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Decontamination of *Aspergillus parasiticus* in rice by dielectric barrier discharge cold plasma: Variable effects and mechanism of degradation

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Abstract: Rice is prone to be contaminated with spoilage or toxigenic fungi during harvest, storage and processing, with *Aspergillus* species being the most frequent. It is crucial to develop effective sterilisation technologies for mycotoxin prevention and food safety. In this study, sterilised rice infected by *Aspergillus parasiticus* strain was treated by dielectric barrier discharge (DBD) cold plasma. Various parameters, including moisture content, oxygen content, treatment time and voltage were tested. Furthermore, sterilisation mechanism of *Aspergillus parasiticus* by cold plasma was also explored. Results indicated that decontamination effect could be significantly affected by moisture content, oxygen concentration, voltage and treatment time. A 99.89% degradation rate against *Aspergillus parasiticus* was achieved at 90 kV after 5 min. Cold plasma could reduce the initial concentration of 6.05 to 2.28 CFU·mL^{-1} within 240 s, and to thoroughly decontamination within 360 s. In addition, cold plasma treatment destroyed the integrity of *Aspergillus parasiticus* cell membrane, resulting in a reduction in mycelium biomass and dry weight, as well as a significant decrease in intracellular $Ca^{2+}Mg^{2+}$-adenosine triphosphatase (ATPase) activity. These findings demonstrate the potential of cold plasma technology for environmentally friendly sterilisation of hazardous fungi in grain system.

Keywords: sterilisation; fungus; grain; food safety

Rice, as the staple food in Asia, plays a crucial role in meeting human energy needs and nutritional intake (Park et al. 2012). In recent years, major rice-producing countries such as Thailand (Amnuaylojaroen et al. 2021), Vietnam, Indonesia, China, India, and Myanmar are experiencing a decline in production due to population growth and land resource occupation (Wu et al. 2021). Additionally, rice is prone to various fungi infections during growth, harvesting, transportation, and storage, with *Aspergillus* species being the most frequent and serious. *Aspergillus* prevalence in rice not only cause a high economic loss due to inferior or unacceptable quality, but also result in mycotoxins contamination, which can pose serious threats to human and animal health (Schuhmacher-Wolz et al. 2010; Liu et al. 2024). Therefore, it is necessary

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to develop rapid and convenient methods to control the invasion of hazardous *Aspergillus parasiticus* before mycotoxins production at an early stage.

Existing sterilisation technologies include high-temperature sterilisation, microwave treatment, ozone fumigation as well as radiation sterilisation (Misnal et al. 2021). However, most of these above methods may result to an uneven distribution inside rice grains, and the uneven heating at high temperatures can alter their physicochemical properties and morphological structure, especially affecting moisture content and grain uniformity (Manickavasagan et al. 2006; Chen et al. 2017; Wang et al. 2019). In addition, these methods can also alter the food matrix and have negative effects on the colour, texture, flavour, and nutritional components of the food (Misra et al. 2015). Thus, cost effective methods are still highly desirable. Cold plasma technology, as a novel non-thermal sterilisation method, has shown significant advantages over traditional methods due to its high efficiency, residue-free process, and minimal impact on the nutritional quality of food (Chiozzi et al. 2022). Past studies have indicated that cold plasma can not only effectively reduce the number of microorganisms in food but also significantly improve the cooking characteristics, hardness, stickiness, as well as nutritional and enzymatic properties of rice (Anuntagool et al. 2023). These findings reveal the substantial potential of cold plasma technology in enhancing the quality of cereal products, especially in terms of transitioning from laboratory research to industrial production applications.

Common cold plasma generation methods include dielectric barrier discharge (DBD), atmospheric pressure plasma jet (APPJ), corona discharge plasma (CDP), and glide arc discharge (GAD) (Ekezie et al. 2017). Among these, DBD cold plasma has been widely applied in food research due to its simple equipment structure and high discharge space uniformity. A key feature of DBD cold plasma is its ability to more readily establish non-thermal equilibrium plasma conditions, facilitating the scale-up from small laboratory reactors to large manufacturing units (Kogelschatz 2003). The sterilisation mechanism of DBD cold plasma involves the generation of highly reactive oxygen and nitrogen species, UV radiation and electric fields (Ouf et al. 2015; Dasan et al. 2016). The production of reactive species in cold plasma can be influenced by various parameters such as voltage, type of working gas, distance between electrodes, and humidity (Shi et al. 2017). Ott et al. (2021) demonstrated that cold plasma technology can utilise air to generate reactive oxygen species (ROS) and reactive nitrogen species (RNS) to degrade *Aspergillus flavus* cultures and deoxynivalenol (DON) mycotoxins produced by *Fusarium graminearum* (Ott et al. 2021). Guo et al. (2023) also discovered that cold plasma treatment of artificially mould-contaminated rice grains significantly inhibited the microbial activity of *Aspergillus niger*, *Rhizopus oryzae*, *Penicillium verrucosum*, and *Fusarium graminearum*. However, there is still a lack of thorough research on the specific sterilisation efficacy of DBD under different conditions, as well as the specific impact of DBD treatment on the internal sterilisation mechanisms of various fungal species.

Therefore, the objective of this study is to explore the effects of DBD cold plasma on the degradation of *Aspergillus parasiticus* in rice under different parameter settings, in order to optimise the parameters. Furthermore, a series of tests were conducted on the *Aspergillus parasiticus* to reveal the inherent mechanism of cold plasma sterilisation, aiming to assess the feasibility of cold plasma in controlling mould in grain.

MATERIAL AND METHODS

Experimental materials. A batch of fresh rice sample (japonica, polished) was obtained from Yangzhou, China. The sample was exposed to 15 kGy of Co-60 gamma radiation for thoroughly sterilisation. The isolate of *Aspergillus parasiticus* strain 144221 was purchased from Beina Chuanglian biotechnology research institute in Beijing, China.

Instrumentation and equipment. Figure 1 is a schematic diagram of the CPS-I type high-voltage electric field cold plasma system used in this study, co-developed by Suzhou 'YiRun' Food Technology Co., Ltd., China. This system is mainly composed of a dielectric barrier, high-voltage electrodes, a high-voltage power supply, and a controller, with the electrodes covered by dielectric material. The dielectric barrier layer, made of polypropylene, acts as the medium for uniform discharge between the two electrodes. During the treatment process, the distance between these two electrodes was maintained at 55 mm. The input power is set to 220 V and 50 Hz.

Cultivation of *Aspergillus parasiticus* strain. In order to propagate the mycotoxin-producing *Aspergillus parasiticus* strain 144221, it was placed in a BSC-250 waterproof constant temperature incubator (Shanghai Jinghong Company, China) with the temperature maintained at 28 °C and the relative humidity at 85% and cultured on potato dextrose agar for 5 days. After the cultivation was completed, the culture was rinsed

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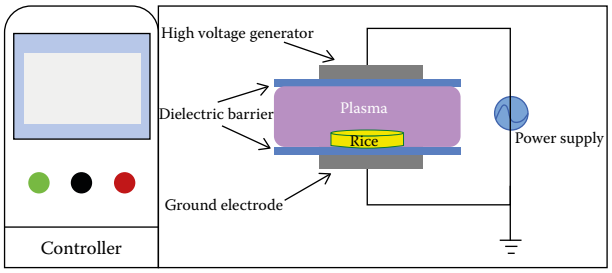

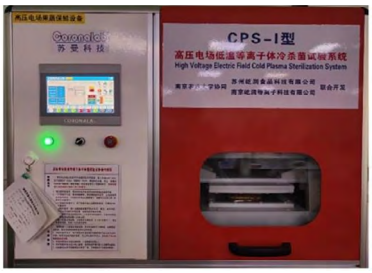

Figure 1. Schematic diagram of cold plasma system

with sterile water to prepare a spore suspension. To ensure the spore concentration is appropriate, the spore density is measured using the plate count method and adjusted to between 10^5 to 10^6 colony forming units (CFU)·mL^{-1}. After adjusting the moisture content of the rice to 20%, 2 mL of the spore suspension was added to each 100 g of the rice sample, and the culture was continued for 12 h under the same environmental conditions. It was detected that the microbial content in the rice at this time was 6.68 log CFU·g^{-1}.

Sterilisation effects of DBD treatment on mouldy rice. For each portion, a 20 g rice sample was weighed and placed in a DBD cold plasma system for treatment to degrade *Aspergillus parasiticus* in rice under different conditions of moisture content (12, 14, 16 and 20%), treatment time (5, 10, 20, 30 and 60 min), voltage (70, 80, 90 and 100 kV) and gas ratios (0% O_2 + 70% N_2 + 30% CO_2, 25% O_2 + 45% N_2 + 30% CO_2, 45% O_2 + 25% N_2 + 30% CO_2 and 65% O_2 + 5% N_2 + 30% CO_2), and to examine the bactericidal effect on it. The moisture content was calculated by measuring the mass difference before and after drying the rice in an oven at 105 °C until it reached a constant weight. The gas ratios were regulated with the help of a MAP-ID 400 modified atmosphere packaging machine (Gangqing Machinery Manufacturing Co. Ltd., China). The selected conditions were determined based on previous studies and preliminary experiments, and each condition was treated in triplicate.

Determination of the sterilisation rate of *Aspergillus parasiticus*. Using the spore suspension obtained from the previous experimental cultivation, the concentration of the spore solution was determined to be 6.05 log CFU·mL^{-1} by the plate count method. Keeping other treatment conditions consistent, the treatment was carried out under a low-temperature plasma at 90 kV for 60, 120, 240, and 360 s respectively. Subsequently, the number of *Aspergillus parasiticus* after treatment was determined, and the sterilisation rate was calculated according to the following formula.

$$\text{Sterilisation rate} = \frac{N_0 - N_t}{N_0} \times 100\%$$

where: N_0 – number of microorganisms before treatment (CFU·mL^{-1}); N_t – represents the number of microorganisms after treatment [after being treated for a certain period of time under specific conditions (CFU·mL^{-1})].

Scanning electron microscopy observation. To further observe and compare the effects of cold plasma treatment on *Aspergillus parasiticus*, untreated and plasma-treated (for 240 s) samples were selected. Initially, both sets of samples underwent CO_2 critical point drying to preserve integrity and stability. Subsequently, Quanta-200 Scanning Electron Microscope (SEM) (FEI Company, USA) was employed to examine the samples at magnifications of 500×, 1 000×, 2 000×, and 10 000×, facilitating the observation of microstructural and morphological changes in both untreated and treated samples.

Determination of cell membrane permeability. In order to evaluate the effect of DBD treatment on the permeability of the cell membrane of *Aspergillus parasiticus* spore suspension, the changes in electrical conductivity were measured using an FE38 conductivity meter (Mettler-Toledo Group, Switzerland) after the treatment. The spore suspension was treated at 90 kV for 60, 120, 240, and 360 s respectively. The electrical conductivity of the suspension was measured after each treatment duration. The changes in the permeability of the cell membrane of the spore suspension were observed by comparing the electrical conductivity data before and after the treatment under different treatment times.

Measurement of intracellular components (nucleic acids, proteins, ATPase). In biochemical and molecular biology experiments, degree of light absorption (OD_{280nm}) is primarily used to determine protein concentration, as proteins absorb ultraviolet (UV) light at 280 nm wavelength and there is a direct proportionality between absorbance and protein content. OD_{260nm} is used to measure nucleic acid (DNA and RNA) con-

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centration, where nucleic acids absorb UV ATPlight at 260 nm wavelength, and the absorbance is directly proportional to nucleic acid content. In this experiment, the spore suspension of *Aspergillus parasiticus* was treated with DBD at 90 kV for durations of 60, 120, 240 and 360 s, and the changes in OD_{260nm} and OD_{280nm} were measured to evaluate the impact of DBD treatment on the permeability of the fungal cell membrane (Hammer et al. 2004).

Intracellular adenosine triphosphatase (ATPase) measurement involves initially taking both untreated and cold plasma-treated *Aspergillus parasiticus* suspensions, centrifuging, washing with PBS, and resuspending. Subsequently, cells are lysed using an ultrasonic cell disruptor (300 W, 10 min, 1.1 s intervals) under ice bath conditions to allow intracellular enzymes to leach out. Subsequently, intracellular ATPase post-treatment levels were measured using the Ultra Trace ATP ($Ca^{2+}Mg^{2+}$) Assay Kit (A070-3-1) produced by Nanjing Jiancheng Bioengineering Institute (China). This method provides data on the changes in the content of intracellular ATPase in *Aspergillus parasiticus* before and after treatment, thereby understanding the effect of cold plasma treatment on the intracellular enzyme activity of the fungus.

Data evaluation and statistics. Upon completion of the experiment, data analysis and graphing were conducted using Origin Pro 2024 and SPSS (version 26.0), with each data point representing the average of three replicates.

RESULTS AND DISCUSSION

Analysis of sterilisation efficacy. Moisture content has been identified as a significant factor affecting plasma degradation. As shown in Figure 2A, for rice samples with 12% moisture content treated by DBD at 90 kV for 30 min, the initial microbial count of 6.68 log CFU·g^{-1} was reduced to 3.00 log CFU·g^{-1}, achieving a sterilisation rate of 99.98% and demonstrating a clear antimicrobial effect. Increasing moisture content initially led to a decrease in antimicrobial effects, which then improved again. The initial reduction in efficiency may be due to the dilution of reactive substances, while further increases in moisture content enhance the generation of ROS

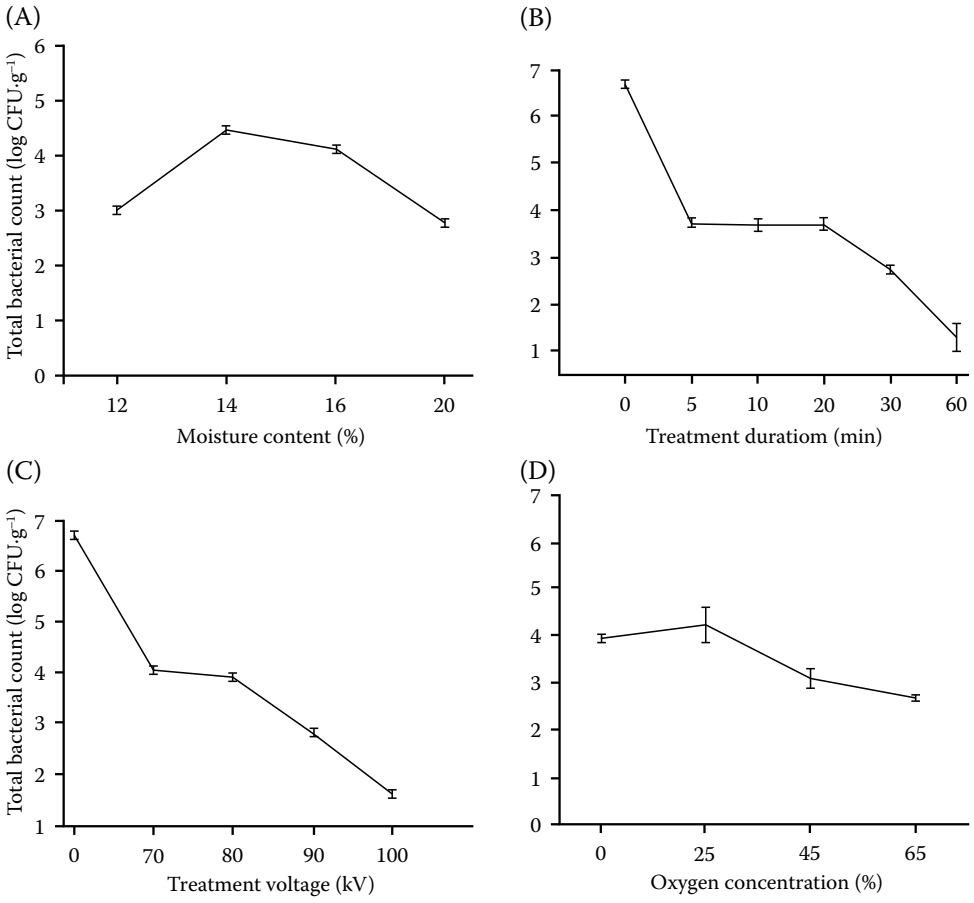

Figure 2. Dielectric barrier discharge efficacy on surface *Aspergillus parasiticus* of rice under varying conditions: (A) different moisture content; (B) different treatment time; (C) different treatment voltage; (D) different oxygen concentration

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in the plasma system, resulting in higher degradation rates (Feizollahi et al. 2020; Qian et al. 2020).

Voltage, time, and oxygen concentration have a significant impact on the sterilisation effect of plasma. Figure 2B illustrates that after treating 20% moisture rice samples with cold plasma at 90 kV for 5 min, the microbial load decreased from 6.68 to 3.73 log CFU·g^{-1}, achieving a 99.89% sterilisation rate. With longer treatment times, the sterilisation rate increased, nearing 100% at 60 min due to the accumulation of more active species (Liu et al. 2024). As shown in Figure 2C, treating rice at 70 kV resulted in a reduction of *Aspergillus parasiticus* to 4.04 log CFU·g^{-1}, and an increase to 100 kV further reduced it to 1.60 log CFU·g^{-1}. Higher voltages enhanced the ionisation and inelastic collisions of gas molecules in the DBD plasma system, leading to higher concentrations of active species corresponding to increased degradation efficacy (Wang et al. 2020). According to Figure 2D, treating rice with cold plasma with an oxygen concentration of 0% reduced *Aspergillus parasiticus* to 3.97 log CFU·g^{-1}, and an increase to 65% oxygen reduced it to 2.69 log CFU·g^{-1}. The increased oxygen concentration improved the sterilisation effect due to the generation and ionisation of ROS such as O radicals, O, and O_3 (Yang et al. 2018).

Sterilisation rate of *Aspergillus parasiticus.* The sterilisation rate is an indicator that measures the effectiveness of sterilisation or disinfection, typically expressed as a percentage, and it describes the reduction in the number of viable microorganisms before and after treatment. As shown in Figure 3, *Aspergillus parasiticus* with an initial concentration of 6.05 log CFU·mL^{-1} was reduced to 5.19 log CFU·mL^{-1} after 60 s of DBD treatment at 90 kV, with a sterilisation rate of 86.28%. Extending the treatment time to 120 s reduced the concentration to 4.08 log CFU·mL^{-1}, achieving a sterilisation rate of 98.94%. Further increasing the treatment duration to 240 s decreased the colony count to 2.28 log CFU·mL^{-1}, with a sterilisation rate of 99.98%. After 360 s of treatment, the *Aspergillus parasiticus* was completely eradicated. It is evident that under the same conditions, the sterilisation effect on the spore suspension is higher than that on the rice samples inoculated with *Aspergillus parasiticus*.

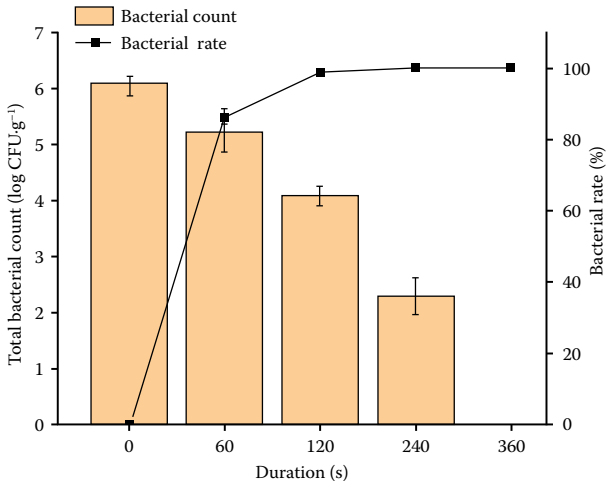

Figure 3. Bactericidal rate of *Aspergillus parasiticus* after dielectric barrier discharge treatment

SEM analysis results. Devi et al. (2017) found through scanning electron microscopy studies that cold plasma treatment can create larger pores on the surface of *Aspergillus flavus* spores, leading to complete spore detachment. Bermúdez-Aguirre's et al. (2013) research indicated that cold plasma could interact with spore surfaces, causing spore death through electroporation, and forming small pores on the surface. After treating *Aspergillus flavus* with a dielectric barrier discharge plasma by Šimončicová et al. (2018), found that the biomass of *Aspergillus flavus* decreased by approximately 55% in just 5 s. After 15 s of plasma treatment, the dry weight of the mycelium dropped to 10%. In the samples treated with plasma for 60 s, obvious differences were detected for the first time, and at this moment, the cell viability of *Aspergillus flavus* was completely or almost completely lost. They observed that compared with the untreated mycelium, the treated mycelium had different depressions on its surface, showing a contracted state with sharp edges (Šimončicová et al. 2018). The DBD cold plasma device used in this paper is different from the research equipment they used, but similar results have also been obtained. As shown in Figure 4, the untreated mycelium of *Aspergillus parasiticus* is clearly distinguishable. The mycelium is plump and tightly intertwined with each other, and there are numerous spores densely arranged. After being treated with cold plasma, the mycelium becomes shrivelled, with reduced volume and sparse distribution, and the number of spores also decreases significantly. Comparing the scanning electron microscope images of *Aspergillus parasiticus* treated with cold plasma and the untreated ones is of great significance. This clearly indicates that the cold plasma treatment can reduce the biomass of *Aspergillus parasiticus*, decrease the dry weight of the mycelium, and simultaneously reduce the activity of the spores.

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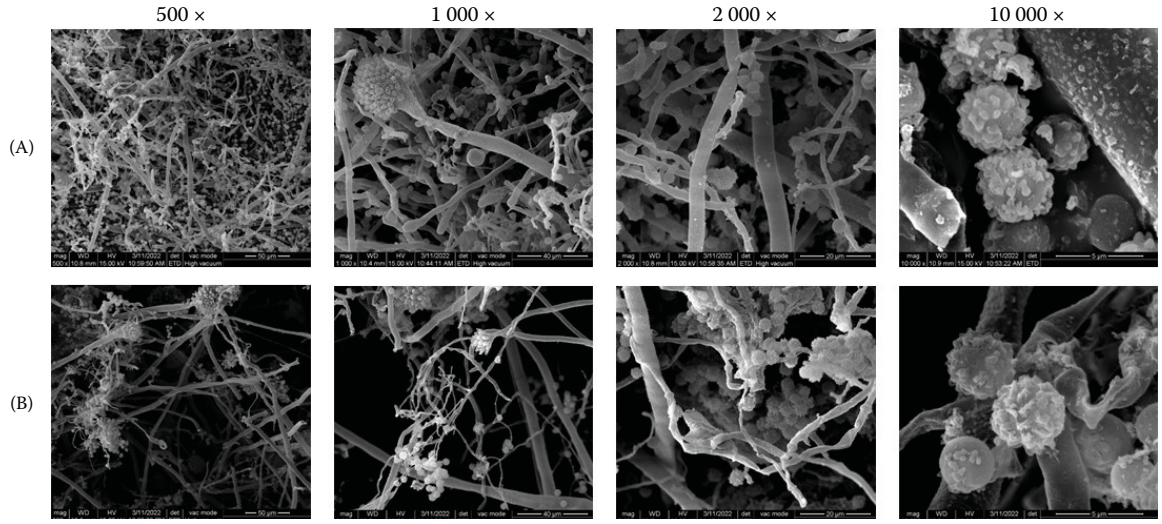

Figure 4. Scanning electron microscopy observation of morphological changes in *Aspergillus parasiticus* strains after dielectric barrier discharge (DBD) treatmen: (A) untreated; (B) after DBD treatment

Analysis of cell membrane permeability assay results. The survival of fungal cells relies significantly on the integrity of their cell membranes, which protect them against harsh external environments. Compromised membrane integrity can lead to leakage of intracellular substances such as ions, proteins, and nucleic acids, disrupting normal metabolic and physiological functions. Consequently, the integrity of fungal cell membranes has emerged as a critical research topic. This study explored the effects of DBD treatment on the disruption of *Aspergillus parasiticus* cell membranes by measuring the conductivity of the extracellular fluid. Results showed that untreated *Aspergillus parasiticus* spore suspension had a conductivity of 19.97 μS·cm^{-1}, which increased to 141.13 μS·cm^{-1} after 60 s of DBD treatment, and reached 536.57 μS·cm^{-1} after 360 s, at which point the *Aspergillus parasiticus* was completely eradicated (Figure 5). Generally, an increase in cell membrane permeability facilitates the leakage of electrolytes and intracellular ions, thereby increasing the conductivity of the spore suspension (Zhang et al. 2016). The results indicated that as DBD treatment time increased, the concentration of microbial colonies decreased, and conductivity gradually increased, confirming that DBD treatment compromised the integrity of the *Aspergillus parasiticus* cell membrane.

The damage to the cell membrane might be attributed to the etching action of various radicals in the plasma or the accumulation of charged particles on the membrane surface (López et al. 2019; Misra et al. 2019). Etching is considered a key deactivation mechanism (Schlüter et al. 2013), promoting the diffusion of secondary reactive species such as NO, OH, H_2O_2, and O_3 within the cell, and causing chemical bond disruption and morphological changes (Bourke et al. 2017). Additionally, electrostatic disruption is a potential damaging mechanism; when the membrane surface's electrostatic force exceeds the membrane's tensile strength, membrane structure damage may occur (Her-

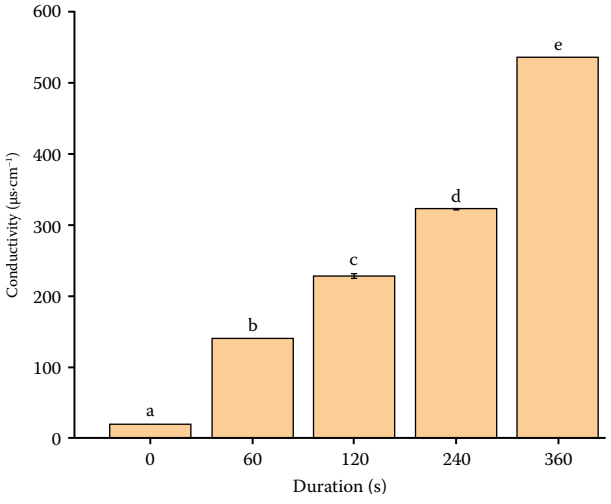

Figure 5. Electrical conductivity values of *Aspergillus parasiticus* spore suspensions treated with dielectric barrier discharge for varying durations

a, b, c, d, and e – significant differences at the significance level of $P < 0.05$

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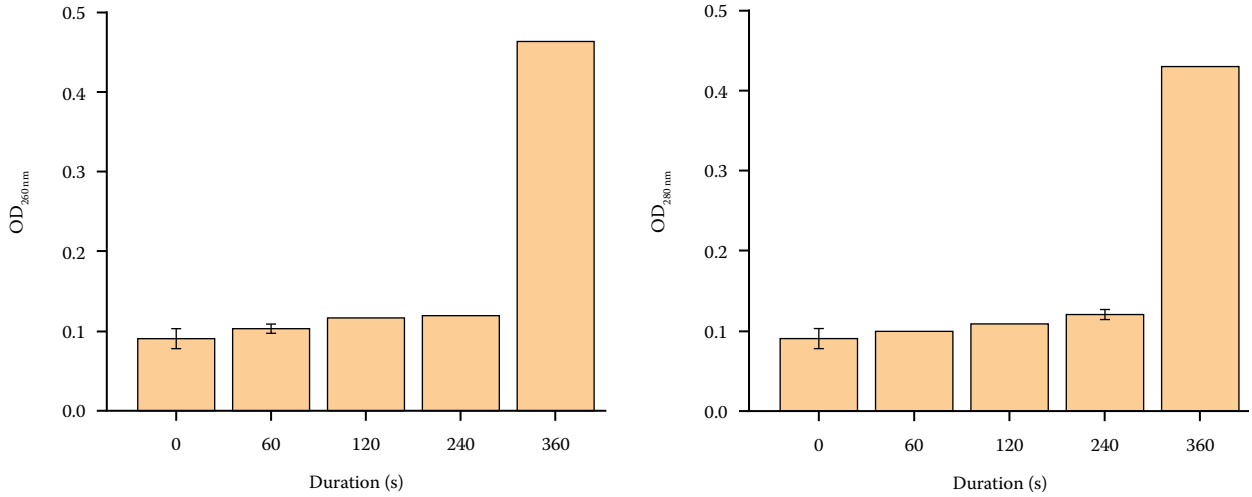

Figure 6. Effects of dielectric barrier discharge treatment on the absorbance values at degree of light absortion (OD_{280nm}) and OD_{260nm} for *Aspergillus parasiticus*

twig et al. 2018). Research by Dasan et al. (2016) has demonstrated that atmospheric pressure fluidised bed plasma has a lethal effect on the membrane integrity of *Aspergillus flavus* and *Aspergillus parasiticus* spores (Dasan et al. 2016). Therefore, the results of this study further confirm that DBD plasma treatment also has a lethal effect on the integrity of *Aspergillus parasiticus* spore membranes, highlighting the significant impact of DBD treatment on spore membrane integrity.

Analysis of determination results of intracellular substances. Nucleic acids, essential structural macromolecules that carry unique genetic information, are located within the cytoplasm (Kohanski et al. 2010). As illustrated in Figure 6, with increasing treatment time, there was a continuous rise in the absorbance values at OD_{260nm} and OD_{280nm}. The initial absorbance values of *Aspergillus parasiticus* spore suspension were 0.09 at both OD_{260nm} and OD_{280nm}, which after 360 s of cold plasma treatment, increased to a maximum of 0.46 and 0.43, respectively. The increase in OD_{260nm} and OD_{280nm} suggests outflow of macromolecules from within the *Aspergillus parasiticus*, impacting cell growth metabolism. Although nucleic acids and proteins are the main components responsible for the absorption at these wavelengths, it should be noted that other factors may also contribute to the observed changes in absorbance. Cell components were continuously released through the leakage of nucleic acids and proteins, resulting in irreversible cellular damage.

Analysis of intracellular adenosine triphosphatase measurement results. ATPase is a globular protein that catalyses physiological metabolism in cells and sustains vital activities in living organisms. Its primary function is to hydrolyse the phosphate ester bond in ATP molecules, breaking them down into adenosine diphosphate (ADP) and inorganic phosphate (Pi). This reaction releases energy for cellular processes. ATPase plays a critical role in various biological processes, such as maintaining the intracellular ATP/ADP ratio, facilitating cellular response to external stimuli, and regulating cell proliferation and apoptosis. ATPase activity and function are regulated by various factors, including enzyme structure, intracellular environment, and metabolic status. As shown in Figure 7, the initial $Ca^{2+}Mg^{2+}$-ATPase enzyme activity of 9.36 U·mg^{-1} pro-

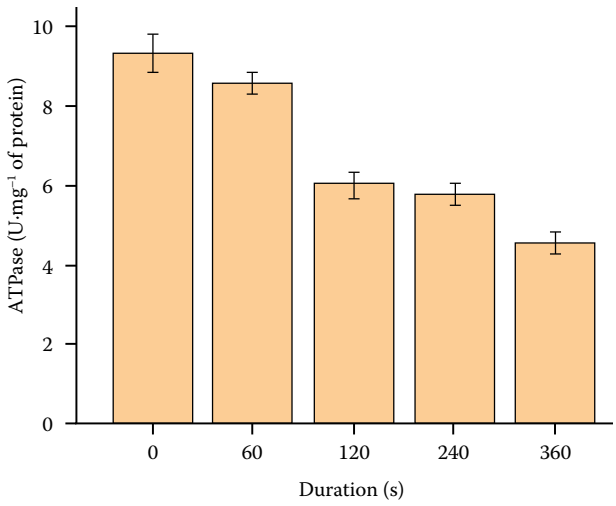

Figure 7. Changes in adenosine triphosphatase (ATPase) activity of *Aspergillus parasiticus* after dielectric barrier discharge treatment

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tein decreased to 8.59 $U \cdot mg^{-1}$ protein after 60 s of cold plasma treatment. With an extended treatment duration of 360 s, the $Ca^{2+}Mg^{2+}$-ATPase enzyme activity further declined to 4.54 $U \cdot mg^{-1}$ protein, a reduction of 51.50%. The results indicate that DBD treatment can denature *Aspergillus parasiticus* proteins, leading to reduced ATPase activity.

CONCLUSION

This study demonstrates the significant efficacy of DBD cold plasma technology in inhibiting the growth of *Aspergillus parasiticus* in mouldy rice. The results indicate that factors such as moisture content, treatment duration, treatment voltage, and gas composition significantly affect the sterilisation effect of DBD cold plasma. DBD treatment notably reduced the number of *Aspergillus parasiticus* colonies and achieved a high sterilisation rate. Additionally, morphological changes in the *Aspergillus parasiticus*, such as shrivelled hyphae and reduced spore count, suggested that DBD impacted not only the survival rate but also inhibited biomass growth. Furthermore, DBD treatment increased cell membrane permeability and induced leakage of critical intracellular substances, including nucleic acids, proteins, and ATPase, suggesting that DBD might exert its sterilising effect by damaging cell membrane integrity and causing leakage of cellular contents. While DBD shows promising application prospects in controlling *Aspergillus parasiticus*, further research is needed to understand its sterilisation mechanisms and potential for practical production. This study not only offers a new perspective on using cold plasma technology to enhance food safety and quality but also lays a solid foundation for the application of related technologies in agriculture and the food industry.

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