Fungal Contamination and Toxigenic *Aspergillus flavus* Strains on Dried Anchovies Sold in North Sumatra

Jesica Simanullang and Kiki Nurtjahja
Department of Biology, Faculty of Science and Technology, Universitas Medan Area, Medan, Indonesia

Keywords: *Aspergillus flavus*, Fungal population, Anchovies, Toxigenicity

Abstract: Dried anchovies at Medan traditional markets commonly sold unpacked and stored in open air, as the result, the dried-salted fish was easily spoiled and contaminated by mycotoxigenic fungi. The aims of the study were to enumerate fungal population and to characterize *A. flavus* strains. Each fungal species were isolated from medan anchovies at five traditional markets. The percentage of fungal contamination was determined by a direct plating using dichloran 18% glycerol agar medium. Fungal population was determined using a serial dilutions and pour plate in DG18 medium. Toxigenic *Aspergillus flavus* strains were characterized by the production of yellow pigment on the reverse side of petri dishes containing coconut agar medium 10%. The presence of regulatory (*aflR*) and structural genes (*nor-1, ver-1 and omt-1*) genes determined aflatoxin biosynthesis was determined by amplifying four sets of specific primers (aflR, nor-1, ver-1 and omt-1). Results showed that medan anchovies were contaminated by storage fungi. *Aspergillus niger* was the most common found followed by *A. flavus* and *A. chevalieri*. Eighty strains of *A. flavus* were isolated from all samples, they are consisted 69 toxigenic and 11 strains were non-toxigenic.

1 INTRODUCTION

Anchovies is dried-salted fish that one of fish products that contain high protein content. Medan anchovies commonly produced traditionally by local fisherman. During processing the fish product was preserved conventionally by adding some salt and dried under the sunlight. Moisture content anchovies between 32 and 34% with salt content between 13 and 17% (Yusra 2017). During storage and high relative humidity the dried-stored anchovies might increase their water activity (a_w) and contaminated by storage fungi.

About 14 of 150 dried-salted fish samples stored at a_w 0.73 to 0.80 were contaminated by *Aspergillus flavus* (Indriati et al. 2017). In addition, *Aspergillus flavus* was the most predominant found on dried salted fish (Youssef et al. 2003) Storage fungi such as *Aspergillus flavus*, *A. candidus*, *A. niger*, *A. wentii*, *A. chevalieri* and *Penicillium islandicum* commonly xerophilic and was able to grow at low a_w (Pitt and Hocking 2009).

Fungal contamination occured n dried fish at moisture content 12.3 to 32% and genus *Aspergillus* particularly *A. niger*, *A. fumigatus*, *A. ochraceus* and *A. flavus* were the most common found (Sam et al. (2015). Fungal contamination on food and feedstuffs may cause change taste, colour, reduce nutrition (Hassan et al. 2011). The purpose of recent study was to enumerate fungal contamination and to determine toxigenic A. flavus strains on anchovies sold by retailers at traditional markets in North Sumatera, Indonesia.

2 MATERIALS AND METHOD

2.1 Anchovies Sampling

Anchovies as sample were bought from five traditional markets and as mush as 100 g anchovies obtained from five retailers at each market. All samples were packed in sterile polyethylene bag and stored in in cold until used.

2.2 Determination of the Percentage of Anchovies Contaminated by Fungi

The percentage of anchovies contaminated by each fungal species was detemined by direct plating on dichloran 18% glycerol agar (DG18) medium in

Simanullang, J. and Nurtjahja, K.
Fungal Contamination and Toxigenic Aspergillus flavus Strains on Dried Anchovies Sold in North Sumatera.
DOI: 10.5220/0010613000002775
In *Proceedings of the 1st International MIPAnet Conference on Science and Mathematics (IMC-SciMath 2019)*, pages 548-551
ISBN: 978-989-758-556-2

petri dish (9 cm diameter). Five replicates were madefor each sample.

2.3 Enumeration of Fungal Population

Each fungal population was determined by serial dilution method followed by pour plate in Dichloran 18% Glycerol Agar ((DG18). One hundred gram of each sample were ground using Mill Powder RT-04 no serial number 980923 (Mill Powder Tech. Co LTD, Taiwan) with speed 25 000 rpm for 30 second and 25 g of fine sample in 500 ml flask was dilute by sterilized distilled water until the volume up to 250 ml. The suspension then was homogenazed and 1 ml of the aliquot in petri dish (9 cm in diameter) was pour plate in DG18 medium, three replicates was made. All plates then were incubated at 28°C for 5 d. Each fungal population were enumerated, isolatesd in Potato Dextrosa Agar (PDA) and identified according to Pitt and Hocking (2009) by subculturing in Malt Extract Agar (MEA) and Czapex Yeast Agar (CYA).

2.4 Determination on the Toxigenicity of *Aspergillus flavus* by Culture Technique

The toxigenicity of each A. flavus strains to produce aflatoxin was determine using coconut agar medium (CAM) according to Lin and Dianese (1976). Each strain of A. flavus was culture in CAM for 5 day at 28°C. The presence of yellow pigment at the reverse side of petri dish indicate toxigenicity.

2.5 Extraction of *A. flavus* Genome

Genome of each *Aspergillus flavus* strain was obtained from two 2 days old mycelial culture on PDA (28°C). As much as 40 g mycelia in 1.5 ml PCR tube containing 600 µl nuclei lysis solution was homogenated using micropestle. Genome extraction kit used was Mini Kit (Promega, Madison, WI, USA). The homogenate then was incubated at 65°C for 15 minute and cooled 5 minute until the temperature 29°C, then, 200 µl protein presipitation was added, vortexed and centrifuged at 13000×g for 3 minute. The supernatan was mixed in 600 µl isopropanol followed by centrifugation 13000×g for 2 minute, the supernatant was discarded and 600 µl ethanol 70% was used to wash the pellet. The mixture was then centrifuged 13000×g for 2 minute. The pellet obtained then rehydrated by 50 µl DNA rehydration solution, vortexed for 5 second and 0.5 µl RNAse was add. The DNA mixture was incubated at 37°C for 15 minute. The DNA concentration was determined using nanophotometer (IMPLEN, Munich, Germany). Electrophoresis DNA was conducted using SCIE-PLAS. Cambridge, England with 1,2% gel agarose and gel was stained with 1 µl ethidium bromide. The photograph used was Gel Doc (Uvitec, Cambridge, Serial) under UV light 303 nm.

2.6 PCR Amplification of Aflatoxin-related Genes

Polymeration chain reaction used was GeneAmp PCR Labcycler Gradient System (Sensoquest, Germany, serial number 1123280105). Specific primer for genes aflatoxin biosynthesis used were nor-1, ver-1, omt-1 and aflR with basepair 400, 895, 1232 dan 1032 bp respectively. Amplification preparation was conducted as follows: Amplification mix for each primer consisted of 5 µl GoTaq Green Master Mix (Promega), 3 µl nuclease free water, 0,5 µl for each primer F (forward) and R (reverse), then 1 µl DNA template and volume of end DNA Mix reaction on PCR tube was 10 µl. Process of DNA mix in PCR was preincubation step at 94ºC for 10 min followed by 35 cycles of denaturation at 94ºC for 10 min, annealing at 65ºC for 2 min and extension at 72ºC for 2 min and final extension at 72ºC for 7 min. The PCR product was analyzed by electrophoresis (SCIE-PLAS, Ltd, Cambridge, England) using 1,5 % agarose gel in 1×TAE 40 mM Tris-acetate, 1mM EDTA (pH 8). Gels stained with ethidium bromide 0.1 mg/l, visualized under *Gel Doc* (Uvitec, Cambridge, serial number 13 200263) under UV light (303 nm).

3 RESULTS AND DISCUSSION

3.1 Percentage of Contaminated Anchovies

Eleven species of storage fungi that contaminated anchovies were isolated from 5 traditional market (Table 1).

Table 1: Percentage of anchovies contaminated by fungi at five traditional markets in Medan, North Sumatera

Fungal species	Percentage of anchovies contaminated by fungi				
	Market 1	Market 2	Market 3	Market 4	Market 5
Aspergillus candidus	6.70	0	0	0	0
A. chevalieri	33.30	46.70	0	0	0
A. flavus	33.30	0	63.30	10.00	3.00
A. fumigatus	0	0	0	0	6.60
A. niger	30.0	23.3	23.30	100,00	43.30
A. oryzae	0	0	0	0	10.00
A. tamarii	3.30	46.70	0	10,00	3.00
A. wentii	3.30	0	0	0	0
Penicillium sp.1	13.30	0	0	0	10.00
Rhizopus oligosporus	33.30	13.30	3.30	0	10.00
Mycelia sterilia	3.30	0	0	0	0

Table 2: Fungal population (cfu/g) on anchovies contaminated by fungi at five traditional markets on Medan, North Sumatera

Fungal species	Fungal population (cfu/g)				
	Market 1	Market 2	Market 3	Market 4	Market 5
Aspergillus candidus	1×10	0	0	0	0
A. chevalieri	8.3×10	1.33×10^{-2}	0	0	0.3×10
A. flavus	0.7×10	0	1.13×10^{-2}	1.7×10	0.3×10
A. fumigatus	0	0	4×10	0.3×10	0.6×10
A. niger	0	0.3×10	3.3×10	3.3×10	0
Cladosporium sp.	0.3×10	0	0	0	0.3×10
Rhizopus oligosporus	0.3×10	0.3×10	0	0	0

CFU = colony forming unit

Table 3: Toxigenicity representative isolates of A. flavus strains isolated from anchovies based on cultural in CAM (coconut agar medium) medium and molecular based on the presence of regulatory (*aflR*) gene and structural (*nor-1, omt-1, ver-1*) genes

Strain	Regulatory gene	Structural genes			The presence of yellow pigment in CAM medium	Toxigenicity
	aflR	*nor-1*	*omt-1*	*ver-1*		
Af1	+	+	-	+	negative	non-toxigen
Af2	+	+	+	+	positive	toxigen
Af3	+	+	-	+	negative	non-toxigen
Af4	+	+	+	+	positive	toxigen
Af5	+	+	+	+	positive	toxigen
Af6	+	+	+	+	positive	toxigen
Af7	+	+	-	+	negative	non-toxigen
Af8	+	+	+	+	positive	toxigen
Af9	+	+	+	+	positive	toxigen

Among the fungal species, the highest percentage anchovies were contaminated by *A. niger* followed by *A. flavus* and *A. chevalieri*, *A. tamarii, Penicillium* sp. and *Rhizopus oligosporus.* The presence of *A. niger* was high and found at all markets. Our result in line with Wheeler et al. (1986) who reported that dried salted fish was common infected by *A. niger*. Hassan (2011) also reported that most salted fish was contaminated by *A. niger* (73.3%) higher than *A. flavus* (66.6%), *Penicillium* spp. (40%) and *Rhizopus* spp. (6.6%).

As shown in Table 1 all anchovies sold by retailers at traditional markets were infected by molds, the contamination might occur processing and fungal population incresed during storage. As reported by Azam (2002) conventional drying on fish products susceptible contaminated by microorganisms. In addition, high relative humidity

leads the dried fish absorb water vapor from air and increase moisture content.

3.2 Fungal Population on Anchovies

Fungal population per gram anchovies (colony forming unit or cfu/g) obtained from relailers at five traditional markets are shown in Table 2). Genus of *Aspergillus* was the most dominant (5 species) followed by *Cladosporium* and *Rhizopus oligosporus*.

Table 2 showed that *Aspergillus chevalieri* was the highest population (8.3×10 cfu/g) followed by *A. niger* (3.3×10 cfu/g), *A. flavus* (1.13×10^{-2} cfu/g), and *A fumigatus* (3.3×10 cfu/g). Among storage fungi *A. chevalieri* is xerophilic and grow at a_w 0.71 to 0.74 (Pitt and Hocking 2009). Previous study by Sam (2015) stated that 23 fungal species were isolated from dried fish and were predominated by *A. flavus* and *A. niger.*

3.3 Toxigenicity of *A. flavus* Strains

Eighty strains of *A. flavus* were successfully isolated. Based on toxigenicity determination using CAM medium, they are consisted of 69 strains are toxigenic (aflatoxin producers) and 11 strains are non-toxigen. Gene analysis determine aflatoxin biosynthesis (Table 3) indicate that regulatory gene (*aflR*) and structural genes (*nor-1, omt-1* and *ver-1*) are key enzyme that determine aflatoxin production as previously reported by Erami et al. (2007) that the presence of the for keys genes determine toxigenicity of *Aspergillus flavus*.

Table 3 showed that the presence of regulatory and structural genes on *A. flavus* determined aflatoxin biosynthesis. The toxigenicity determined byn culture technique method in CAM medium and molecular using specific primers showed similar results.

4 CONCLUSION

High fungal contamination on anchovies are potential to spoile and contaminated aflatoxin. Good handling practices was reqiured to reduce fungal growth during storage.

ACKNOWLEDGEMENT

The research was funded by Universitas Sumatera Utara, contract TALENTA Reseach grant no. 136/UN5.2.3.1/PPM/KP-TALENTA USU/2019.

REFERENCES

Azam, K., 2002. Fisherman community of Kuakata Bangladesh: Fisherman Activities and Quality of Fish. Sufer Project (DFID- UGC), Khulna University.

Erami, M., Hashemi S.J., Pourbakhsh S.A., Shahsavandi S., Mohammadi S., Shooshtari S., Jahanshiri Z. 2007. Application of PCR on detection of aflatoxigenic fungi [short communication]. Arch Razi Institute 62(2):95-100.

Hassan, A.A., Hassan M.A., Howayda M., Shafei E.I., Rasha M.H., El-Ahi H.S Abd Ei-Dayem R.H., 2011. Detection of aflotoxigenic moulds isolated from fish and their products and its public health significance. Nature and Science, 9(9): 106-114.

Indriati, N., Hermana I., Hidayah I., Rahayu E. 2017. Prevalence of aflatoxin B1 in commercial dried fish from some regions of Java. Squallen Bull. of Mar.And Fish. Postharvest and Biotechnology 12:107-115.

Lin, M.T., Dianese J.C. 1976. A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. Phytopathology. 66:1466-1469.

Pitt, J.I., Hocking A.D. 2009. Fungi and Food Spoilage. New York (US): Springer.

Sam, J.F., Jeyasanta K.I., Edward J.K.P. 2015 . Aflatoxin investigation on dried fishes of Tuticorin, South East Cost of India. Journal of Foodborne and Zoonotic Diseases. 3:49-62.

Yusra, 2017. Analisis kandungan formalin ikan asin kering di Gasan Gadang, Kabupaten Padang Pariaman Sumatera Barat. Jurnal Katalisator, 2(1):20-28.

Youssef, M.S., Abo-Dhab NF, Farghaly R.M. 2003. Studies of mycological status of salted fish Moloha in Upper Egypt. Mycobiology, 31:166-172.

Yuliar, Abidin Z., Mangunwardoyo W. 2011. Potency of biocontrol agents isolated from compost and peat soil of tropical peat swamp forest in Kalampangan zone, Central Kalimantan. Journal of Forestry Research 8(2): 144-157.

Warren, W., Hergoualch, Kauffman J.B Murdiyarso D., Kolka R. 2017. Ann appraissal of Indonesia's immense peat carbon stockusing national peatland maps: uncertainties and potential lossesfrom conversion. Carbon Balance and Management 12 (12): 1-12.

Wheeler, K.A., Hocking A.D., Pitt J.I., Anggawati A.M. 1986. Fungi Associated with Indonesian Dried Fish. Food Microbiology, 3:351-357.