Decontamination of aflatoxin producing fungi on agriculture products by atmospheric glow discharge plasma

Smit Preechayan[1*]
Kittipong Tonmitr[2]
Amnart Suksri[3]
Pooncharassami Siriputthaiwan[4]

Abstract

Fungal contaminations on agricultural products have been a detrimental problem especially for agro-industrial country like Thailand. Such contamination could lead to economic instability and decremental of product quality. Some species of fungi can develop Aflatoxin; this severe toxin is poisonous to consumer as well as livestock. Reduction of pathogenic microorganism is crucial. A good method to decontaminate toxic fungi is to reduce the amount of viability toxin producing fungal spore from the beginning of food chain in the raw material and agricultural products. This paper study the ability of constructed one-atmospheric glow discharge plasma on a reduction of contaminated aflatoxin producing fungi from agricultural products. Plasmsa was generated at low frequency in the range of 400-800 Hz and applying the voltage at 30 kV. Experimental test have been carried out with 4 kinds of commonly found agricultural products which are; corn, bean, garlic, and shallot. Prior to the test, there were number of naturally contaminated fungi and it was counted to 380, 510, 710 and 7×10^4 CFU/g, respectively. After the test, it was found that corn and bean can be sterilized and the total mold was completely reduced. In the case of garlic and shallot, the amount of fungi was reduced but still remained at 97 and 2×10^4 CFU/g, respectively. Apart from agricultural products, the method of one-atmospheric glow discharge plasma can sterilize the *Aspergillus flavus* spores specie that is coated on a glass bead with the ability to inoculate of approximately 1.8×10^7 CFU/g within less than 30 minutes.

Keywords : Atmospheric plasma, Aflatoxin-producing fungi, Agriculture products

Introduction

Being an agro-industrial country, Thailand produces a great number of agricultural products. However, many of them are damaged by fungal contamination which occurred mostly during harvesting or storage. In addition, Thailand is located on the tropical climate zone where the weather

[1] *Graduate Student, Master of Engineering, Department of Electrical Engineering, Faculty of Engineering, Khon Kaen University*
[2, 3] *Associate Professor, Department of Electrical Engineering, faculty of Engineering, Khon Kaen University*
[4] *Lecturer, Department of Microbiology, Faculty of Science, Khon Kaen University*
* *Corresponding author, e-mail : smith_1410@yahoo.com*

is hot and humid and it is suitable for fungi to grow and difficult to control. Such contamination could lead to economic instability and decrement of product quality. Ultimately, some species of fungi can develop aflatoxin, a severe toxin which is poisonous to consumer as well as livestock.

Aflatoxin is developed from natural spore species called *Aspergillus flavus* and *Aspergillus parasiticus*, which can be found in several agricultural products, e.g. corn, bean, garlic, shallot, and other grains which are mostly used as ingredients in Thai food. It is a type of severe toxin, causing liver cancer, and can be poisonous to human and livestock. It is difficult to decontaminate because it can endure very high temperature; thus cannot be decontaminated with normal cooking temperature.

Lately, there has been a continuous demand in finding the method which can efficiently restrain and decontaminate the toxic fungi. On the other hand, such method must be suitable and safe for agricultural products. Thus, a suggested method for the purpose is the reduction of the amount of viable toxin producing fungal spores from the beginning of the food chain in raw materials and agricultural products.

Currently, plasma technology is used in microorganism decontamination in several industries, such as air conditioning, food manufacturing, and health and medicine. In fact, there has been interest and application in one-atmospheric glow discharge plasma because the vacuum process, which consumes a lot of time and cost in each cycle, is not required. There are many types of atmospheric plasma generated, e.g. Corona Discharge, Dielectric Barrier Discharge, and Glow Discharge Plasma. The most suitable type for the sterilization or inoculation purposes is the Glow Discharge Plasma (Montie et al., 2000)

This paper aims to study the ability of constructed one atmospheric glow discharge plasma at low frequency on the reduction of contaminated aflatoxin producing fungi from agricultural products in Thailand. The four kinds of agricultural products which are commonly found, and regularly contaminated, were used in the experiment, e.g. corn, bean, garlic, and shallot. The equipment used in the experiment was the one atmospheric glow discharge plasma for fungal decontamination on agricultural products. Its construction was uncomplicated while the parts were inexpensive and could be found locally. Ultimately, it should be used as a prototype for the development of plasma generator and applied for the industrial use in the future.

Methodology

According to the objectives of the study, the following are the steps to study the ability of a constructed one atmospheric glow discharge plasma to reducing contaminated aflatoxin producing fungi

1. Construction of the test equipment
2. The total mold reduction test on the surface of agricultural products
3. The test of effect of plasma on aflatoxin producing fungi on tested agricultural products

1. Construction of the test equipment

The construction of the plasma generator for the reduction of contaminated agricultural products can be divided into 2 steps as follow

1.1 Generating glow discharge plasma

1.2 Designing the equipment structure for fungi decontamination

1.1 Generating Glow Discharge Plasma

The glow discharge plasma generates plasma on metal electrodes, designed with basic principle

of Roth et al. (1998) as One Atmosphere Uniform Glow Discharge Plasma. It is an electrical discharge in air at atmospheric pressure without requiring an impedance matching circuit or transformer or high frequency electric fields. Instead, plasma was generated by high voltage AC between solid and gas dielectric, causing electric field to the surface of such dielectric. In such area, high voltage AC will pass through gas (which is air in this experiment), turning gas into plasma.

The plasma generated with high voltage AC was generated with pulse signal generator and switching circuit which connects AC signals through induced secondary coil of the high voltage transformer. This secondary high voltage created were connected with 100 cm^2. electrode to generate plasma.

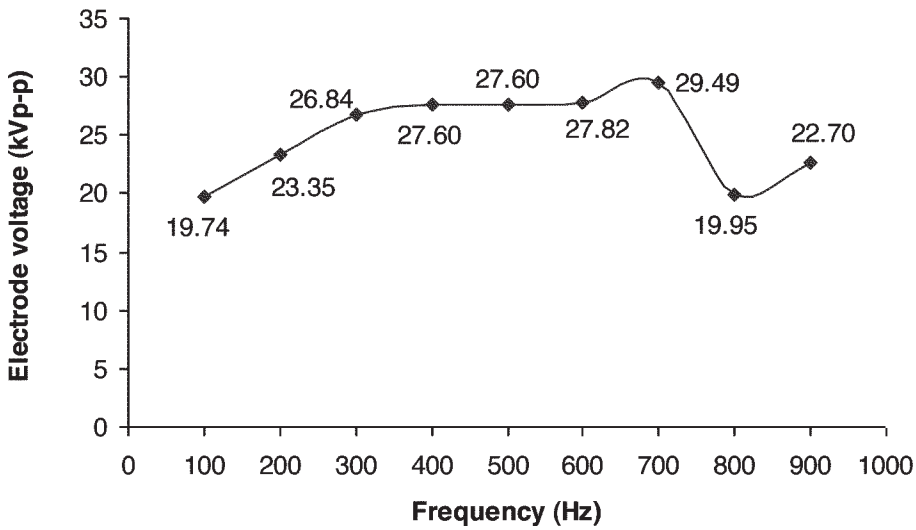
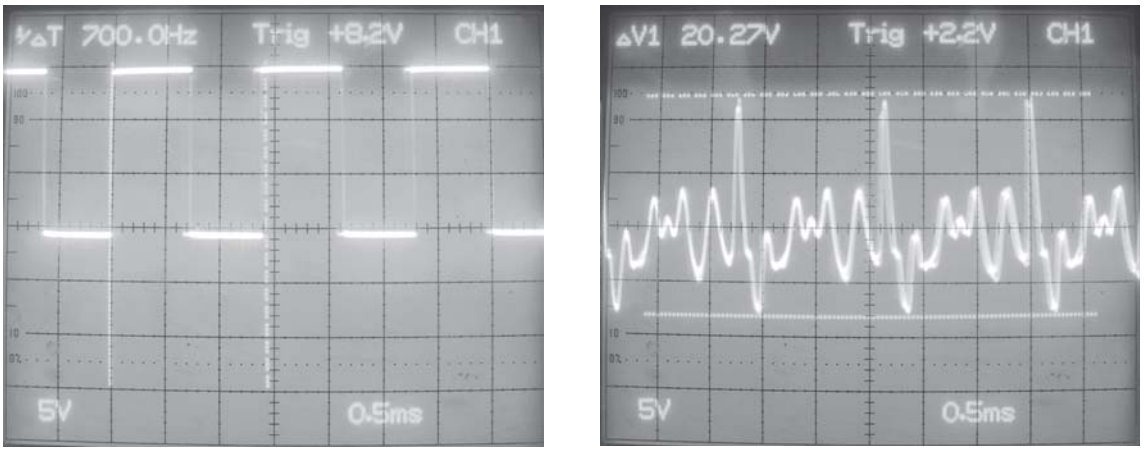


Figure 1. Electrode voltage versus applied frequencies

When the plasma generator was done, we started testing the electrical discharge at frequency starting from 100 Hz up to 900 Hz and by incrementally adjusting at 100 Hz in each step. The frequency adjustment resulted in changes in electrode voltage shown in Figure 1, which is the measurement of maximum peak voltage. In addition, we could also observe the difference in brightness of plasma (Glowing Discharge) on electrode at each level of adjustment. For the purpose of fungal decontamination test in this study, we select the appropriate frequency according to the characteristics and brightness of the discharge, along with the resulting electrode voltage.



A. Typical Pulse Signal **B.** Typical Electrode Voltage

Figure 2. Typial Pulse Signal measured from the equipment

In this research study, we have set the circuit frequency at 700 Hz. The signals measured from the plasma generator are shown in Figure 2, where Figure 2A shows the typical pulse signals read from the pulse signal generator and switching circuit, while Figure 2B shows electrode voltage from the resistive voltage divider at 1:1455 ratio, connecting with an oscilloszcope and isolation transformer, which can measure high electrode voltage from the $\pm$ 29.5 kV_{MAX}

1.2 Designing the equipment structure for sterilization purpose

After generating plasma on electrodes, the equipment structure for fungal decontamination was designed. Such equipment consisted of: a cylindrical acrylic containing the electrode pad for plasma generator; PVC pipe with the top for feeding raw material samples; fans for ventilating the plasma generating area. The fans were used because while generating plasma, several active species were also generated for microorganism resistance. In addition, they can also carry the active species to the decontamination area as well as cool down the temperature and let out steam within. Figure 3 shows the equipment arrangement and the plasma generator structure.

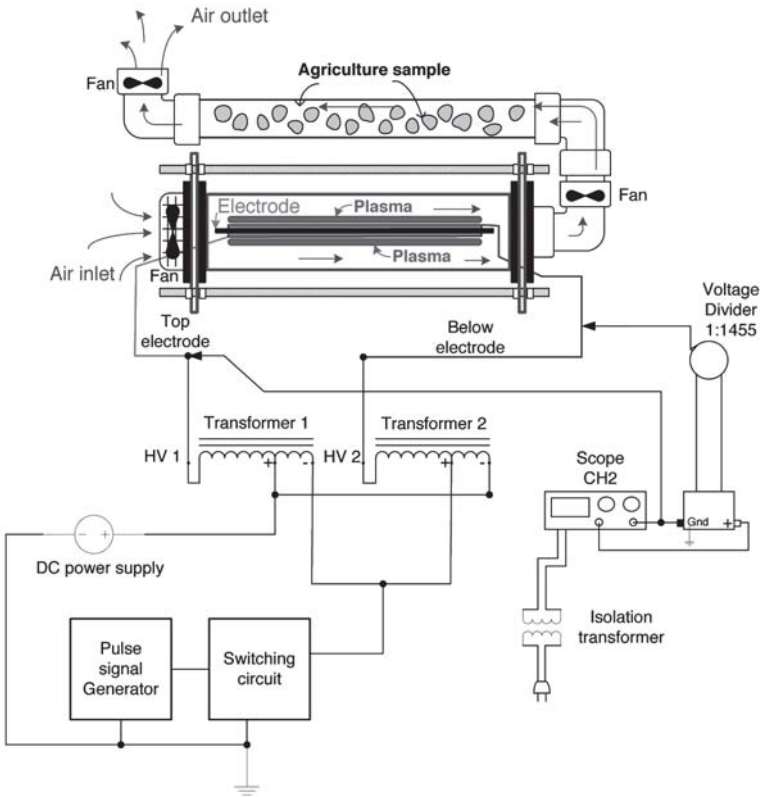


Figure 3. Test equipment arrangement and plasma generator structure

The electrode generating plasma were made of square-weaved copper electrode placed parallel to each other. The two sides of electrode where placed so that there was overlapping between the copper wire of the top and bottom electrode. This, in turn, generated electrical discharge or plasma between them. Figure 4. shows the alignment of electrode.

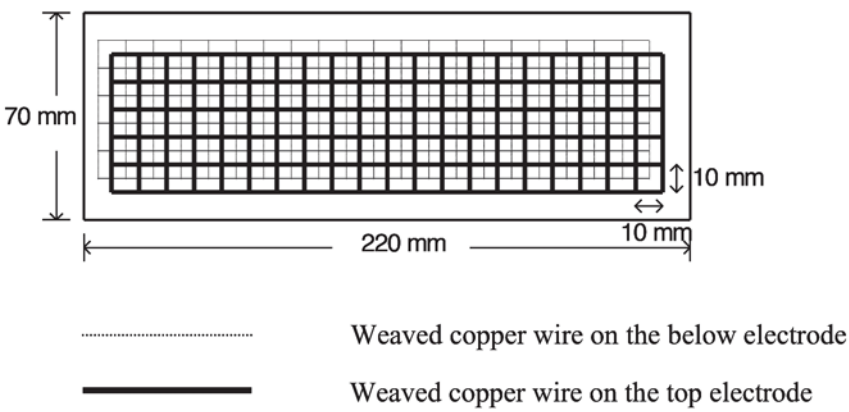


Figure 4. Top view of electrode for plasma generation

Electrodes (Figure 4) were made of double-sided copper Printed Circuit Board (PCB) with the size of 20 x 80 x 0.2 centimeters (width x length x depth). The copper PCB was corroded resulting in 100 squares of 1x1-centimeter cavities. From the top view, it could be seen that the top and bottom electrode were overlapping, as shown in Figure 4 (thin and think lines). The copper wires were approximately 1 millimeter-thick. Plasma was generated on the surface of the two sides of electrode.

2. The total mold reduction test on the surface of agricultural products

This study aims to test the ability of a constructed plasma generator to decontaminate or reduce the amount of viability fungal spores on agricultural product surface. The agricultural products selected for the experiment were corn, bean, garlic, and shallot, all with difference surface physical characteristics and were frequently found with high amount of fungal spores. The samples were randomly chosen from suppliers and tested with actual fungi on their surfaces.

Test Procedures

The same procedures were used in testing all 4 types of agricultural products: corn, beans, garlic, and shallot; the test was conducted on each type separately and one at a time, as follow

(1) Samples in each type of products were divided into 2 groups. The first group was controlled and did not go through the process for decontamination, whereas the second group was the test group going through the decontamination process with the constructed equipment.

(2) The test group was tested for fungal decontamination by the constructed glow discharge plasma, with the pulse signal while generating plasma as shown in Figure 2 & 3. The equipment arrangement is shown in Figure 4 and the test duration was 20 minutes for each sample.

(3) The samples in both groups were microbiological tested to determine the amount of total mold according to the Bacteriological Analytical Manual (BAM)(1998).

(4) Decontamination evaluation was done by comparing the amount of fungi in the two groups of samples: between the naturally contaminated samples in controlled group and the remaining total mold in the tested group.

3. The test of effect of plasma on aflatoxin producing fungi on tested agricultural products

The study of the effect of plasma on the viable toxin producing fungal spores on agricultural product surfaces consisted of: Procedure 3.1, The determination of fungal spores producing toxins on tested samples was done by through microbiological test to distinguish and identify *Aspergillus flavus* spore species which produces aflatoxin; and Procedure 3.2, The test for decontamination of *Aspergillus flavus* spore species with the constructed plasma generator. The details of the test are as follow.

3.1 Identification of *Aspergillus flavus* from samples

The objective is to identify whether *Aspergillus flavus* was found on the randomly selected samples, since it is the fungal species which mainly produces aflatoxin.

Test Procedures

The colony sample from the selected agricultural products were subjected to identification as *Aspergillus flavus* (section flavi) using both slide

culture technique and morphological characterization using CU (Modified Rose bengal) medium (Cotty, 1994). This modified medium is composed of 3 grams of Sucrose, 2% Agar, 3 grams of $NaNO_3$, 0.75 gram of KH_2PO_4, 0.5 gram of $MgSO_4.7H_2O$, 10 grams of NaCl, 1 milliliter of A&M Micronutrients, pH 6.5 complemented with chloramphenicol 5 mg/L.

The fungal isolates which showed positive result to be identified as *Aspergillus flavus* were further examine microscopicly.

3.2 The effect of plasma on *Aspergillus flavus* spore species

The objective of this procedure was to verify the effect of plasma from the constructed test equipment on the decontamination or reduction of the amount of toxin producing *Aspergillus flavus*

Test Procedures

The *Aspergillus flavus* spores species from procedure 3.1 were coated on a glass bead, starting at the amount of 10^7 CFU/g. It was then sterilized by the plasma generator. Afterwards, the before and after comparison was done by comparing the amount of colony on the culture medium plate.

Sample Preparation

(1) A glass bead sterile was placed on a plate sterile, containing glycerol sterile for maintaining *Aspergillus flavus* spores species on the surface .

(2) The glass bead sterile in (1) was picked with forceps and placed on plate sterile with *Aspergillus flavus* spores species, then the plate was rotated, in order to evenly distribute the spores over the plate surface.

(3) 10 glass beads in (2) were picked with forceps (1 gram) and dropped in a tube sterile containing approximately 9 milliliter diluents (dilution 10^{-1} to find the initial amount of spores). Another 2 plates sterile of 10 glass beads sterile were prepared for the next procedure (plasma decontamination test).

The Yeast and Molds Analysis

(1) The experimental samples were diluted at the appropriate dilution, depending on the type of sample, with 0.1% Peptone as a diluent.

(2) Melt the culture medium, DG18, then warm in a water bath at 45 degree Celsius.

(3) A 1.0-milliliter pipet was used to draw the diluted sample at each dilution as well as the original sample (in the case of liquid sample) onto the culture medium plate. The culture medium, DG18, was poured onto the plate at 20-25 milliliter. Triplicate the process for each dilution. Rotate clockwise and counter-clockwise, then up and down along the plane to blend the sample and culture medium (Pour Plate Method).

(4) Let it mature at 25 degree Celsius for 5 days without inversing the plate.

(5) Once it is mature, count the number of colony in the range of 10-150 colonies per plate.

(6) The number of colony found was used for calculating the amount of fungi.

(7) In the case of fungi identification, the colony would be stained on glass slides and examined with a microscope to identify its species.

Results

1. Amount of fungi before and after the glow discharge plasma decontamination

Each type of agricultural products, i.e. corn, bean, garlic, and shallot, were divided into 2 groups: controlled and tested. The tested groups went through the decontamination process under the plasma generation at 29.5 kilovolt and 700 Hz, for 20 minutes, the time duration which the plasma effect on beans was expected to be clearly visible (Smit, 2008). Afterwards, the amounts of total mold were calculated and the results were then compared with those of

the controlled groups. Figure 5 shows the amount of fungi found from the experiments.

From the results shown in Figure 5, corn and bean samples initially had the total mold on the surface of 510 and 380 CFU/g, respectively, whereas the glow discharge plasma could reduce the amount to less than 10 CFU/g on both types of samples. There was, in other words, no colony on the culture plate. On the other hand, the total mold in garlic was initially 730 CFU/g, and after sterilization, by the glow discharge plasma, it was down to only 97 CFU/g. shallot were the type of samples with relatively high fungal contamination of 7×10^4 CFU/g, and the decontamination brought it down to only 2.1×10^4 CFU/g.

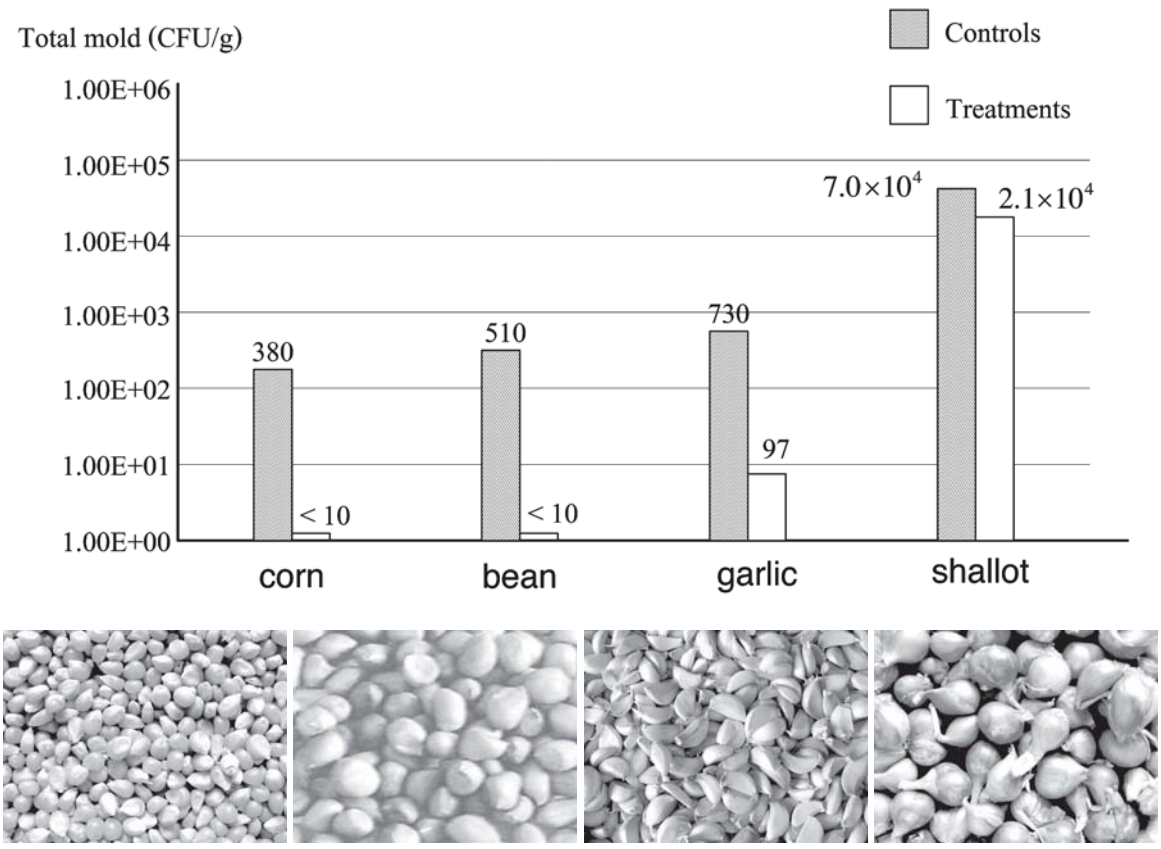


Figure 5. The amount of total mold on the surface with (Treatments) and without (Controls) the glow discharge plasma decontamination

2. Effect of plasma on toxin producing fungi on agricultural product samples

The result from the experiment of plasma effect on toxin producing on agricultural product samples consist of

2.1 Investigation of aflatoxin producing fungal contamination on agricultural samples

2.2 The decontamination of *Aspergillus flavus* spore species with the constructed plasma generator

2.1 Investigation of aflatoxin producing fungal contamination on agricultural samples

The four agricultural products were subjected to isolate of viable colony-forming units (spores). Then result shown in Table 1 indicates the number of colony with morphological characteristics similar to those of *Aspergillus flavus*. We then further investigated by means of randomly examination of aflatoxin producing fungi especially *Aspergillus flavus* using methods according to Procedure 3.1. The obtained results revealed that fungal colonies isolated from Sample 1 & Sample 2 were not identified as *Aspergillus flavus* however some colonies from Sample 3 (1) and Sample 4 (2) were identified as *Aspergillus flavus*. The results suggested therefore that selected agricultural product samples carried toxin producing fungal contamination , especially shown here as aflatoxin producing fungus : *Aspergillus flavus*.

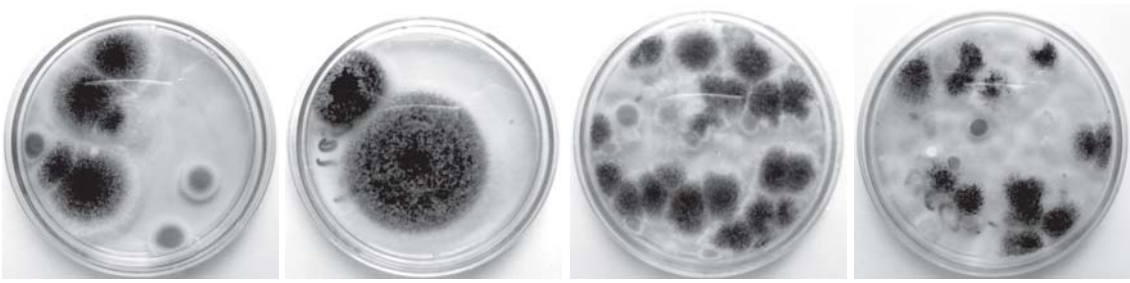


Figure 6. Culture plates from samples tested for *Aspergillus flavus* spore species.

Number of fungal Isolates from agricultural product	Sample 1	Sample 2	Sample 3	Sample 4
Putatively classified as *Aspergillus flavus*	27	6	3	4
Confirmly identified as *Aspergillus flavus*	0	0	1	2

Table 1. Spore species identification results of shallot and garlic samples.

2.2 The decontamination of *Aspergillus flavus* spore species with the constructed plasma generator

Glass beads were surface-coated by *Aspergillus flavus* spores in the order of 1.8×10^7 CFU/g. The fungal spore coated beads were then subjected to sterilization with the constructed plasma generator. Sterilization capacity was determined by comparison of the number of fungal colony (viable count) from the fungal spore coated beads either with or without sterilization. Since we started at a rather high volume of fungi of 1.8×10^7 CFU/g, the treatment time was set at 30 minutes.

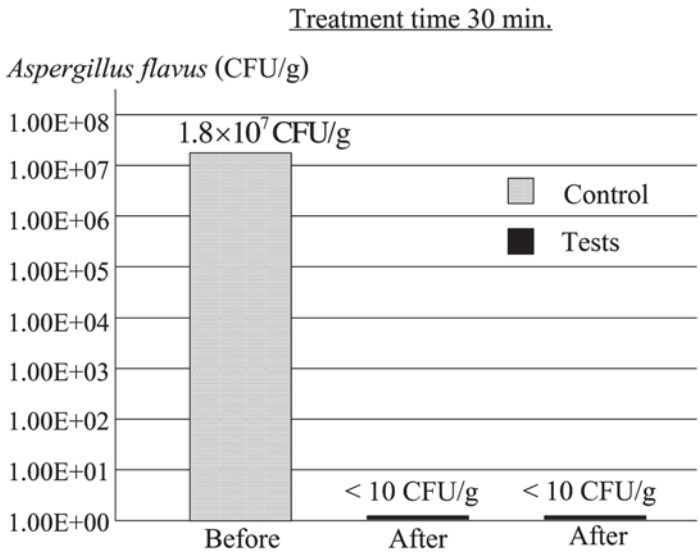


Figure 7. Number of *Aspergillus flavus* spore on the glass bead surface, before (Control) & after (Tests) the decontamination by glow discharge plasma.

Figure 8A. Without (before) the decontamination by glow discharge plasma

Figure 8B. With (after) the decontamination by glow discharge plasma

Figure 8. *Aspergillus flavus* culture plate of the two set of samples

The experiment condition used for decontamination of *Aspergillus flavus* coated beads were 700 Hz, 30 kVP-P, 30 minutes. The viable counts of fungal spore were only less than 10 CFU/g from the plasma treated *Aspergillus flavus* coated beads and 1.8×10^7 CFU/g from those of the control group, as shown in Figure. 6.

Conclusion and Discussion

In order to study the ability of the one-atmospheric glow discharge plasma generator for fungi decontamination on agricultural products, we have constructed an equipment prototype for the experiment on fungal decontamination on 4 types of agricultural products: corn, bean, garlic, and shallot. The experimental results are as follow

(1) The atmospheric discharge plasma generator was successfully constructed by applying the glow discharge based on basic electrode designed of "One Atmosphere Uniform Glow Discharge Plasma". The equipment operated at low frequency of lower than 1 kHz, which is suitable for the available materials and selected equipments. In addition, the plasma generated could be further studied for its decontamination ability in the next step.

(2) Plasma generated from the constructed equipment could produce active species which efficiently reduces the amount of total mold in a short period of time. The experiment was done with the naturally contaminated agricultural product samples.

(3) Plasma generated from the constructed equipment could produce active species which efficiently reduces the amount of *Aspergillus flavus*, aflatoxin producing fungi.

(4) Although each type of samples had undergone the same treatment time, there were noticeable differences in the reduction of total mold. This is due to the difference in nature of surface of each type of agricultural products, which affected the ability of plasma decontamination. It could be observed that plasma could efficiently reduce the amount of fungi on the surface of corn and bean. On the contrary, although plasma could reduce more than half of the original amount of fungi in shallot, there was still high number of fungi remaining. This is because shallot have several skin layers, making it more difficult to decontaminate. Therefore, the treatment time needs to be increased.

According to the experimental results from this study, it is affirmative that the atmospheric glow discharge plasma is an efficient method in controlling fungal contamination on agricultural products. The experimental equipment was constructed with simple and low-cost circuits and materials. Thus, the equipment can be used as a prototype for local use because most researches abroad use plasma at high frequency which requires expensive parts and equipment construction. As a result, this study can be used as a basic prototype for developing plasma generator for domestic use. Finally, it can also be used for further development of agricultural industry in fungal contamination and aflatoxin control in agricultural products, in compliance with the standards for export products.

References

Bacteriological Analytical Manual US Food and Drug Administration Center for Food Safety and Allied Nutrition edition 8, reversion A, (BAM). 1998

Cotty PJ. 1994. Comparison of Four media for the isolation of Aspergillus flavus group. **Fungi Micropathologia** 125 : 57-62

Montie, T.C., Wintenberg, K.K., and Roth, J.R. 2000. An Overview of Research Using the One Atmosphere Uniform Glow Discharge Plasma (OAUGDP) for Sterilization of Surfaces and Materials. **IEEE Transactions on plasma science.** Vol.28, NO.1 (February): 41-50.

Roth, J.R., Sherman D.M., and Wilkinson, S.P. 1998. Boundary layer flow control with one atmosphere uniform glow discharge. **AIAA** 98-0328, 36th Aerospace Sciences Meet. (January 12-15) : 1-28.

Smith Preechayan, Kittipong Tonmitr, Amnart Suksri, Pooncharassami Siriputthaiwan.2009. Application of Atmospheric Plasma : Fungal decontamination on Grains. **KKU Research Journal.** 14 (2): 213-223.