Optimum cold plasma generating device for treatment of *Aspergillus flavus* from nuts surface

A. H. Ghorashi¹ · M. A. Roosta Tasouji² · A. Kargarian¹

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Abstract The objective of this research is to avail an optimum cold plasma generating device for inactivating *Aspergillus flavus* from nuts surface. For this purpose, a variety of tests were carried out using three applicable plasma generating devices such as atmospheric pressure capacitive coupled plasma (AP-CCP), direct current diode plasma (DC-DP), and inductively coupled plasma (ICP) for different values of input power, pressure, and irradiation time, using Argon gas. The experimental results were achieved upon performing irradiation of sample pistachio nuts by the aforementioned three devices. Finally, after data analysis based on two factors of toxin inactivation amount and cost feasibility for large scale industrial applications, the AP-CCP device was found to be as an optimum device with an acceptable 4 Logs fungi reduction. However, on the basis of single factor, i.e. toxin reduction rate, the DC-DP device works better with a highest fungi reduction rate of 5 Logs, using Argon in 1 Torr vacuum pressure, 300 W and 20 min irradiation.

Keywords Cold plasma devices · *Aspergillus flavus* · Nuts · Fungi reduction · Industrial relevance

Introduction

The fungus *Aspergillus flavus* is one of the most acute formation sources of *Mycotoxins*. It is known as a serious threat to agricultural products; particularly those containing fat and high carbohydrates like oil seeds and nuts. Hence, Pistachios, almonds, peanuts, corn, wheat and rice can be good hosts for the fungus. In order to suppress fungal growth and reducing *mycotoxin* formation, many remedies have so far been suggested (Goryacheva et al. 2007; Martins et al. 2008; Tsitsigiannis et al. 2012). These methods of treatments are costly, not completely effective, can be harmful, and impractical or even if not so, they are ultimately not economically feasible and relevant for big scale industrial applications.

In recent years, interests on potential applications of cold plasma have been increased for microbial decontamination and treatments of *Mycotoxins*, including inactivation of *Aspergillus flavus* infecting nuts shell (Dasan et al. 2016; Kim et al. 2014, 2017; Lee et al. 2018; Los et al. 2017; Misra et al. 2014; Nikiforov et al. 2016; Oh et al. 2015; Schnabel et al. 2015; Shama and Kong 2012; Tasouji et al. 2018; Thirumdas et al. 2015; Ziuzina et al. 2014). The results of these valuable research works differ from one another depending on treatment time, input power, pressure and the gas medium through which the plasma is generated. Factors effecting the formation of plasma mainly are: plasma forming gas (Argon, Helium, Nitrogen, Sulfur hexafluoride etc.), gas pressure and flow rate, electric and magnetic fields, shape and arrangement of electrodes and finally, the type of plasma generating device (Tasouji et al. 2018).

The present work is carried out in the same laboratory and on the same experimental set ups where the earlier research work was done (Tasouji et al. 2018); but this work

✉ A. H. Ghorashi
aghorashi@aeoi.org.ir

¹ Plasma and Nuclear Fusion Research School, Nuclear Science and Technology Research Institute, Tehran, Iran

² Chemical Engineering Group, Faculty of Engineering, Azad University, Marvdasht, Iran

is concentrating on the type of plasma forming device used. In other words, we have experimentally proved that the type of device has a key role in fungi reduction rate. Such cumulative research data are presented for the first time, in this paper. Hereby, whilst introducing three applicable plasma generating devices, namely AP-CCP, DC-DP, and ICP; the numerous tests are undertaken using these devices. Finally, after comparing the obtained results, the optimum means of treatment is determined, based on two objectives i.e. toxin inactivation amount and the methods relevancies for large scale industrial applications. Moreover, achievement of a higher fungi reduction of 5 Logs with DC-DP device, exceeding 1 Log more fungus reduction as compare with Authors' earlier report (Tasouji et al. 2018) can be claimed as another contribution of the present research, based on the single factor of toxin reduction rate only. It is to be noted that the tests were performed with different time, input power and pressure, using Argon gas, whilst preserving the nuts overall appearance, texture, and odor after the plasma irradiation. Argon gas was used because of its advantages such as low cost, less power consumption, and high sterilization efficacy. Pistachio is an expensive nut and most people prefer to eat it fresh and either unroasted. Pistachio brain contains high fat and a soft shell as compared with other nuts and hence it is the best host for *Aspergillus flavus* contaminant. Today, *Aflatoxins* (AFB1, AFB2, AFG1, and AFG2) as the secondary metabolite of fungus *Aspergillus* are the most prevailing *mycotoxins* that contaminate a wide range of foods and crops. AFB1 is the main and most common toxin threat to pistachio nuts (Mojtahedi et al. 1979; Sarrami et al. 2019). For instance, the concentration rate of AFB1 is 0.2894 ppm in a total amount of 0.4452 ppm comprising the concentration rates of all four aforementioned *Aflatoxins* (Sarrami et al. 2019). Moreover, pistachio as an export product, is a source of foreign income for the countries wherein cultivated. Furthermore, with respect to export/import of nuts, stringent regulatory limits are imposed for the level of contamination by these toxins. In view of the said facts, the authors are focusing on pistachios as their research specific sample nut, considering it as a relevant would be representative of all nuts as a research subjected.

Materials and methods

The specifications of materials, sample nuts and methods used in this research were almost similar to ones as reported for the earlier work (Tasouji et al. 2018). However, the performed microbial cultures are detailed, hereafter. The pure isolates of *Aspergillus flavus* (PTCC-5004), pistachio isolates pathogen with ability to produce AFB1 135 µg/kg (Mojtahedi et al. 1979) from Persian Type

Culture Collection (PTCC) gene bank, were provided to us via faculty of animal husbandry of Tehran University. The isolates were cultured according to the streak technique (Beuchat 1993; Grainger et al. 2001) on Yeast Extract Glucose Chloramphenicol (YEGC) Agar (Merck KGaA, Germany Code No. MC1160000500) plates at Pastor Microbiology Laboratory, Tehran. The Agar plates were kept in a CO₂ incubator at 37 °C (simulation of human body temp.) for the first 24 h and then at 25 °C for the next 48 h (Belli et al. 2004; Beuchat 1993; Dehghan et al. 2008; Lahouar et al. 2016; McClenny 2005; Samapundo et al. 2007; Suganthini et al. 2009). After 3-days incubation, the fungal culture was ready for samples contamination. A total of ten sterile pistachio nuts were placed on each 3-day-old plates and gently rolled around for few minutes on a rotor platform to distribute spores uniformly. In order to insure the fungal contamination, the sample nuts were incubated at 20 °C overnight (13 h). Before applying plasma, the fungus inoculated samples were kept under a laminar flow hood for about 3 h; to remove excess moisture from nuts shell (Tasouji et al. 2018).

After irradiation process, the fungal spore survivors on the inoculated nuts surface were evaluated with reference to the un-inoculated control samples. The fungi separation from nuts surface was done using 0.1% peptone (Merck KGaA, Germany) water in a beaker and vibrated well on a vibrator. A fixed amount of the obtained inoculum was then serially diluted in 0.1% peptone solution according to the serial dilution technique (Grainger et al. 2001). Different dilution ratios were used depending on the level of colony forming units per gram of sample. A 100 µl of each diluted sample was spread uniformly onto YEGC Agar medium and the plates were incubated at 25 °C for 72 h (Beuchat 1984, 1992; Tasouji et al. 2018). Once the colonies were counted, the results are expressed as Log Colony Forming Units per gram (Log CFU/g). Decimal reduction values are determined for each treatment conditions. All obtained control counts were within the CFU values of 6 Logs. To insure complete fungi separation, the nuts were kept at room temperature for 7 days but no further fungi growth was observed. The irradiation, separation, colony growth and counting processes were repeated for each test by every device. Finally, the obtained results are presented and discussed in “Results and discussion”.

In this research, in order to evaluate overall acceptance and appearance of the irradiated nuts, the statistical method of Analysis Of Variance (ANOVA) tests (Tasouji et al. 2018) with respect to the obtained treatment results by each of the three plasma devices were also performed, during three respective dates. The ANOVA results are accordingly discussed in “Results and discussion”.

Experimental cold plasma generating devices

The basic method of a simple and a cost effective cold plasma generation is ionization of a medium gas by the electric discharging between electrodes of such generating devices. Application of such cold plasma is actually the most feasible one in food industries as well as bio-medicals, considering its non-toxicity, easy approach as well as economic cost effectiveness. The referred cold plasma is generated either in an open air at an atmospheric pressure or in a vacuum with different ranges of pressures. The three relevant devices used in this research work represent both types of cold plasma generations; i.e. in an open air or in a vacuum. The devices and methods of experiments, undertaken, in this research are hereafter described accordingly.

AP-CCP generating device

The AP-CCP generating device consists of a radio frequency (RF) power supply machine, Argon gas cylinder, a glass made cylindrical movable container and the AP-CCP generating apparatus which works like a plasma torch (jet). The AP-CCP device consists of two concentric electrodes through which the medium gas flows; one is RF biased and the second one is grounded. In order to prevent arcing, a quartz tube is placed between the electrodes. By applying RF power to the inner electrode the discharged gas is ignited and the ionized gas flows through a nozzle with a flow rate of 50 SCCM (Tasouji et al. 2018). The sample nuts were easily irradiated by a plasma jet which was manually controlled to ensure irradiation all over the pistachios, placed on a plastic plate in atmospheric pressure. Frequency of RF was 13.65 MHz and at 50 Ω on standby mode.

ICP generating device

Argon gas enters the chamber from top end of this device and RF power supply is applied to the gas by using the coil wrapped around the chamber. The vacuum pump makes the suction from the bottom end of the chamber and the ionization process is finally done by producing a powerful inductive current using the coil. The produced plasma is concentrated by a central core placed on the device top. After putting the sample carrying container on the metal sheet within the coil, the required vacuum of 2 Torr was maintained and then Argon gas was entered into the chamber. Finally, plasma was irradiated on the samples at a power of 250 W for 20 min. These values of input power and irradiation time are the maximum permissive amount for this device since no results could be obtained for lower

Fig. 1 Inductive coupled plasma device

ranges of input power and lesser irradiation time. Figure 1 shows the experimental ICP device.

DC-DP generating device

In the DC-DP generating device, the cable of RF power supply and Argon gas enter the cylindrical metallic chamber from the top end of the device. The Pirani gauge set up at the right side and a vacuum pump provides vacuum from the bottom end of the chamber. Two metallic plates, in one of which the sample is kept, act like two charged capacitors, discharging Argon gas frequently to ionize the gas and to produce plasma. After putting the sample between the plates and setting up the Pirani gauge, the required vacuum and pressure are provided using a rotary pump. Then the gas is injected into the system and the required power is applied using RF power supply. The tests are performed by the DC-DP generating device within the pressure ranges of 0.5–2 Torr, 50–300 W power input and 5–20 min irradiation time. Figure 2 depicts DC-DP device.

Results and discussion

It is mentioning that this research report contains a number of tables of data which are omitted from this manuscript which is due to the journal's requirements for limited numbers of tables and figures.

The various tests were performed on the AP-CCP device at input powers of 50, 75, 100 and 150 W, each one at durations of 2, 6 and 10 min, respectively. After irradiation process with this device, counting the irradiated sample fungus and also the control fungi count, the obtained detailed results are as reported in Reference (Tasouji et al. 2018). As reported, the acceptable rate of fungi reduction

Fig. 2 Direct current diode plasma device

of 4 Logs was obtained at 100 W and irradiation time of 10 min. It is worth noting that, at 150 W and 10 min irradiation by the plasma torch, the fungus was totally reduced (6 Logs), but according to ANOVA test, some minor alteration were observed on pistachio shells. Moreover, the plasma jet device was slightly heated up during irradiation. It is expected at commercializing stage, where the devices are machine made and work more accurately, such type of shell damage would probably be prevented. The average of all three tests at three different times of 2, 6 and 10 min is depicted in Fig. 3 for both control sample (blue line) and irradiated sample (red line) (Tasouji et al. 2018).

The coefficient average of three repetitions is 2.2 CFU/g for 2 min, 7.5 CFU/g for 6 min, and 3.8 CFU/g for 10 min. The tests error rates on AP-CCP device are 5%, 32% and 34% for 2, 6 and 10 min, respectively. Please note that in accordance with the United States Food and Drug Administration (US FDA) standards, the standard error rate

Fig. 3 The rate of fungi reduction by AP-CCP device at 100 W

is acceptable up to 33.3% (Tasouji et al. 2018). The irradiated samples were examined by ANOVA test and no alterations in color, odor, texture, overall appearance were observed and the results acceptability was finalized.

In the case of ICP generating device, after irradiation of samples and counting the fungus amount on the irradiated samples and the control samples, the average fungi reduction rates by this device are indicated in Fig. 4. For more accuracy, the experiments were repeated three times.

As it is shown in Fig. 4, at input power of 250 W, time of irradiation 20 min, and pressure of 0.5 Torr, the remained fungi is 2.2×4 Logs CFU/g for the first test, 2.9×4 Logs CFU/g for the second test, and 2.4×4 Logs CFU/g for the third test. Subtracting the logarithmic data related to the irradiated fungus (red line) and the fungus control (blue line) leads to only 2 Logs fungus reduction and the error rate obtained by ICP device is 20%. Moreover, there are some limitations using the ICP device such as: applying the inductive force, creation of the uniform distribution of plasma, also having a strong chamber against the pressure.

The tests on the DC-DP plasma generating device were carried out in different pressures, input powers, and time durations. Averaging the data obtained at input power of 100 W and 1 Torr pressure for different time durations results in a fungi reduction of 2 Logs. The error rate in this experiment is 16% for 2 min, 14% for 10 min and 18% for 20 min which is an acceptable rate in accordance with the standard error rate (33.3%). Although the effects of time lapse on the fungi reduction is hardly observable, the results indicate that the fungus reduces a little more by increment of irradiation time. In order to indicate the effects of plasma pressure, the fungi reduction rates versus pressure with an input power of 300 W is shown in Fig. 5. This figure indicates that the fungi reduction rate increases with pressure. The reduction for 0.5 Torr is 4 Logs and for 1 and 2 Torr is 5 Logs which are completely in accordance with the most appropriate reduction rate certified by US

Fig. 4 The rate of fungi reduction by ICP device at 250 W

Fig. 5 The rate of fungi reduction by DC-DP device at 300 W, 20 min and different pressures

FDA. The reason for fungi reduction rate with pressure is the increment of plasma penetration depth, resulting in more fungi reduction. It should be noted that for preserving the nuts appearance the maximum pressure in this experiment was 2 Torr. Moreover, comparing the data obtained from input power of 300 W with those of 100 W shows that at higher input power the fungi reductions are higher than the lower input power. The error rates are 10%, 12% and 11% for 0.5, 1 and 2 Torr pressure, respectively. Here also, the irradiated samples were examined by ANOVA test and no alterations in color, odor, texture, overall appearance were observed and the treatment acceptability was approved. It is to be mentioned that, since at above 1 Torr pressure the fungi reduction rate remained almost the same (differing in 18 CFU/g. Only) but at higher pressures (two and above) the nuts appearance was inclined to be altered, hence the result obtained at 1 Torr pressure is preferred and is a safer one.

Finally, to introduce the optimum device for inactivating the *Aspergillus flavus* from the nuts surface, based on the two proposed objectives of toxin inactivation amount and economic feasibility for large scale industrial applications, the results obtained on the three plasma generating devices

discussed above are compared. More detailed information including the optimum condition for each device is indicated in Table 1. As can be seen, the best results for each device are as follows: the fungi reduction for the ICP generating device is 2 Logs, for the AP-CCP generating device is 4 Logs and for the DC-DP generating device is 5 Logs which is the highest rate of reduction, and if one is aiming at toxin treatment only, then the DC-DP device would be the best option for inactivation of *Aspergillus flavus* from nuts shell. However, since for DC-DP generating device the irradiation is carried out in a vacuum chamber as against of AP-CCP device, where the irradiation is performed at atmospheric pressure, hence it is obvious that the fungi treatment by DC-DP in large scale will be too expensive. Therefore, in view of the commercial importance of cost effectiveness and considering the acceptability of 4 Logs fungi reduction in accordance with US FDA as well as the acceptability of results by ANOVA tests, the AP-CCP device is ultimately considered to be the optimum device, fulfilling both objectives of this research.

Conclusion

In this research report, three plasma generating devices namely AP-CCP, ICP, and DC-DP as applicable cold plasma candidate devices for inactivating *Aspergillus flavus* from nuts surface are briefly introduced. Pistachio has been chosen as the sample nut and experiments on all devices were carried out using Argon as the main gas.

The experimental results reveal that for the ICP generating device at 250 W input power, 0.5 Torr pressure, and 20 min irradiation, the maximum obtained fungi reductions was 2 Logs (33%) only. For AP-CCP generating device, various tests were carried out in atmospheric pressure at different input power and irradiation times. Ultimately, the fungus reduction of 4 Logs (about 67%) was obtained at 100 W input power for irradiation time of 10 min, observing no alteration in color, odor, texture, overall

Table 1 The best results of ICP, AP-CCP and DC-DP devices, indicating optimum device

Device	Time (Min.)	Power (W)	Pressure (Torr)	Temp. (°C)	Reduced fungi (Log (CFU/g))	Total fungi (Log (CFU/g))	US–FDA and ANOVA	Economic feasibility
ICP	20	250	0.5	Amb.	2	6	NA	NA
AP-CCP	10	100	Atm.	Amb.	4	6	Passed	Positive
DC-DP	20	300	1	Amb.	5	6	Passed	Negative

Temp. temperature, Amb. ambient, Atm. atmosphere, NA not applicable

appearance of the nuts. Finally, for DC-DP device at 300 W input power, 2 Torr pressure, and 20 min irradiation, the highest fungi reduction of 5 Logs (83%) was achieved. Therefore, on the basis of the amount of fungus reduction rates solely, such percentage of inactivation of nuts contaminant *Aspergillus flavus* can be claimed as an innovative achievement.

According to the US FDA standards as well as ANOVA tests, results obtained by the two AP-CCP and DC-DP devices are acceptable. However, considering the practical objectives for treatment of the nuts in large scale, AP-CCP being operative at atmospheric pressure is qualified as the optimum plasma generating device for the proposed fungi treatment. Whilst, the inoculation by the DC-DP device which requires extra expenses, due to a vacuum chamber, would not be economically feasible.

Finally, it is suggested that the AP-CCP device would be commercially designed such that it could irradiate the nuts more accurately to prevent the nuts shell alteration, as compared to the prototype handmade device used in this research work. It is hoped that the industrial relevance of recent research work would encourage the related food industries to utilize such results in order to ensure nuts health and safety.

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