Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.8, No.6, 2018
www.iiste.org

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

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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 consummers. 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 10^7 CFU/g (Singapurwa *et al*., 2017b) and 5.5 x 10^4 CFU/g (Singapurwa *et al*., 2017d), and after packaging of 15.22 x 10^3 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 Kuning, 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

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. Indentification 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'TCCTCCGCTTATTGATATGC3 ') and ITS4 (5' TCCGTAGGTGAACCTGCGG 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; 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 Contaminat 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
Population of *Aspergillus* sp. in *pedetan* of lemuru fish

Sampling Location	Number of Sampel	Population of *Aspergillus* sp. (CFU/g)			
		Isolate AyS-A	Isolate AyS-B	Isolate AyS-C	Isolate AyS-D
Perancak	10	6.14×10^5	5.27×10^6	3.47×10^6	-
Pengambengan	10	3.28×10^6	-	4.72×10^6	-
Baler Bale Agung	10	6.34×10^6	3.26×10^6	5.64×10^6	-
Air Kuning	10	3.46×10^6	-	4.87×10^6	-
Baluk	10	3.59×10^6	8.16×10^6	-	8.28×10^6
Pangkung Gayung	10	6.82×10^6	3.24×10^6	2.58×10^6	-
Melaya	10	5.25×10^6	6.71×10^6	-	-
YehSumbul	10	2.55×10^6	1.87×10^6	8.75×10^5	-
Banyubiru	10	7.92×10^5	-	-	3.82×10^6
Berangbang	10	5.62×10^5	-	-	-

- : 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 2.00×10^3 - 3.09×10^4 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. flavu*s 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.

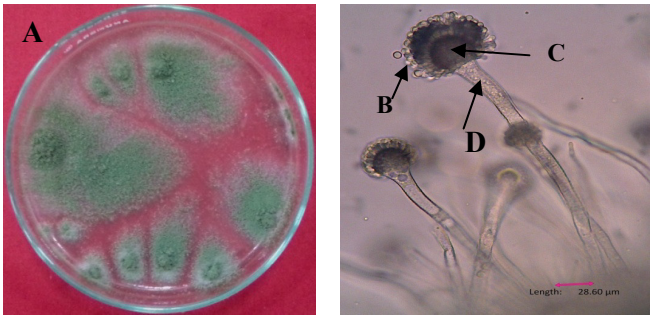

Figure 2
Colonies and microscopic structures *A. flavus* isolates AyS-A. A: *A. flavus* colony isolate AyS-A; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

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.

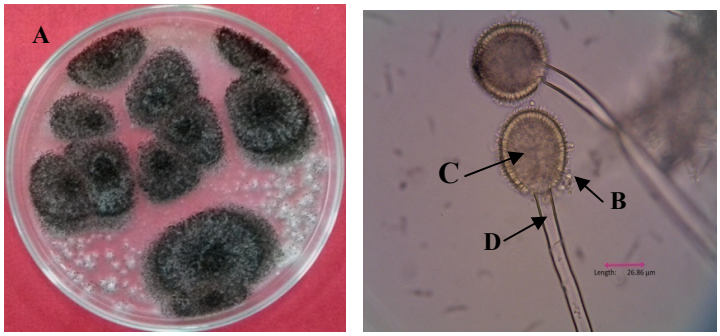

Figure 3
Microscopic colonies and structures *A. aculeatus* isolate AyS-B. A: colony of *A. aculeatus* isolate AyS-B; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

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.

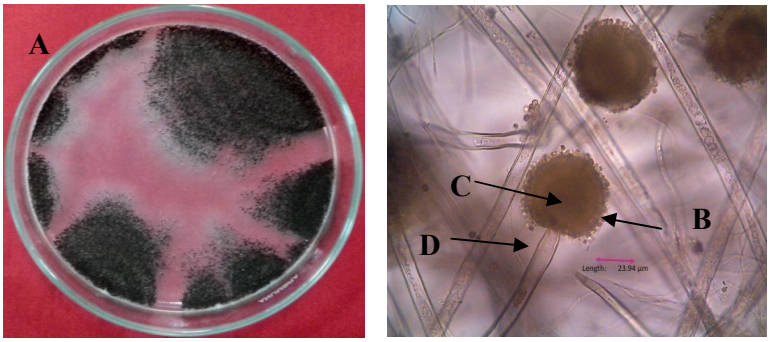

Figure 4
Colony and microscopic structure of *A. niger* isolate AyS-C. A: *A. niger* colony isolate AyS-C; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

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.

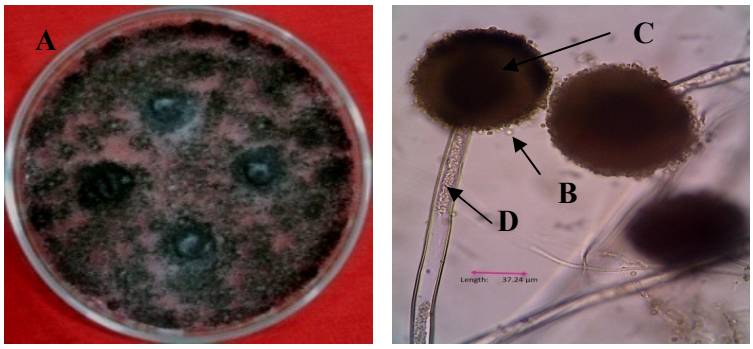

Figure 5
Colonies and microscopic structures of *A. tubingensis* isolat AyS-D. A: *A. tubingensis* colony isolate AyS-D; B: conidia; C: vesicles; D: conidiofor. 400X magnification.

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

The amplification of the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) using primer ITS1 (5 'TCCTCCGCTTATTGATATGC 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.

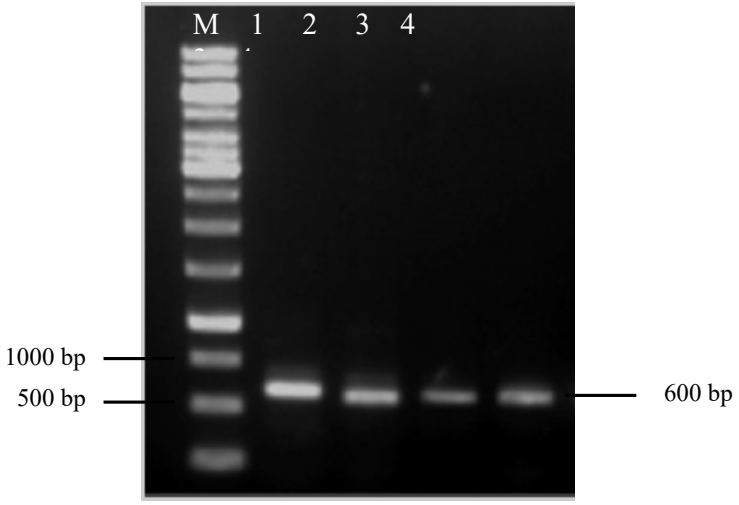

Figure 6
Amplikon gene 18S rRNA 4 mold isolates. M. 1 Kb DNA marker,
1. Isolate AyS-A, 2. Isolate AyS-B, 3. Isolate AyS-C, 4. Isolate AyS-D.

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
Comparison of percentage similarity of 18S rRNA gene of mold isolates AyS-A, AyS-B, AyS-C, and AyS-D with some DNA sequences in GenBank using the BLAST program

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

Phylogenic 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).

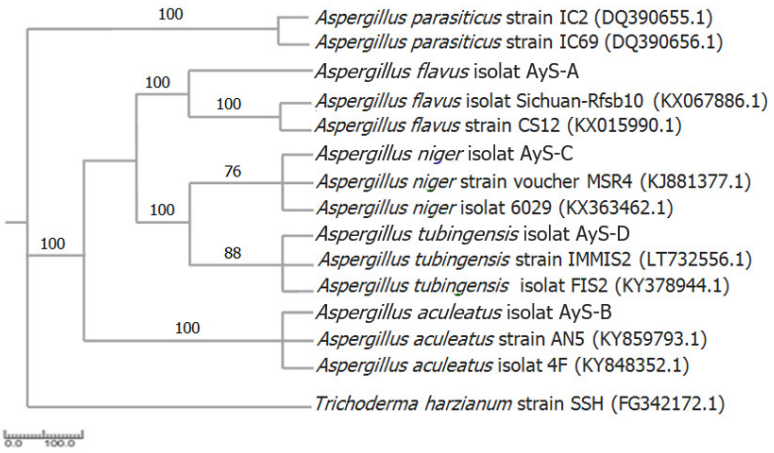

Figure 7
Maximum Parsimony Phylogenetic Tree 18S rRNA shows genetic relationship between 4 isolates of fungi and previously identified fungi available in Genbank Database.Value of Bootstrap derived from 1,000x replicates.

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 33.26 x 10^6 CFU/g, *A. aculeatus* of 28.51 x 10^6 CFU/g, *A. niger* of 11.16 x 10^6 CFU/g, and *A. tubingensis* of 12.10 x 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.

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