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Influence of Non-Thermal Atmospheric Pressure Plasma Jet on Extracellular Activity of α-Amylase in *Aspergillus oryzae*

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Abstract: In a previous study, we found that plasma can enhance spore germination and α-amylase secretion in *A. oryzae*, a beneficial fungus used in fermentation. To confirm this, in the current study, we investigated the effects of plasma on development and α-amylase secretion using an enlarged sample size and a different plasma source: a plasma jet. There was a ~10% ($p < 0.01$) increase in spore germination upon non-thermal atmospheric pressure plasma jet (NTAPPJ) treatment for 5 min and 10 min, as compared with the control (no plasma treatment). The activity of α-amylase detected in potato dextrose broth (PDB) media during incubation was significantly elevated in plasma-treated samples, with a more obvious increase upon 10 min and 15 min treatments and 24–96 h incubation periods. The levels of the oxidation reduction potential (ORP) and NO_X (nitrogen oxide species) were higher in the plasma-treated samples than in the control samples, suggesting that these two variables could serve as standard indicators for enhancing α-amylase activity after plasma treatment. Genome sequencing analysis showed approximately 0.0016–0.0017% variations (changes in 596–655 base pairs out of a total of 37,912,014 base pairs) in the genomic DNA sequence of *A. oryzae* after plasma treatment. Our results suggest that NATPPJ can enhance the spore germination and extracellular activity of α-amylase, probably by increasing the levels of ORP and NO_X to an optimum level.

Keywords: *Aspergillus oryzae*; α-amylase; enzyme secretion; non-thermal atmospheric pressure plasma

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1. Introduction

At present, the demand for enzymes is increasing in different industrial activities, such as chemistry, food, textiles, and pharmaceuticals. In particular, the food industry is continuously demanding enzymes for use in fermentation and other processes. α-amylase is one such enzyme with high demand in starch-processing industries, such as those involved in the production of glucose syrups, bread, and alcoholic beverages, because it is used for the hydrolysis of starch into simple sugar components. In addition, α-amylase is widely used as an additive for making soaps, detergents, and animal feeds, as well as in treating paper and textiles [1]. To be used on an industrial scale, the efficient and environmentally friendly production of enzymes is essential. Wild-type and genetically modified microorganisms have been considered good producers of food enzymes because those are more stable and steadily active, compared with plant and animal enzymes [2–4]. However, there are still many technical barriers that have to be overcome for the industrial-scale production of enzymes by microorganisms, such as low levels of enzyme production and secretion, contamination of enzymes with other components, and the instability of enzymes under certain conditions. Therefore, technology development is necessary to improve the efficiency of enzyme production and to ensure that the process is simple, fast, safe, and involves a low cost.

Non-thermal atmospheric pressure plasma (NTAPP) is a cutting-edge technology that has been gaining attention for numerous applications, including environmental, medicinal, agricultural, decontamination, and food bioprocessing [5–8]. Plasma, an ionized gas

that is known as the fourth state of matter, can generate free electrons, neutral reactive oxygen and nitrogen species (RONS), charged gas ions, and active gas species [9]. Since RONS are known to produce deactivating and activating effects, depending on their concentration, plasma treatment can also possibly generate these dual effects based on the treatment dose. The application of NTAPP to microorganisms has been mostly focused on the deactivation of microbes harmful to human life [10–12]. In food industries, NTAPP has proven to be extremely useful for food preservation by mediating the deactivation of microorganisms, including pathogens, spoilage fungi, and bacterial spores [13]. The plasma-mediated activation of vitality and enzyme production in microorganisms has recently been reported in several studies [14–18]. Since a broad spectrum of plasma effects can be expected, depending on factors such as the types of microorganisms, dosage of plasma, and RONS produced, NTAPP is a potential technology that can be applied to improve enzyme production and the vitality of beneficial microorganisms. However, the fundamental processes involved in the effects of NTAPP on the activation of microbial cells are poorly understood.

In a previous study, we observed that there was an increase in the germination and secretion of α-amylase by *A. oryzae* post plasma treatment [18]. As a continuous investigation, in the present study, we examined the effect of plasma on the germination, growth, and α-amylase production in *A. oryzae* by using a scaled-up sample size and NTAPP jet (NTAPPJ). *A. oryzae*, considered to be generally recognized as safe (GRAS), is a good model microbe for studying the production of food enzymes, because it is known to have the capability of producing and secreting high-value industrial enzymes [19–21].

2. Materials and Methods

2.1. Fungal Strain and Culture Conditions Used in this Study

The fermenting fungus *A. oryzae* (strain KACC47488) used in this study was kindly provided by the Korean Agriculture Type Collection at the National Agrobiodiversity Center (Wanju-gun, Jeollabuk-do, Korea). The fungus was maintained on a potato dextrose agar (PDA) medium (KisanBio, Seoul, Korea) at 30 °C in the dark. In the experiment, PDA or potato dextrose broth (PDB) was used to culture the fungus at 30 °C.

2.2. Plasma Device and Treatment of Fungal Spores

Spores of *A. oryzae* were treated with an NTAPP jet (Figure 1). The electrode structure and physical properties of the plasma jet device were described in a previous study [22]. The feeding gas was ambient air, provided at a flow rate of 1.5 L min^{-1}. Approximately 0.05 W of plasma energy was generated.

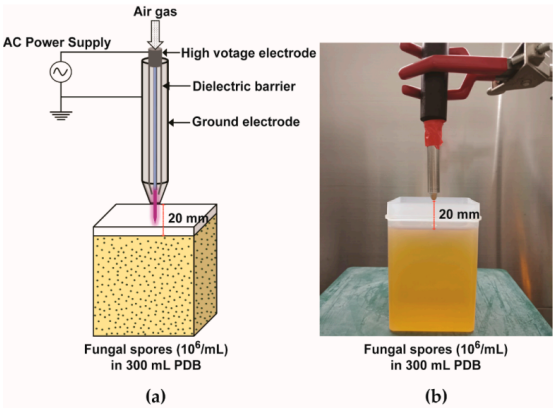

Figure 1. Non-thermal atmospheric pressure plasma jet device. (**a**) Schematic view of the plasma jet device and the experimental set-up for fungal spore treatment with plasma. (**b**) Photograph of treatment of the fungal spore suspension with the plasma jet.

A. oryzae spores were harvested by adding 15 mL of sterile phosphate-buffered saline (PBS) to 1-week-old culture plates and scraping the fungal mycelia using a spreader. The fungal suspension was filtered through 4 layers of sterile miracloth (Calbiochem, Darmstadt, Germany), and the filtrate was centrifuged at 3134× *g* for 5 min. After the supernatant was discarded, the spore pellet was resuspended in a new PDB solution such that the final concentration was 10^6 spores per mL. Then, 300 mL of the spore suspension (10^6 fungal spores per mL) was placed in a square jar (72 mm × 72 mm × 100 mm). The fungal spores were treated with an NTAPP jet at a distance of 20 mm for 5, 10, 15, and 20 min (Figure 1). Untreated spores (0 min) were used as a control. Following treatment, the spore suspension (50 mL) was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C while shaken for the indicated times.

2.3. Fungal Germination and Growth Measurement

Following treatment with the plasma jet, the spore suspension was serially diluted using a PBS solution, and 100 µL of the diluted suspension was spread onto PDA plates. The plates were incubated at 30 °C in the dark for 2 days, and following this, the number of colonies (germinated spores) was counted. The relative spore germination percentage, compared to that of the control, was calculated as follows: relative germination (%) = (number of germinated spores post plasma treatment/number of germinated spores in the untreated control) × 100. The average germination percentage was calculated from three replicate plates per experiment, and the experiment itself was repeated two times.

For growth, the treated fungal spores (50 mL in an Erlenmeyer flask) were incubated at 30 °C while shaken for 48 h. The fungal mycelia were collected by filtering the fungal culture through 4 layers of sterile miracloth. After the water was squeezed out, the fungal mycelia were maintained at 60 °C until completely dry. The weight of the completely dried mycelial mass was then measured. The average dry weight was assessed from three replicates per experiment, and the experiment itself was repeated three times.

2.4. Measurement of the Total Protein Level and α-Amylase Activity in the Media

Following treatment with the plasma jet, the spore suspension (50 mL) was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C while shaken for the indicated times. At each time point, only cultured media was collected, and the total protein concentration was determined using the Bradford protein assay kit (Bio-Rad, Hercules, CA, USA).

For measuring the α-amylase activity, the diluted culture medium (100 µL) was added to a reaction mixture containing 100 µL of 1% (*w*/*v*) soluble starch in a 0.1 M acetate buffer (pH 5.6). The reaction mixture was incubated at 50 °C for 30 min [23], and the liberated reducing sugars (product: maltose equivalent) were measured using the 3,5-dinitrosalicylic acid method [24]. A separate blank (cultured media without soluble starch) was used for each sample to eliminate the non-enzymatic release of sugars. Maltose was used to construct a standard curve. One unit (U) of amylase activity was defined as the amount of the enzyme that released 1 µg of maltose (as a reducing sugar equivalent) per mL per minute under the assay conditions. Specific activity was expressed as amylase activity (U) per mg of protein.

2.5. Measurement of H_2O_2, NO_X, NO_2^-, and NO_3^- Levels, Oxidation Reduction Potential, and pH in PDB Media

PDB (300 mL) containing fungal spores (10^6 per mL) was treated with a plasma jet for 5, 10, 15, and 20 min or left untreated (0 min, i.e., the control). Following treatment, the PDB solution (50 mL) was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C while shaken for the indicated times. The H_2O_2 concentration was measured using an Amplex™ Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes, Eugene, OR, USA), while the NO_X levels were quantified using a QuantiChrom™ Nitric Oxide Assay Kit (BioAssay Systems, Hayward, CA, USA), according to the manufacturer's protocols. The levels of NO_2^- and NO_3^- were estimated using MQuant™ Nitrite and Nitrate test

strips (EMD Millipore, Billerica, MA, USA). The oxidation reduction potential (ORP) was measured using an ExStik™ Model RE300 waterproof ORP meter (Extech, Nashua, NH, USA), while the pH was analyzed using a portable pH meter (Oakton Instruments, Vernon Hills, IL, USA).

2.6. Quantitative Polymerase Chain Reaction of Secretion-Related Genes

To measure the mRNA levels of the genes associated with protein secretion, the fungal spores (300 mL, 10^6 fungal spores per mL) were treated with plasma for 5, 10, 15, and 20 min or left untreated (control). Following treatment, the spore suspension (50 mL) was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C while shaken. Fungal mycelia were harvested after 48 h by filtering the culture through 4 layers of sterile miracloth, washed twice with deionized (DI) water, and then stored at −80 °C until use after removing the water. Total RNA extraction, cDNA synthesis, and RT-PCR were performed as described in a previous study [15]. The expression levels of the two putative genes involved in vesicle trafficking and transport in *A. oryzae*, which showed increased transcription levels post plasma treatment (*SAR1* and *YPT* homologs) in a previous study [18], were measured using a quantitative polymerase chain reaction (qPCR) (Table 1). The relative mRNA levels were determined as a ratio compared to the expression level of a reference gene (18S ribosomal RNA) (ratio = $(2)^{\Delta Ct\ target\ (control-sample)}/(2)^{\Delta Ct\ reference\ (control-sample)}$) [25]. The primer sequences of the putative genes are listed in Table 1. An average of six replicate measurements was considered.

Table 1. List of primers used in the quantitative polymerase chain reaction (qPCR).

Genes	Primer sequences
Vesicle trafficking and transport	
GTPase (ER to Golgi), SAR1 homolog	Forward-5′CGAAGTGAGCGGTATCGTTT3′ Reverse-5′CCCTTTCCTGTGGTCTGGTA3′
GTPase (cis to medial Golgi), YPT1 homolog	Forward-5′TGATGGCAAGACAGTGAAGC3′ Reverse-5′TTGACACCCTCAGTGGCATA3′
Reference gene	
18S ribosomal RNA	Forward-5′GGAAACTCACCAGGTCCAGA3′ Reverse-5′AGCCGATAGTCCCCCTAAGA3′

2.7. Genomic DNA Analysis

To determine the plasma-induced changes in the genome of *A. oryzae*, the whole genome was sequenced post plasma treatment. The fungal spores (300 mL, 10^6 fungal spores per mL) were treated with plasma for 5 and 20 min or left untreated (control). The spore suspension (50 mL) was transferred to a 100 mL Erlenmeyer flask and incubated at 30 °C while shaken for 48 h. The fungal mycelia were harvested, washed twice with DI water, and stored at −80 °C until use. The genomic DNA was extracted using a high-salt cetyltrimethylammonium bromide (CTAB) extraction protocol [26], and its concentration was measured using a NanoDrop™ system (Biotek Instruments, Winooski, VT, USA).

DNA library construction and sequencing were carried out by Macrogen (Seoul, Korea). For library construction, the genomic DNA was randomly fragmented, and 5′ and 3′ adapters were ligated to the DNA fragments. Adapter-ligated DNA fragments were amplified using PCR and purified on gel. The DNA library was loaded into a flow cell and captured using oligos complementary to the library adapters on the surface of the flow cell. Each DNA fragment was amplified into clonal clusters using bridge PCR amplification. Sequencing was performed using Illumina SBS (sequencing by synthesis) technology (next-generation sequencing) (Macrogen, Seoul, Korea). The sequence reads were aligned to each other to identify variants.

2.8. Statistical Analysis

All data were presented as the average of 6–9 replicates. Statistical analysis of the data was performed using the Student's t-test, and statistical significance was indicated as follows: * denotes $p < 0.05$, and ** denotes $p < 0.01$.

3. Results

3.1. Plasma Jet Enhances Spore Germination in A. oryzae

After the *A. oryzae* spores (10^6 spores/mL, 300 mL) in PDB were treated with an NTAPP jet (Figure 1), there was a significant increase ($p < 0.01$) in the number of germinated spores per mL of suspension in the samples treated for 5 and 10 min (Figure 2a). The relative germination percentage was significantly higher in the spores treated with plasma for 5 and 10 min, compared with that in the control (0 min) (Figure 2b). An increase in germination of approximately 8% and 9% was observed in the spores treated with an NTAPP jet for 5 and 10 min, respectively, compared with that in the control (Figure 2b).

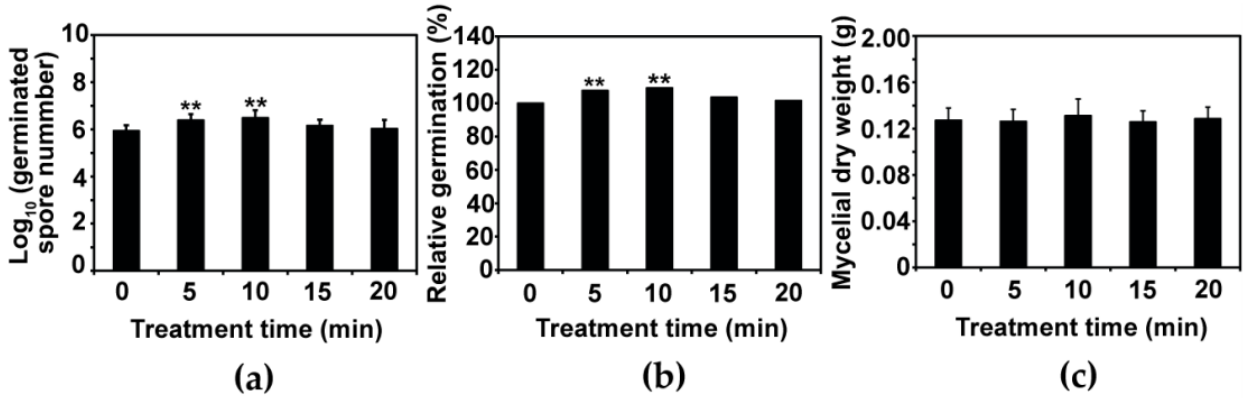

Figure 2. Spore germination and growth of *A. oryzae* post treatment with a plasma jet for 5, 10, 15, and 20 min, or without treatment (0 min = control). (**a**) Log-scale number of germinated spores in a 1 mL suspension with or without treatment with a plasma jet. (**b**) The relative germination percentage (%) of plasma-treated samples compared with that of the control (0 min, 100%). (**c**) The dry weight of the fungal mycelia grown for 48 h in a 50 mL culture. Each value is indicated as the mean and standard deviation of 6–9 replicate measurements. ** $p < 0.01$.

Furthermore, we also investigated the effect of plasma on fungal mycelial growth after germination. No significant differences were observed in the mycelial dry masses between the control (0 min) and the plasma-treated samples after 48 h. Approximately 0.15–0.16 g of dry mycelial mass was obtained from the 50 mL culture of the control (0 min) and each plasma treatment time point sample (Figure 2c).

3.2. Plasma Jet Increases α-Amylase Secretion in A. oryzae

The secretion of α-amylase into PDB media by *A. oryzae* was analyzed by measuring the extracellular levels of total protein secretion and α-amylase activity post plasma treatment of the fungal spores. Generally, an increase in total protein concentration in the PDB media of up to 0.029 mg/mL was observed in all samples during incubation (144 h of incubation in the 15 min plasma treatment group), regardless of plasma treatment (Figure 3a). However, no significant differences in total protein concentrations (mg/mL) were observed between the control (0 min) and the plasma jet-treated samples at each incubation time (24–168 h) (Figure 3a).

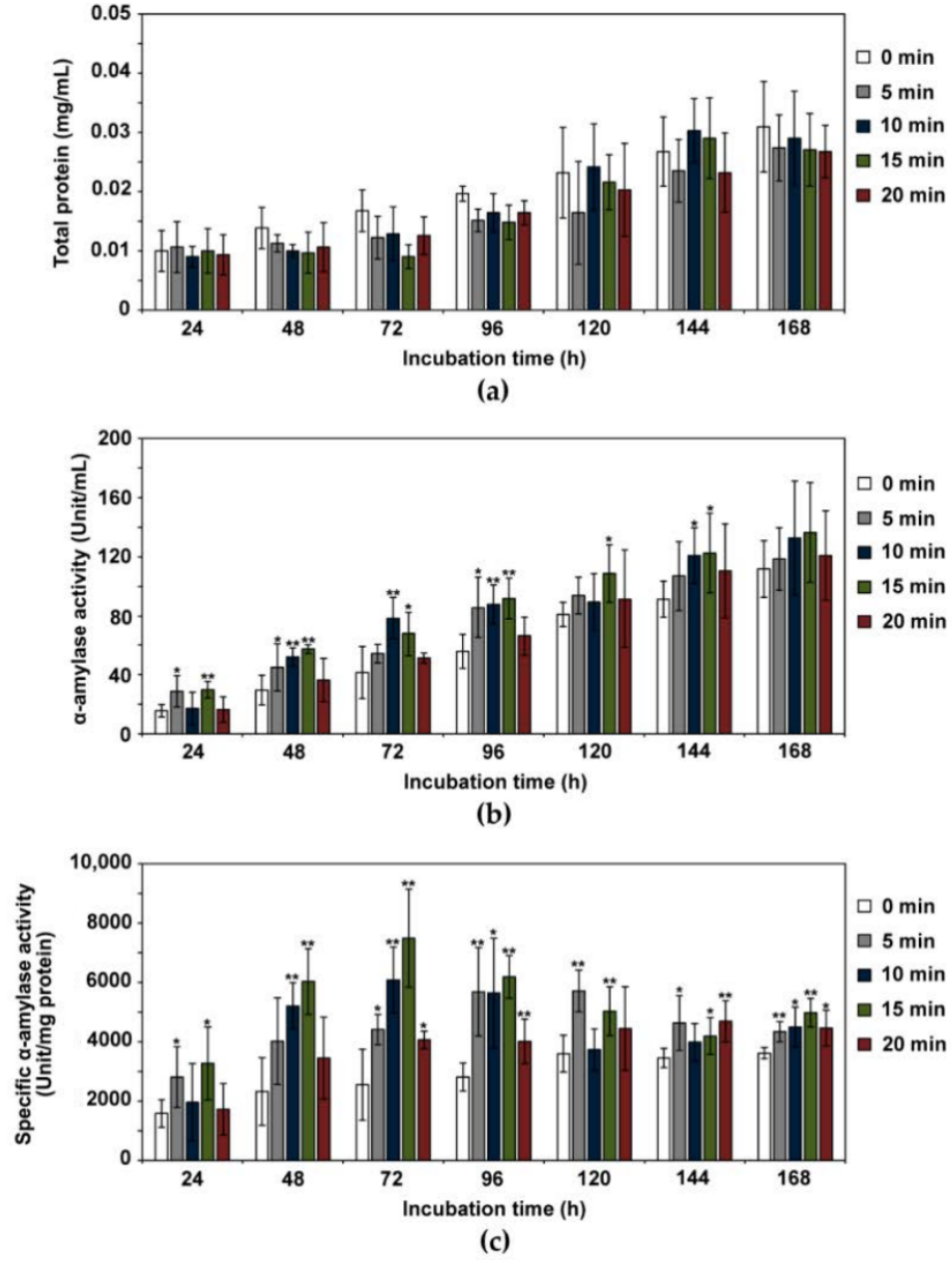

Figure 3. Total protein secretion and α-amylase activity of *A. oryzae* during incubation post treatment with a plasma jet for 5, 10, 15, and 20 min, or without treatment (0 min = control). (**a**) The concentration of total protein in the potato dextrose broth (PDB) media (mg/mL) post incubation for the indicated time periods. (**b**) The α-amylase activity (Unit/mL) detected in the PDB media post incubation for the indicated time periods. (**c**) The specific α-amylase activity (Unit/mg of total protein) estimated in the PDB media post incubation for the indicated time periods. All values were calibrated with the background (0 h incubation) values. Each value is indicated as the mean and standard deviation of 6 replicate measurements. ** $p < 0.01$ and * $p < 0.05$.

An increase in α-amylase activity per mL of PDB media was observed in all samples following incubation (Figure 3b). At all incubation times except 168 h, the α-amylase activity was significantly higher ($p < 0.05$ and 0.01) in the media of the plasma-treated samples than in that of the control (Figure 3b). A significant increase was more frequently observed under the conditions of 10 min and 15 min of plasma treatment and shorter incubation times (24–96 h) (Figure 3b). Treatment with the plasma jet for 20 min did

not seem to have a significant effect on α-amylase activity in the media over the entire incubation period (Figure 3b).

The specific activity of α-amylase (enzyme activity expressed per milligram of total protein) in the PDB media was significantly greater ($p < 0.05$ and 0.01) in the plasma-treated samples than in the control over all incubation periods (Figure 3c). Plasma treatment for 20 min showed a relatively lower increase in specific activity, compared with other treatment times (Figure 3c). The highest levels of fold increase in specific activity were observed in the conditions of 10 min and 15 min of plasma treatment after incubation for 72 h, which were approximately 2.38-fold and 2.93-fold, respectively (Figure 3c).

3.3. Level of NO_X Species and Oxidation Reduction Potential in PDB Increases after Plasma Treatment

Previously, we found that the plasma-induced elevation in α-amylase secretion by *A. oryzae* was closely associated with the NO_X species, particularly NO_2^- and NO_3^- generated in the media post plasma treatment [18]. Thus, we investigated the levels of NO_X, NO_2^-, NO_3^-, and H_2O_2 in the PDB media post plasma treatment. Since no suitable methods were available for measuring the levels of NO_2^- and NO_3^- in the PDB, they were quantified in water and not PDB in our previous study [18]. In this study, however, the levels of NO_2^- and NO_3^- in the PDB media were estimated using colorimetric strips.

Following plasma treatment on day 0, there was a slight increase (0.074–0.093 mg/L) in the levels of H_2O_2 in the PDB solution over the treatment time. However, the levels dramatically decreased after 3 and 7 days of incubation and showed no significant differences among treatment times (Figure 4a). The overall concentration of NO_X was distinctly higher than that of H_2O_2 in the PDB and continually increased with the increasing treatment time, displaying the maximum level after 20 min of treatment (1.993–2.228 mg/L) (Figure 4b). No significant changes in the levels of NO_X in the PDB were observed during incubation at each treatment time (Figure 4b). The levels of NO_2^- and NO_3^- in the PDB solutions were estimated from the intensity of the color that appeared on the test strips. The intensity of the pink color on the strip indicated that the NO_2^- level was generally higher than that of NO_3^-; the levels of NO_2^- and NO_3^- increased over the treatment time during incubation, with a more obvious elevation in the NO_2^- level (Figure 4c). The NO_2^- concentration was estimated to be less than 5 mg/L, while the NO_3^- concentration was much lower than that. There was a slight decrease in both the NO_2^- and NO_3^- levels after incubation for 3 and 7 days, as compared with that on day 0 (Figure 4c).

To understand the overall oxido-reduction state of the PDB media, the ORP was measured in the PDB media during fungal culture post plasma treatment. An increase in the ORP value indicates that the PDB solution has an elevated oxidizing capability. The ORP level of the PDB media with or without fungal spores displayed a positive value of approximately 249.2 mV before plasma treatment (Figure 5). A slight increase of up to 265.7 mV was observed right after treatment with the NTAPP jet for 20 min (day 0) (Figure 5). During incubation, the ORP continued to increase up to 96 h of incubation (with a maximum ORP of 331.7 mV at 20 min of treatment) and then decreased in both the plasma-treated samples and the control (Figure 5). Generally, the ORP levels were higher in the plasma-treated samples than in the control (0 min) at all incubation times (Figure 5). No significant differences in pH levels (pH = 4.83–4.87) were observed between the plasma-treated samples and the control (0 min) (Figure 6).

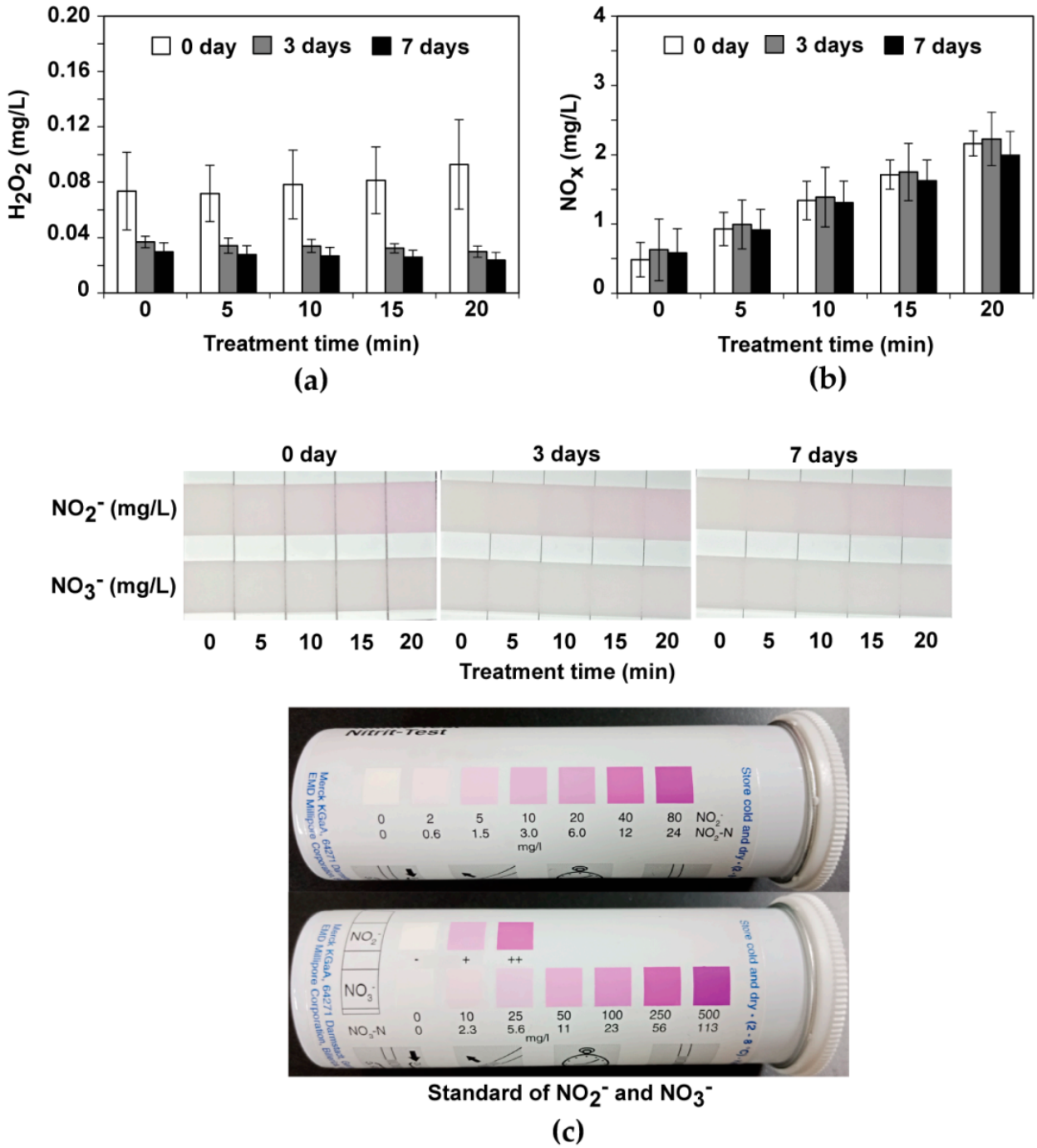

Figure 4. Evaluation of the reactive oxygen and nitrogen species (RONS) level in the PDB solution post plasma treatment for 5, 10, 15, and 20 min, or without treatment (0 min = control). Following plasma treatment, the PDB was incubated for 0, 3, and 7 days. (**a**) Concentration of H_2O_2. (**b**) Concentration of NO_X. (**c**) Color change in the NO_2^- and NO_3^- strips after soaking in the control and plasma-treated PDB (upper panel), as well as the standard concentrations of NO_2^- and NO_3^- according to the color of the strips (bottom panel). In (**a**,**b**), each value is indicated as the mean and standard deviation of 6 replicate measurements.

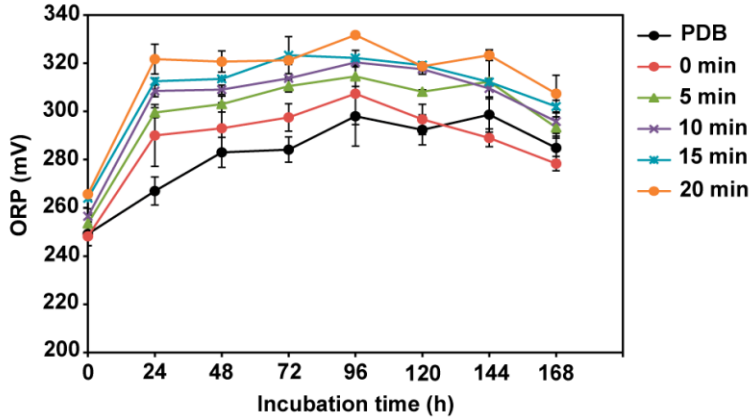

Figure 5. Oxidation reduction potential (ORP) measurements in the PDB media post plasma treatment for 5, 10, 15, and 20 min, or without treatment (0 min). Post plasma treatment, the fungal spores in the PDB solution were cultured for 168 h, and the ORP was measured every 24 h. Each value is indicated as the mean and standard deviation of 9 replicate measurements.

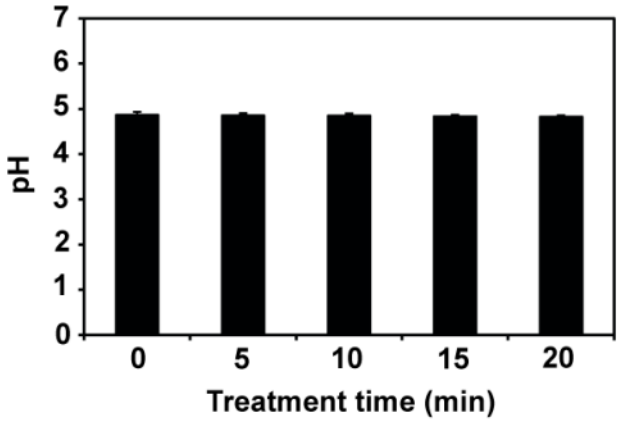

Figure 6. The pH measurement in the PDB media post plasma treatment of the fungal spores for 5, 10, 15, and 20 min. Untreated (0 min) fungal spores were used as a control. The pH was measured immediately after plasma treatment. Each value is indicated as the mean and standard deviation of 9 replicate measurements.

3.4. Plasma Increases the mRNA Levels of Genes Involved in Vesicle Trafficking

Genes homologous to *SAR1* (a GTPase that controls transport from the endoplasmic reticulum to the Golgi) and *YPT1* (a GTPase that controls transport from the cis to the medial Golgi), two putative genes that showed increased transcription in the plasma-treated samples in our previous study (Table 1) [18], exhibited significantly ($p < 0.05$ and 0.01) higher levels of mRNA transcription in the fungal samples cultured for 48 h post plasma treatment of the spores for 15 and 20 min (Figure 7). This indicates that NTAPP jet treatment can activate the mRNA transcription of genes involved in vesicle trafficking of *A. oryzae*.

3.5. The Effect of Plasma on the Genomic DNA of A. oryzae

To determine the plasma-induced changes in the genomic DNA of *A. oryzae*, whole genome sequencing was performed on fungal samples cultured for 48 h after the spores in PDB were treated with an NTAPP jet for 5 min (short time) and 20 min (long time) or left untreated (control). The genome size of *A. oryzae* is ~37 Mb and contains 12,074 genes on 8 chromosomes [27,28]. In our analysis, only variations observed in most reads were considered mutations.

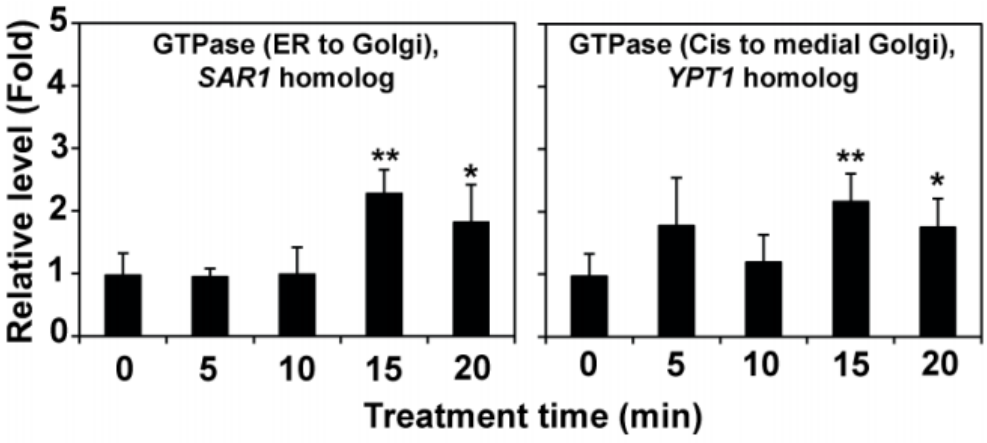

Figure 7. The mRNA levels of two putative genes involved in vesicle trafficking and transport in fungal hyphae cultured for 48 h post plasma treatment of the spores. Each value is indicated as the mean and standard deviation of 6 replicate measurements. ** $p < 0.01$ and * $p < 0.05$.

Compared to the control DNA (no plasma treatment), the genomic DNA from the plasma-treated samples (5 min and 20 min treatments) displayed approximately 0.0016–1.0017% variations in sequence (single base pair changes, deletions, and insertions) (Table 2 and Supplementary Tables S1 and S2). No dramatic differences in sequence were observed between the samples treated with plasma for 5 min and 20 min, although a slightly higher mutation rate was exhibited in the samples treated for 5 min (0.0017%) than in those treated for 20 min (0.0016%) (Table 2). The total mutation percentage in the exon area was about 0.0001–0.0002% in the samples treated with plasma for 5 min and 20 min, which was lower than that in the intron area (Table 2). Missense mutations (changes in the amino acid sequence of a gene) occurred at approximately 0.00004–0.0001% positions in 17 and 10 genes upon plasma treatment for 5 min and 20 min, respectively (Table 2 and Supplementary Table S3).

Table 2. Total mutations in the genomic DNA of *A. oryzae* after treatment with plasma for 5 min and 20 min.

	Treatment Time (min)	Mutation Position Number	Mutation (%)
Total mutations	5	655	0.0017
	20	596	0.0016
Total mutations in exons	5	62	0.0002
	20	25	0.0001
Total mutations in introns	5	593	0.0016
	20	571	0.0015
Total missense mutations	5	35 (17 [a])	0.0001 (0.1377 [b])
	20	17 (10 [a])	0.00004 (0.0810 [b])

[a] Number of genes; [b] Relative percentage of the total number of genes; (number of mutated genes/total number of genes) × 100.

Variations in the genomic DNA appeared on each chromosomal DNA with a similar proportion (Table 3 and Supplementary Tables S1 and S2). The percentage of mutations was slightly higher in chromosome 4 than in the other chromosomes. No change was observed in the mitochondrial DNA (Table 3 and Supplementary Tables S1 and S2).

Table 3. The percentage of mutations in each chromosome and mitochondrial genome of *A. oryzae* after treatment with plasma for 5 min and 20 min.

	Mutations (%)		Mutations in Exons (%)		Missense Mutations (%)		Missense Mutationsin Genes (%)	
Treatment Time (min)	5	20	5	20	5	20	5	20
Chromosome 1	0.00186	0.00160	0.00005	0.00000	0.00002	0.00000	0.04808	0.00000
Chromosome 2	0.00168	0.00115	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Chromosome 3	0.00125	0.00142	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
Chromosome 4	0.00262	0.00309	0.00027	0.00012	0.00016	0.00010	0.19280	0.25707
Chromosome 5	0.00126	0.00128	0.00007	0.00013	0.00007	0.00011	0.13193	0.19789
Chromosome 6	0.00131	0.00138	0.00017	0.00007	0.00010	0.00002	0.22539	0.07513
Chromosome 7	0.00150	0.00112	0.00051	0.00024	0.00027	0.00014	0.31847	0.10616
Mitochondria	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000

4. Discussion

As compared with our previous study [18], in the present study, we scaled up the number of fungal spores (from 7.5×10^7 to 3×10^8) and the media volume being treated (from 15 mL to 300 mL), in addition to changing the plasma source from a micro-DBD (dielectric barrier discharge) plane-type plasma to a plasma jet. Our results demonstrated that the plasma jet, like the micro-DBD plant-type plasma used in our previous study, can promote the germination of fungal spores and α-amylase activity in media. In addition, plasma treatment resulted in similar effects on fungal germination and enzyme activity under the scaled-up condition of the samples. Together with our previous results [15], we concluded that plasma treatment (micro-DBD plane-type plasma or plasma jet) was clearly effective for fungal spore germination, although the subsequent hyphal growth was not always enhanced. Spore germination was improved upon plasma treatment for a shorter time (5–10 min), while longer treatments did not cause significant changes. Dose-dependent activation and inactivation have been observed in mammalian cells, with increased cell proliferation being observed at low doses of plasma and cell death being observed at high doses [29].

Compared to spore germination, an increase in α-amylase activity in the media was observed upon a relatively longer period of plasma treatment (10–15 min). The plasma-induced enhancement effect was more dramatic during early incubation (24–96 h) than later. This may be because of the aging of the culture media and fungal cells. A promising implication from our results is that α-amylase activity in media can be promoted by plasma treatment without using induction media for enzyme secretion, as our experiments were carried out in PBD media, not induction media. The enhanced α-amylase activity in media may be a result of the increased secretion of enzyme protein from fungal hyphae, or the enhanced activity of α-amylase already secreted into the media, or both. Intriguingly, we did not observe a significant difference in the total protein amount in the media between the control and the plasma-treated samples. This indicates that the enhanced α-amylase activity in the media may not be a result of enzyme secretion from fungal hyphae. However, we did observe a slight increase in the α-amylase protein levels in the PDB media in our previous study, and it is possible that the protein levels were possibly too small to be detected using a Bradford assay. Thus, other methods for the quantification of α-amylase protein levels may need to be identified. The elevated secretion of enzymes upon plasma treatment has also been demonstrated by Farasat et al. [14], who showed that treatment with plasma caused an increase in the protein amount and activity of the phytase enzyme in culture media. Their study also demonstrated that plasma could enhance the activity of isolated enzymes by unfolding the tertiary structure of the enzyme slightly. Although the protein levels of the α-amylase secreted into the media were not quantified in our study,

a plasma jet is likely to promote the secretion of α-amylase and the activity of secreted α-amylase in *A. oryzae*.

The oxidative state of the PDB media seems to be closely associated with fungal activation. The highest elevation of α-amylase activity was observed in plasma-treated samples, where PDB media exhibited ORP values of ~310–320 mV. This value was higher than that of the control (0 min, 290–305 mV). H_2O_2 and other reactive oxygen species generated upon plasma treatment may have contributed to the elevation in the ORP level in the PDB media, because H_2O_2 can behave as an oxidant or a reductant in redox reactions [30]. Our results suggest that a certain level of oxidizing conditions outside the cells (310–320 mV in our case) may be needed for activation of the enzyme or enzyme secretion. Studies have shown that increased ORP values in plasma-treated water have a high impact on the oxidizing capacity and anti-microbial potential [31–34]. However, our study demonstrated that microbial activation can also be induced under certain levels of the ORP. As observed in our previous study, NO_X was stably detected in the PDB media during incubation post plasma treatment in this study as well. This indicates that NO_X may be crucial for enhancing enzyme activity. In addition, the long-lived species of NO_2^- and NO_3^- were detected in the PDB media upon plasma treatment, indicating that these could be major players in the plasma-mediated activation of the enzyme or enzyme secretion. Although NO_X levels were higher in the plasma-treated samples, no significant changes were observed in the pH levels, which were close to the optimal pH range of 5.0–6.0, which is suitable for α-amylase enzyme production by *Aspergillus sp.* in a submerged fermentation culture [1].

Treatment with a plasma jet caused a small portion of variations in the genomic DNA of *A. oryzae* in our study. Some of these variations occurred in the protein coding region. The plasma treatment time did not seem to affect the extent of genetic variations because similar levels of variation were observed upon plasma treatment for 5 min and 20 min. The soft rays and free radicals generated from NTAPP are known to damage DNA and RNA with increasing treatment times [35,36]. This may lead to a mutational change in the sequence. In addition, recent studies using *A. oryzae* and *C. albicans* have demonstrated that plasma-induced mutations enhance the activity of salt-tolerant proteases and xylose reductases without the occurrence of mutations in those enzyme genes themselves [16,17]. Since most of the genes found to be mutated upon plasma treatment in our study were uncharacterized, it is not clear whether the increased activity of α-amylase resulted from mutations or not. However, if the mutations that result in amino acid changes in genes are not critical for modifying the protein tertiary structure, the protein function can remain unaltered. In this case, plasma-induced mutations may not be related to the elevation of the extracellular activity of α-amylase in *A. oryzae*. Furthermore, a recent study on human stem cells demonstrates that NTAPP can induce epigenetic modifications and activate the expression of genes involved in cell differentiation [37]. Likewise, plasma treatment can possibly induce the epigenetic changes that activate the expression of genes involved in the metabolic pathways, germination, and secretion of enzymes in *A. oryzae*.

5. Conclusions

In this study, we demonstrated that the scaled-up treatment of *A. oryzae* spores with an NTAPP jet could enhance the spore germination and activity of α-amylase in the PDB media at the hyphal stage. Different treatment times were required for the highest elevation of spore germination and enzyme activity. These results indicate that similar plasma effects on fungal activation, as observed in our previous study, were obtained with a large sample size and different plasma sources. The enhanced activity of α-amylase in the PDB media is likely to be associated with the levels of the ORP and NO_X in the media. This suggests that the levels of the ORP and NO_X in the media resulting from plasma treatment can serve as standards for assessing microbial activation. Plasma jet treatment induced mutational variations in the genomic DNA of *A. oryzae*, but it remained to be elucidated whether these genetic changes might affect enzyme secretion.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-3417/11/2/691/s1: Table S1: total mutations in 5 min ofplasma treatment; Table S2: total mutations in 20 min of plasma treatment; and Table S3: missense mutations in 5 and 20 min of plasma treatment.

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