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Isolation of Fungal Species and Aflatoxin Detection in Fermented Products

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Abstract

The study was done to determine the presence of aflatoxin-producing fungi from two batches of six commercial soy sauce products. The level of aflatoxin present was also detected and determined using Enzyme Linked Immunosorbent Assay (ELISA). Two species of Aspergillus were isolated namely Aspergillus parasiticus and Aspergillus niger Aspergillus parasiticus together with Aspergillus flavus, grow on improperly stored grains and produce aflatoxin known to be one of the most potent carcinogens. Aspergillus niger on the other hand, is a non-aflatoxin producing fungi and is rarely reported to cause pneumonia in humans. Fusarium species are recognized as being pathogenic to man and animals, some species are considered to be common allergen causing Type I allergies, and other species cause storage rot and important mycotoxin producers. Results of the ELISA revealed a low level of aflatoxin ranging from 0.3-1.8 ppb as based on the US FDA standards. Future and further tests and analysis can be done to supplement the results of this study.

Keywords: *Aspergillus*, *Fusarium*, aflatoxin, soy sauce, ELISA.

Introduction

Aspergillus oryzae has been used in the production of many traditional fermented foods like sake (rice wine), miso (soybean paste) and shoyu (soy sauce). This fungal species has also been used to obtain many kinds of hydrolytic enzymes like amylases, proteases and nucleases. In 2005, *A. oryzae* was sequenced and it was found to be significantly larger and contains more genes than the genomes of other *Aspergillus* species[1]. Unlike *A. flavus*, *A. oryzae* does not produce aflatoxin although both species belongs to same genus and its long history of use in food industry proves its safety[2]. Although this lack of aflatoxin production is not observed in *A. oryzae*, however, there have been reports that aflatoxin pathway regulatory gene, *aflR* gene is present in single strains of *A. oryzae* and *A. sojae*[3], as well as presence of *aflR* and *omtA* in several strains of *A. oryzae* and *A. sojae*[4] and sequence variability of part of *aflR* in strains of *A. parasiticus*, *A. flavus*, *A. oryzae* and *A. sojae*[5]. Aflatoxins contained in these fungi, can enter human food chain starting from growing to storage (prior to processing), to processing then to storage and lastly to the consumers. The moisture during the storage, the area's sanitary protocols and other amenities often cause the growth of molds in corn, wheat and other food stuffs. According to a review done by Bennett and Klich (2003)[6], the economic consequences of mycotoxin contamination are intense. Those crops that are contaminated with mycotoxins are often destroyed and some are diverted into animal feed. As a result, there are a reduced growth rates, illness and even death to susceptible animals by giving them these mycotoxin-contaminated feed. Furthermore, these animals can produce meat and milk that contains residues of the toxins and biotransformation products. Thus aflatoxins in cattle feed can be metabolized by cows into aflatoxin M1 which will then be secreted in milk, ochratoxin in pig feed can be collected in porcine tissues. Complete eradication of toxins from foods and feeds is an impossible objective[6]. Hence, naturally occurring toxins such as mycotoxins are regulated differently from food additives. The United States Food and Drug Administration, the European Union, the Japan Institute of Public Health and other government agencies all over the world test the products for mycotoxins and established guidelines for safe doses[7]. These toxins are of particular public health importance because of the diseases they cause to humans. Ingestion of higher doses of aflatoxin can cause acute aflatoxicosis, or in severe cases, liver failure[8]. An outbreak in Kenya in 2004 has shown that contaminated home-grown maize bough from local farms in the affected area entered the distribution system, which results to the widespread contamination of the market maize[9]. Moreover, sorghum and maize samples collected in Deccan plateau in India contained *Fusarium* sp. which caused abdominal pain and diarrhea in people of 27 out of 50 villages surveyed[10]. Recently Okoth and Koala (2012)[11] reported that 120 out of 144 samples collected had levels greater than the regulatory limit (10ppb). Thus, determining its presence in food products produced by non-aflatoxigenic fungi, like that of *A. oryzae* and *A. sojae* has to be investigated and analyzed to assess that these fungi are still safe to use in the fermentation process and for human consumption. This study aimed to isolate *A. sojae* and *A. oryzae* from commercial fermented product (particularly soy sauce) and to determine the level of aflatoxin present in these products.

Material and Methods

Collection of Samples: Two batches of six commercial soy sauce samples were collected and were brought to the laboratory for analysis. Samples were serially diluted and 0.1 ml was spread onto the surface of Potato Dextrose Agar. Plates were incubated for 3-5 days for identification. *Aspergillus flavus* and

A. oryzae strains brought from University of the Philippines-Diliman, Philippines were used as positive controls for the identification of isolates.

ELISA Analysis: Prior to analysis using ELISA reader, 5ml of samples were placed to different sterile beakers containing 25ml of 70% MetOH, then placed on a shaker for 2-3 minutes and filtered using a clean filter paper. Extracts were analyzed using Veratox® Total Aflatoxin ELISA Kit. First, 100µl conjugate was added to red-marked mixing well using 12-channel pipettor, then 100µl of controls and samples were added unto it. From the red-marked mixing well, 100µl was obtained and transferred to the antibody-coated wells, mixed three times and incubated for 2 minutes. After that, contents were dumped into a waste container then the antibody-coated wells were washed 5 times with sterile distilled water and excess water was tap dabbed out on an absorbent paper towel. Next to that, 100µl substrate from reagent boat was transferred to antibody-coated wells, mixed thoroughly and incubated for 3 minutes. Lastly, 100µl Red Stop solution was added to antibody-coated wells and mixed thoroughly. Results were read using StatFax 303 ELISA reader.

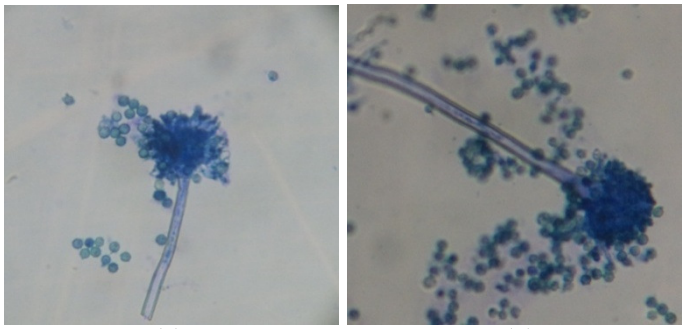
Results and Discussion

Two batches of six commercial soy sauce products were collected, tested and analyzed. Batch numbers of the samples were recorded. Samples were diluted and spread unto potato dextrose agar medium and antibiotic was previously added in the medium to prevent bacterial contamination. After 3-5 days of incubation, species of *Aspergillus* and *Fusarium* were isolated and identified from the two batches of six soy sauce samples used as shown in the table below.

Table-1
Fungal species isolated and identified from two batches of six soy sauce samples

Soy Sauce Samples	Fungi Isolated
1	*Aspergillus* sp.
2	*Fusarium* sp.
3	*Aspergillus* sp., *Fusarium* sp.
4	*Fusarium* sp.
5	*Aspergillus* sp., *Fusarium* sp.
6	*Fusarium* sp.

One of the *Aspergillus* species is closely related to that of *Aspergillus parasiticus* as revealed by its massive green conidial growth (figure-2A) on potato dextrose agar with spherical spores as viewed in HPO (figure-1A). The other was identified as *A. niger* based on its black conidial growth (figure-2B) and globuse brownish-black spores under HPO (figure-1B). The former is widely known for its toxin production[7] while the latter is economically important in the production of citric acid in which it is one of the most efficient, highest yield bioprocesses currently used in the industries[12].



(a) **(b)**

Figure-1
Fungal spores viewed under HPO after stained with methylene blue

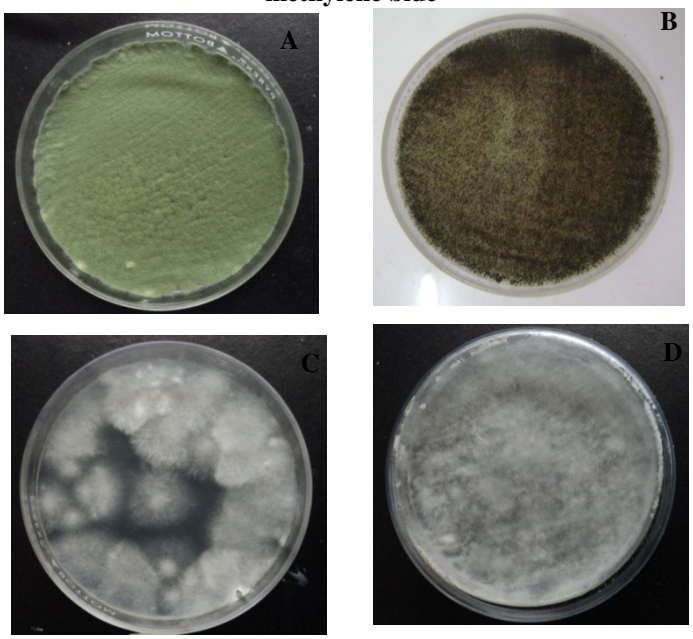


Figure-2
Four fungal species isolated from the six soy sauce products. A and B were the two *Aspergillus* species while C and D were the two *Fusarium* species.

Furthermore, *Fusarium* species has a cottony-white conidial growth which would become light brown and produces moist after several days of incubation (figure-2C) and sickle-shaped spores, the other one turns pinkish after several days of incubation (figure-2D). This Genus is also known to produce various toxins such as trichothecenes (T-2 toxin, HT-2 toxin, deoxynivalenol (DON) and nivalenol) and some other toxins (zearalenone and fumonisins). Contamination of these toxins is worsen whenever shipping, handling and storage practices are advantageous to mold growth[6]. Presence of the toxin-producing fungi in the soy sauce samples implies the possible presence of toxins produced by these fungi in the food samples which could affect humans as it enters the food chain during storage and processing of the product. Thus, further test was done to the products that showed positive results for the presence of *Aspergillus* and *Fusarium* to determine the level of toxin present and results were compared to the allowable limit of toxin in

food samples. This was done using Enzyme-linked Immunosorbent Assay (ELISA) to determine the level of possible presence of toxin in the soy sauce samples. Results revealed a low aflatoxin level in the samples that meet the allowable aflatoxin in foodstuffs (20 ppb) based on the US FDA Standards.

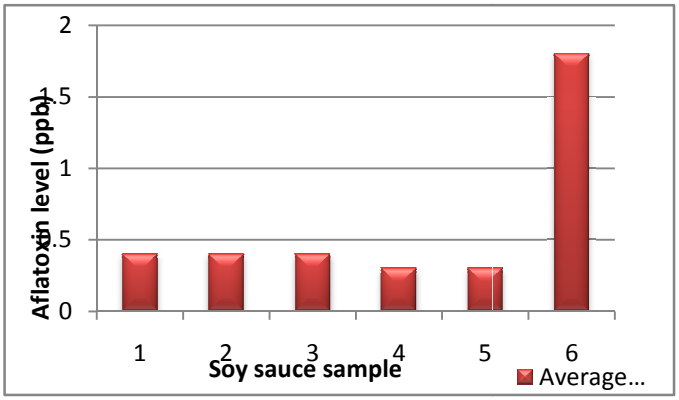


Figure-3
Average aflatoxin level in two batches of six soy sauce samples detected using Veratox ® ELISA Kit and StatFax Elisa Reader

As shown, samples 1, 2 and 3 has an aflatoxin level of 0.4ppb and samples 4 and 5 had 0.3ppb. Sample 6 showed a very high level compared to that of the 5 other samples having 1.8ppb aflatoxin level but still it is below the allowable aflatoxin standards. Although the soy sauce samples contain *Aspergillus* and *Fusarium* species which are known to produce toxins, the level of aflatoxin of the samples revealed that they are still safe for the consuming public since the allowable aflatoxin level is 20ppb in food stuffs. A study done by Gorham and Hokeness (2012)[14] showing that mold exposure leads to altered health in mice.

However, farmers, processors, traders and transporters of these products must have continuous mycotoxin awareness so as minimize if not to eliminate the contamination in these and other food stuffs.

Conclusion

This study was done to determine the presence of aflatoxin-producing fungi form two batches of six commercial soy sauce products. The level of aflatoxin present was also detected and determined using ELISA. Two species of *Aspergillus* were isolated namely *Aspergillus parasiticus* and *Aspergillus niger*. *Aspergillus parasiticus* together with *Aspergillus flavus* grow on improperly stored grains and produce aflatoxin known to be one of the most potent carcinogens. *Aspergillus niger* on the other hand, is a non-aflatoxin producing fungi and is rarely reported to cause pneumonia in humans. *Fusarium* species are recognized as being pathogenic to man and animals, some species are considered to be common allergen causing Type I allergies, and other species cause storage rot and important mycotoxin producers. Results of the ELISA revealed a low level of aflatoxin ranging from 0.3-1.8 ppb as based on the US FDA standards. Although the soy sauce samples contains *Aspergillus* and *Fusarium* species which are known to produced toxins, results revealed that samples are safe for the consuming public. However, further tests and analysis can be done to supplement the results of this study. Since it is normally impractical to prevent the development and persistence of aflatoxins before and after processing of the food stuffs, it is important to have policies to ensure that these products will not pose any significant hazard to human health.

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