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Plasma-based Stimulation of Biotechnological Processes in *Ganoderma lucidum* Mycelia as Example for a Eukaryotic Organism

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ABSTRACT: In biotechnological processes, high-yielding production strains are the condition for economic production of biomass and desired metabolites. Plasma with its components like UV radiation and highly reactive radicals could possibly generate and improve new production strains. Indeed, plasma was able to generate high-yielding bacterial strains. A candidate for eukaryotic cells for improving metabolite production is the medicinal mushroom *Ganoderma lucidum* used for prevention and treatment of many different diseases. Mycelia and fruit bodies contain more than 150 structurally different triterpens. The aim of our investigation was to check whether plasma influences growth and productivity of the fungus. Mycelial cultures were exposed to plasma for 2–20 min by using three different plasma sources: the plasma jet kINPen 09 and two dielectric barrier discharge plasma sources (surface-DBD, volume-DBD). Yield of biomass and extracts were determined. HPLC fingerprints of organic extracts were recorded. Content of triterpens, ganoderic acid A, ergosterol, and β-1-3 D-glucan was determined. Volume-DBD increased extract yield and β-1-3 D-glucan content, while whole triterpenoid content was increased by the plasma jet. Both dielectric barrier discharge plasma sources led to elevated ergosterol contents. The effects seemed to be related to energy input by the plasma sources. In conclusion, plasma could be principally useful for optimization of biotechnological processes not for only prokaryotic but also eukaryotic cells.

KEY WORDS: nonthermal physical plasma; volume dielectric barrier discharge plasma; surface dielectric barrier discharge plasma; plasma jet; medicinal mushroom; high-yielding production strains; ergosterol

I. INTRODUCTION

High-yielding production strains are important for economic production of biomass and desired metabolites. Normally they are generated by a combination of mutagenesis and selection using genetic engineering, x-ray, gamma-ray, UV ray, or radical forming chemicals. The plasma glow contains different types of radiation, as, e.g., UV radiation and highly reactive species which could possibly work synergistically in generation and improvement of production strains. There are few investigations demonstrating the efficiency of atmo-

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spheric-pressure plasma for breeding high-yielding bacterial and cyanobacterial strains.[1–5] Different principles for the generation of plasma were used as, e.g., dielectric barrier discharges (DBD) or glow discharges. All plasma sources worked at atmospheric pressure and room temperature. Ambient air, argon, or helium were used as process gas. Thereby, mutant strains were obtained from *Bacillus subtilis*,[2] *Klebsiella pneumoniae*,[1] *Streptomyces avermitilis*,[3] and *Spirulina platensis*.[5] While in the case of *Bacillus subtilis* plasma glow discharge sources with helium as operating gas have been used to generate the mutant strains, a DBD plasma source with ambient air caused the mutation of *Klebsiella pneumoniae*. Until now such approaches have been limited to prokaryotic cells. Interesting eukaryotic candidates for improving metabolite production are medicinal mushrooms. They are used in traditional Asian medicine for many centuries and meanwhile their importance is also strongly increasing in the Western hemisphere.[6,7] One potent candidate is the basidiomycete *Ganoderma lucidum* (Japanese "Reishi," Chinese "LingZhi"), belonging to the Ganodermataceae family.[8] *G. lucidum* is used in the prevention and treatment of asthma, bronchitis, tumors, and liver, kidney, or heart diseases.[9] The mushroom is characterized by a broad variety of bioactive compounds. Mycelia and fruit bodies contain more than 150 structurally different triterpens, e.g., ganoderic acids, and immunomodulating polysaccharides.[10] These bioactive compounds have, among others, antibacterial, antiviral, antifungal, antioxidant, antitumor, immunomodulating, hypocholesterolemic, histamine release-inhibiting, apoptosis-inducing, and cell growth-inhibiting properties.[9,11–18] It has been shown that biomass and compound productivity of *G. lucidum* can be enhanced by optimizing culture conditions including application of several inducers of triterpenoid biosynthesis,[19–28] by inducing fungal apoptosis through treatment of the cultures with acetyl salicylic acid (aspirin)[29] or by genetic manipulation.[30] A high concentration of hydrogen peroxide (H_2O_2) or the pro-oxidant 1-chloro-2,4-dinitrobenzene (CDNB) is also able to induce the synthesis of individual ganoderic acids. Besides an influence on MAPK (mitogen-activated protein kinase), signaling in fungal cells could be shown. While high ROS concentrations reduce fungal biomass, low concentrations have no effect on production of biomass and ganoderic acids.[31]

Our study aimed at investigating the influence of nonthermal plasma on growth and productivity of *G. lucidum* as an example for eukaryotic cells. For that, mycelial cultures of the mushroom treated with three different plasma sources were examined for production of ergosterol as pro-vitamin D2, total triterpenoids, ganoderic acid A as one individual triterpenoid, total phenolics, and β-1-3 D-glucan.

II. MATERIAL AND METHODS

A. Materials

1. Biological Material

Fresh fruiting bodies of *Ganoderma lucidum* were collected in 1996 near Greifswald, northeastern Germany, and identified by Prof. Hanns Kreisel, Institute for Microbiology,

University of Greifswald. A voucher specimen is deposited at the Department of Pharmaceutical Biology, University Greifswald (no. 104). Small mushroom pieces were removed from the inside of a fresh and cleaned fruiting body under sterile conditions and put on Hagem agar[32] in 60-mm plastic petri dishes. Incubation at room temperature for 3–4 weeks allowed the outgrowth of mycelium. The cultures were controlled microscopically for identity and purity and stored at 5°C. For further assays, mycelium from petri dishes was harvested, pooled, homogenized, and distributed on fresh Hagem agar in petri dishes.

2. Culture Media

Hagem agar consists of ammonium succinate 0.5 g (own production of ammonia 25%, Carl Roth, Karlsruhe, Germany, and succinic acid, Carl Roth), KH_2PO_4 0.5 g (Carl Roth), $MgSO_4 \times 7H_2O$ 0.5 g (Carl Roth), $FeCl_3$ 1% 0.5 mL (Carl Roth), glucose 5.0 g (Carl Roth), malt extract R2 (Carl Roth), agar-agar 15.0 g (Carl Roth), Aqua dest. to 1000 mL. Hagem medium consists of the same components except agar-agar. After pH adjustment to 5.4, both agar and medium were autoclaved at 121°C for 25 min.

B. Mycelial Culture

For S-DBD treatment, from petri dishes (TPP, Trasadingen, Switzerland) with Hagem agar of a thickness of 5 mm, a circular hole (diameter 1 cm) was punched out in the middle and replaced by a stamped-out piece of precultured mycelium (on Hagem agar) of the same diameter. Two days later, inoculation samples were subjected to plasma treatment (Table 1) and then cultivated for 14 days at 21°C. Samples of the grown mycelium were cut into small pieces and then transferred into a 50 mL Erlenmeyer flask filled up to 40 mL with Hagem medium for an ongoing cultivation of 14 days under the same conditions. To increase biomass yield, each culture was then homogenized and transferred equally into four 500 mL Erlenmeyer flasks filled up to 200 mL with Hagem medium and cultivated for another 21 days on a horizontal shaker at 125 rpm at 21°C.

For V-DBD treatment, ten stamped-out pieces (diameter 1 cm) of precultured mycelium on Hagem agar were homogenized in 100 mL Hagem medium to a final concentration of 0.9 mg/mL. Three milliliters of these stock suspensions were transferred into 60-mm petri dishes followed by plasma treatment as shown in Table 1. Seven days after inoculation each suspension was pipetted equally and quantitatively into four 500 mL Erlenmeyer flasks and filled up to 200 mL with Hagem medium. These flasks were cultivated for 14 days at 125 rpm and 21°C.

For treatment with kINPen 09, 10 mL of the mycelial suspension prepared as for V-DBD experiments (0.9 mg/mL) was transferred into 50 mL Erlenmeyer flasks, and plasma treated medium was added according to Table 1. After 7 days of incubation, stock suspensions were divided into four equal volumes in 500 mL Erlenmeyer flasks that were filled up to 200 mL with Hagem medium and cultivated at the same conditions as samples for V-DBD.

TABLE 1: Technical data of the three plasma sources used for treatment of *Ganoderma lucidum* mycelia

	Surface DBD	Volume DBD	kINPen 09
Voltage	10 kV	9–10 kV	2 – 6 kV
Applied frequency	20 kHz	33 kHz	1.1 MHz
Plasma on/off time (s)	0.413/1.223	—	—
Process gas	Ambient air	Argon	Argon
Argon gas flow (sL/min)	—	0.5	3.8
Energy (J/min)	18	360	<60
Treatment time	2x 20 min	3x 2 min	3x 2 min
Treatment days	2, 3	0, 2, 4	0, 2, 4
Total energy input (J)	720	2,160	<360
Total culture time (d)	49	21	21
Mycelia	Solid	Suspension	Suspension

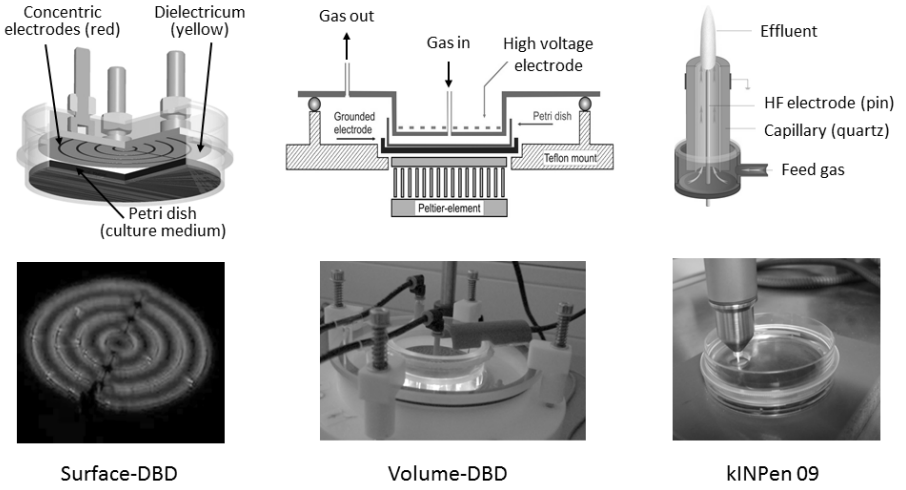

FIG. 1: Scheme of plasma sources used and the burning plasmas

C. Plasma Sources

All plasma sources used were developed and built at the Leibniz Institute for Plasma Science and Technology e.V. (INP), Greifswald, Germany.[33–36]

1. Surface Dielectric Barrier Discharge Plasma Source (S-DBD)

The surface DBD plasma arrangement based on a setup described by Hähnel et al.[34] and Oehmigen et al.[35] was used. A schematic representation of the surface DBD is

shown in Fig. 1. The electrode system was made of material for circuit boards and had a thickness of 1.5 mm and a diameter of 50 mm. Both electrodes had an outer diameter of 37 mm and were separated by an epoxy-glass fiber bulk that functioned as a dielectric barrier. On the other side of the dielectric, the electrode was a concentric ring-shaped structure. The electrode system was mounted into the upper shell of a 60-mm petri dish. The height of the electrode system in the upper shell of the petri dish could be adjusted between 2 and 5 mm above the liquid sample in the lower shell of the petri dish. A sinusoidal high voltage was supplied between both electrodes with a peak voltage of 10 kV and 20 kHz (Table 1). To keep the power input low, the high voltage was pulsed with a ratio of 0.4/1.2 ms plasma-on/plasma-off time. Ambient air was used as process gas. Energy of 18 J/min was dissipated into the plasma (Table 1). The plasma was formed in a thin layer above the structured side that faced towards the liquid sample (Fig. 1).

2. Volume Dielectric Barrier Discharge Plasma Source (V-DBD)

The volume DBD used is described in detail by Straßenburg et al.[37] Briefly, the plasma source consists of two flat, round copper electrodes (A=16.6 cm²). The perforated high-voltage electrode is integrated in the chamber lid. The lid is made of 2.4-mm-thick acryl glass and acts as dielectric barrier. It has two openings for gas exchange and is sealed against the Teflon base plate by a rubber ring. Therefore, a gas gap of 5.6 mm between lid and grounded electrode is realized. The grounded electrode is cooled by means of a Peltier element. Electrical power is supplied trough a homemade transformer, an amplifier (T&C Power Conversion, Inc. AG1021), and a function generator (Tektronix AFG 3101). An energy of 360 J/min was achieved by the setup described (Table 1). Argon gas was used as process gas and therefore, to accomplish an air-free atmosphere, all samples were locked air-tight and incubated for 1 min with argon at a flow rate of 0.5 standard liters (sL)/min before plasma treatment. Physical parameters of the plasma source obtained during treatment of the culture medium are listed in Table 1.

3. Plasma Jet kINPen 09

In the center of a quartz capillary, a pin-type electrode was mounted. Argon as the feed gas flowed through the capillary (gas flow 3.8 sL/min). A radio frequency voltage (1.1 MHz, 2–6 kV) was coupled to the center electrode. The plasma was generated from the top of the center electrode and expanded to the surrounding air outside the nozzle. Treatment was done spirally for 2 min using an *X*-*Y*-*Z* table (Fig. 1). An apparent plasma jet cone was positioned at liquid level (Fig. 1). For technical data of the plasma jet see Table 1.

D. Plasma Treatment

All plasma treatments were done in 60-mm plastic petri dishes. Mycelial cultures of the fungus were exposed to nonthermal plasma using the three different plasma sources, kINPen 09, S-DBD, and V-DBD. The mycelium was treated as solid piece inoculated in a 60-mm petri dish with Hagem agar or as a 5 mL suspension in Hagem medium (0.9 mg/mL). Treatment times varied from 2 to 20 min. Table 1 gives an overview about treatment days and treatment times with resulting total energy input by plasma exposure as well as total culture times. S-DBD and V-DBD were used for direct treatment of the mycelia. Direct and indirect treatment were combined in the case of treating mycelial suspensions with the kINPen 09. Briefly, after the first direct exposure in 60-mm petri dishes the suspensions were transferred into 50 mL glass flasks and afterwards 5 mL kINPen 09 treated Hagem medium was added. Additionally, as a first trial, solid mycelium was treated indirectly by addition of 5 mL kINPen 09 treated Hagem medium (not shown in Table 1).

E. Extraction of Mycelial Biomass and Compound Determination

After cultivation mycelial biomass was separated from the culture medium by filtration, lyophilized, and extracted by different solvents. Yield of biomass and of extracts was determined. To get an overview of chemical composition HPLC fingerprints of organic extracts were taken. Dichloromethane extracts were further analyzed for content of whole triterpens by chemical methods as well as for quantity of ganoderic acid A and ergosterol by HPLC. Total phenolics were quantified according to the method described in Ph. Eur. 6.0 and the determination of total triterpens as ergosterol (Sigma-Aldrich, St. Louis, MO) and lanosterol (ICN Biomedicals, Eschwege, Germany) was carried out by using the Liebermann-Burchard reaction and subsequent photometric detection. The β-1-3 D-glucan content was determined in hot aqueous extracts by the "Mushroom and yeast beta-glucan kit" from Megazyme International (Bray, Ireland), a specific enzyme kit.

All HPLC procedures were performed on a Shimadzu system (Kyoto, Japan) with a diode array detector, together with a RP18 column (LiChroCART, 250×4 mm, Merck, Darmstadt, Germany). Gradient elutions were applied for fingerprints of DCM extracts and quantification of ganoderic acid A (both at 254 nm) using MeOH (VWR, Darmstadt, Germany) and 0.1% orthophosphoric acid (Merck) as solvents and, furthermore, for the quantification of ergosterol (at 282 nm) MeOH and acetonitrile (VWR) were used. Reference standards were supplied from Phytolab, Vestenbergsgreuth, Germany (ganoderic acid A) and Sigma-Aldrich (ergosterol).

III. RESULTS AND DISCUSSION

Biomass production of mycelial cultures of *Ganoderma lucidum* was not influenced by plasma, neither after S-DBD, V-DBD, nor after kINPen 09 treatments (Table 2). How-

ever, yield of DCM extracts was found to be higher if mycelium was exposed to V-DBD (Table 2). Although DCM extract yield after S-DBD treatment was not influenced, the HPLC fingerprint revealed differences compared to untreated mycelia. The peak distribution between control and S-DBD treated mycelia was similar (Fig. 2); however, the peak at 36 min appeared higher in plasma treated probes. Calculation of the peak area confirmed this impression (Fig. 3A). As it was assumed that this peak reflects ergosterol, HPLC with ergosterol as reference substance was performed and confirmed the hypothesis. Plasma treatment changed ergosterol content considerably (Table 2; Fig. 3B). Both dielectric barrier discharge plasma sources, S-DBD and V-DBD, led to elevated ergosterol contents. Although total energy input was three times higher by using the V-DBD arrangement compared to S-DBD, the change of ergosterol content was about three times less. Solid-medium cultures seem to be superior to submerged cultures,[24] which could be one other reason for the different results obtained with S- and V-DBD. Surprisingly, the treatment of mycelia in suspension with the plasma jet kINPen 09 caused a decrease in ergosterol content (Table 2). Further investigations are necessary to check whether the higher ergosterol content leads to higher content of vitamin D2. The exposure of *Lentinula edodes*, the Shiitake mushroom, and *Agaricus bisporus*, the white button mushroom, with UVB radiation increased the vitamin D2 content of the mushrooms.[38]

The content of ganoderic acid A and total phenolics was not influenced by using the three plasma sources. β-1-3 D-glucan as polysaccharide was increased by exposing *G. lucidum* mycelia to V-DBD (Table 2). Both S-DBD and the kINPen 09 did not cause any change in β-glucan content. In contrast, total triterpenoid content was increased by using the plasma jet. Enhanced production of ganoderic acids was reported by You et al.[24] and by Xu et al.[27] who only changed the culture condition as described above. By varying the culture medium Zárate-Chaves et al.[25] found improved production of phenolic compounds. Changing culture conditions also led to improvement of polysaccharide production.[24,28]

Taken together, the different plasma sources influenced the *G. lucidum* cultures dif-

TABLE 2: Comparison of results obtained with three different plasma sources

	Surface DBE	**Volume DBE**	**kINPen 09**
DCM extract yield	0	⬆ (19%)	0
Content of:			
Ergosterol	⬆ (66%)	⬆ (19%)	⬇ (38%)
Total triterpenoids	Ø	Ø	⬆ (24%)
Ganoderic acid A	Ø	Ø	Ø
Total phenolics	Ø	Ø	Ø
β-Glucan	Ø	⬆ (17%)	Ø
Total energy input	720 J	2,160 J	<360 J
Mycelia	Solid	Suspension	Suspension

Ø no change; ⬆/⬇ increase/decrease by the given percentage in parentheses

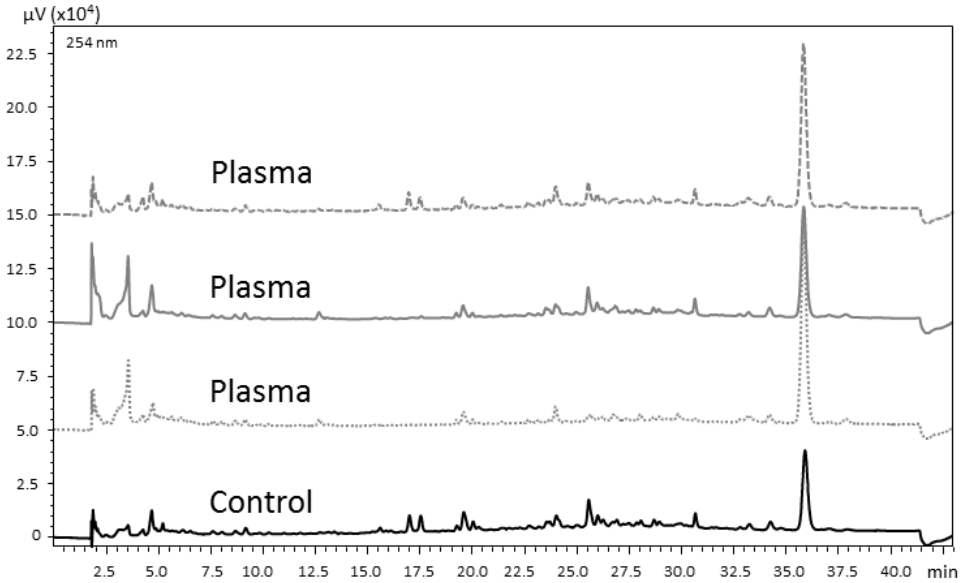

FIG. 2: Fingerprint of DCM extracts following treatment of solid mycelia with surface-DBD. Black: Untreated control; gray: three different DBD-treated mycelia

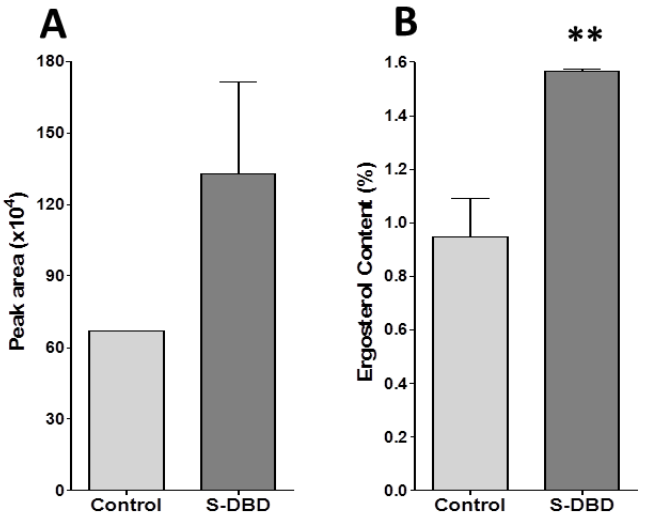

FIG. 3: Peak area (A) of the highest peak identified in the HPLC fingerprint of DCM extracts (see Fig. 2) and content of ergosterol (B). Mean ±SD, *N*=1 to 3 (peak area) and *N*=2 to 5 (ergosterol); $^{**}p<0.01$

ferently. Most changes were induced by using the V-DBD and, therefore, a correlation to total energy input is assumed. For this reason and since the solid-medium culture seemed to be better than the submerged culture, three probes of solid mycelia grown on Hagem agar were covered with 5 min kINPen 09 treated Hagem medium on days 0, 3, 5, and 8. Total energy input was thereby increased from about 360 to 1,200 J. Within 14 days in one probe, significant increased growth was observed (Fig. 4). From this probe a reserve was generated and a reculture confirmed the enormous growth on Hagem agar.

Summarizing, treatment of the mycelial cultures of *G. lucidum* with plasma resulted in positive effects on extract yield (V-DBD), content, and spectrum of some metabolites. All effects were dependent on the treatment time, plasma equipment, operating gas, and treatment conditions. V-DBD with the highest energy input caused the most changes. These first results show that plasma could be principally useful for optimization of biotechnological processes not only for prokaryotic but also for eukaryotic cells. A detailed comparison between our results and those obtained with several prokaryotic organisms is not possible mainly because of big differences in plasma sources used and treatment regimes. Further investigations with other organisms, and to get more insight into mechanisms, are in progress. One important task would be the identification of plasma components responsible for the observed effects. It is well known that reactive oxygen and nitrogen species (ROS and RNS) play an important role in the effects of plasma on mammalian cells.[39,40] Otherwise ROS itself have been proved to affect physiological functions in fungi, e.g., ascospore germination and hyphal growth.[41,42] The reports of You et al.[29,31] about the influence of ROS on *G. lucidum* cultures lead to the conclusion that ROS and RNS in plasma could be important for the influence on mushroom cultures. There are also some similarities between the effects of plasma on MAPK kinases

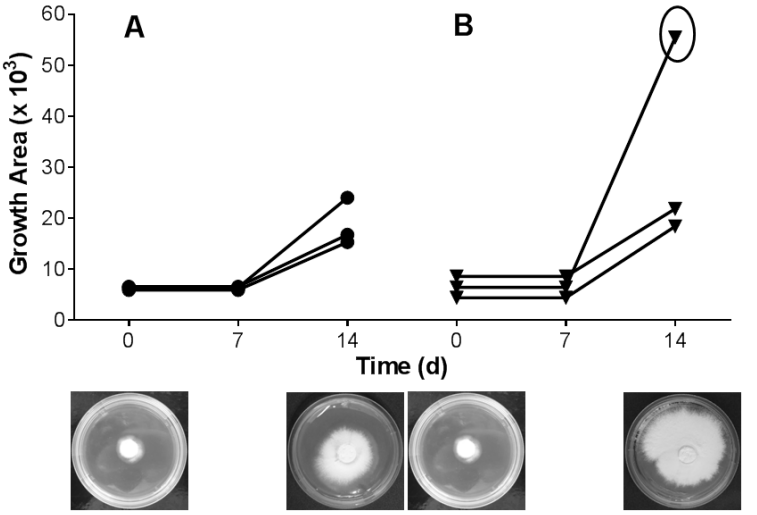

FIG. 4: Growth area of control (A) and kINPen 09 treated (B) *Ganoderma lucidum* mycelia

of human cells[43] and the effects of ROS on related fungal enzymes from *G. lucidum*.[29,31] Besides, the known influence of UV radiation on mushrooms lets us assume that radiation components of plasma could also contribute to the observed effects.

IV. CONCLUSION

Plasma with its different types of radiation and generation of ROS and RNS was able to enhance extract yield (V-DBD), content of ergosterol (V-DBD, S-DBD), total triterpenoids (kINPen 09), and β-glucan (V-DBD). Effects observed seem to be related to total energy input by the different plasma sources. Thereby, treatment of *Ganoderma lucidum* mycelia as example for a eukaryotic organism with nonthermal atmospheric-pressure plasma demonstrates the potential of plasma to generate high-yielding production strains.

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