscopic structure of *A. niger* isolate AyS-C. A: *A. niger* colony isolate AyS-C; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

The results of macroscopic observation show that *A. tubingensis* isolate AyS-D has a characteristics black colony. Microscopic observation showed that the size of conidia *A. tubingensis* isolate AyS-D was 4.48 μm x 2.53 μm, vesicle diameter 80,62 μm, and conidiofor length 143,08 μm (Figure 5). According to Cheng *et al.* (2016), *A. tubingensis* is a mold that often contaminates black brick tea so as to reduce the quality of tea.

Figure 5
Colony and microscopic structures of *A. tubingensis* isolat AyS-D. A: *A. tubingensis* colony isolate AyS-D; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

3.3. Identify *Aspergillus* sp. based on 18S rRNA sequencing

The amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using primer ITS1 (5 'TCCTCCGCTATTGATATGC 3') and ITS4 (5 'TCCGTAGGTGAACCTGCGG 3') results in a 600 bp fragment as seen in electroforegram (Figure 6). The size of the amplicon DNA that has been obtained in accordance with the primary research design conducted by Mulyatni *et al.* (2011) that the amplification of ITS4 and ITS5 regions results in a 600 bp DNA fragment. Henry *et al.* (2000) identify *Aspergillus* sp. with the amplification of ITS1 and ITS2 regions resulting in DNA fragments of 565-613 bp.
Based on the alignment of the 18S rRNA gene sequence with the GenBank database using the BlastN program, the isolates of AyS-A were included in the *Aspergillus flavus* group because the isolates were homologous with *A. flavus* Sichuan-Rfsb10 isolate (KX067886.1) and *A. flavus* strain CS12 (KX015990.1) with a maximum identity level of 99%. Mold isolates of AyS-B were included in the *Aspergillus aculeatus* group because of the homologous AyS-B isolate with *A. aculeatus* strain AN5 (KY859793.1) and *A. aculeatus* isolate 4F (KY848352.1) with a maximum identity level of 99%. Mold isolates of AyS-C were included in the *Aspergillus niger* group because the isolates were homologous with *A. niger* strain voucher-MSR4 (KJ881377.1) and *A. niger* strain isolate 6029 (KX363462.1) with a maximum identity level of 99% while the isolate AyS-D was included in the *Aspergillus tubingensis* group because of the homogeneous AyS-D isolate with *A. tubingensis* of IMMIS2 strain (LT732556.1) and *A. tubingensis* isolate FIS2 (KY378944.1) with 100% maximum identity level (Table 2).

**Table 2**

<table>
<thead>
<tr>
<th>Mold isolate AyS-A, AyS-B, AyS-C, dan AyS-D</th>
<th>Similarity Percentage (%)</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus flavus</em> isolat Sichuan-Rfsb10</td>
<td>99</td>
<td>KX067886.1</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em> strain CS12</td>
<td>99</td>
<td>KX015990.1</td>
</tr>
<tr>
<td><em>Aspergillus aculeatus</em> strain AN5</td>
<td>99</td>
<td>(KY859793.1)</td>
</tr>
<tr>
<td><em>Aspergillus aculeatus</em> isolat 4F</td>
<td>99</td>
<td>(KY848352.1)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> strain voucher-MSR4</td>
<td>99</td>
<td>(KJ881377.1)</td>
</tr>
<tr>
<td><em>Aspergillus niger</em> strain isolate 6029</td>
<td>99</td>
<td>(KX363462.1)</td>
</tr>
<tr>
<td><em>Aspergillus tubingensis</em> strain IMMIS2</td>
<td>100</td>
<td>(LT732556.1)</td>
</tr>
<tr>
<td><em>Aspergillus tubingensis</em> isolat FIS2</td>
<td>100</td>
<td>(KY378944.1)</td>
</tr>
</tbody>
</table>

Phylogenetic tree analysis using 1,000 times Bootstrap replication showed that the isolate of AyS-A was *Aspergillus flavus*, the isolate of AyS-B was *Aspergillus aculeatus*, the isolate of AyS-C was *Aspergillus niger*, and the isolate of AyS-D was *Aspergillus tubingensis* because of isolate AyS-A, isolate AyS-B, isolate AyS-C, and isolate AyS-D one clade with *Aspergillus flavus* mold sequences, *Aspergillus aculeatus*, *Aspergillus niger*, and *Aspergillus tubingensis* in GenBank database with 100% Bootstrap Support (BS) (Figure 7) (Samson *et al*., 2014).
Conclusion

Four species of fungi as contaminants are found on lemuru fish *pedetan*, they are *Aspergillus flavus*, *A. aculeatus*, *A. niger*, and *A. tubingensis*. The population of each species of fungi is for *A. flavus* of $3.326 \times 10^6$ CFU/g, *A. aculeatus* of $2.851 \times 10^6$ CFU/g, *A. niger* of $1.116 \times 10^6$ CFU/g, and *A. tubingensis* of $1.210 \times 10^6$ CFU/g.

Acknowledgments

On this occasion, the authors would like to thank Udayana University Graduate Program and Biopesticide Laboratory of Udayana University Faculty of Agriculture for all the support and facilities that were used during the research.

References


