

# High-Voltage Atmospheric Cold Plasma Treatment of Yeast for Spoilage Prevention

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**ABSTRACT:** Yeast cells were killed when exposed to high-voltage atmospheric cold plasma (HVACP) in dry air and oxygen-rich modified air (MA65). HVACP was most effective in MA65, resulting in  $> 2 \log_{10}$  colony forming unit (CFU)/mL reductions versus  $0.5\text{--}2.0 \log_{10}$  CFU/mL reductions in air. Other variables contributing to cell death included applied voltages, exposure times, cell densities, and suspension volumes. Viable cell reductions were observed at all exposure levels. Yeast sensitivity to cold plasma was higher at lower cell densities and suspension volumes. Cell death determined by plate counts was corroborated using Trypan blue, which stains dead cells. Cell death was mediated by plasma-generated reactive gas species (RGS), for example,  $O_3$ ,  $NO_x$ , and  $H_2O_2$ , detected in treated yeast suspensions. Yeast were less sensitive to plasma treatment in grape juice compared to water, owing to the possible consumption of RGS by the organic matter in juice. Cold plasma reduced the pH of treated yeast suspensions, caused the release of soluble protein from the cells, and inactivated cell-wall-bound yeast invertase, all as a function of voltage and exposure time. These data indicate damage to yeast at the cellular level. Results were supported by electron microscopy, which showed that yeast exposed to plasma are misshapen, compared to untreated cells.

**KEY WORDS:** cold plasma, yeast viability, enzyme inactivation, nonthermal plasma, juice spoilage

## I. INTRODUCTION

High-voltage atmospheric cold plasma (HVACP) is a novel, nonthermal technology that has potential for microbial decontamination of foods and beverages.<sup>1,2</sup> The term plasma refers to a quasineutral ionized gas, primarily comprised of photons, ions, and free electrons, plus atoms in their fundamental or excited states with a net neutral charge.<sup>3,4</sup> HVACP uses high voltage to energize gas molecules and generate reactive gas species (RGS) that can kill food-spoilage microorganisms. Dielectric barrier discharge (DBD) is one of the most common methods to generate cold plasma, wherein plasma is generated between two plane-parallel metal electrodes, and at least one of which is covered by a dielectric layer.<sup>5</sup> The advantages of HVACP over conventional decontamination methods include environmental friendliness, low-energy usage, low-temperature processing, scale-up versatility, absence of chemical residues, and minimal effect on nutrient value and organoleptic properties of food.

Food spoilage, which causes significant economic loss each year, is a problem in the brewing and fruit juice industries. The spoilage occurs is caused by wild yeast such as

*Kloeckera apiculata*, *Candida stellata*, *Brettanomyces intermedius*, and *Saccharomyces cerevisiae*.<sup>6</sup> The latter produces phenolic off-flavors in beer and wine from decarboxylation of ferulic and coumaric acids to 4-vinylguaiacol and 4-vinylphenol, respectively.<sup>7</sup> The aim of this study was to explore the use of HVACP to eliminate costly yeast-mediated spoilage encountered during brewing and juice production. *S. cerevisiae* was chosen as the target organism for this investigation.

## II. MATERIALS AND METHODS

### A. Chemicals

Sucrose was obtained from Sigma-Aldrich. The bicinchoninic acid protein assay kit was purchased from the Pierce Chemical Company. HiVeg peptone was supplied by HiMedia. Sodium arsenate was obtained from Alfa Aesar. Dry air and modified air (MA65) were purchased from Praxair. All other chemicals used in this study were obtained from Fisher Scientific and were of reagent grade.

### B. Yeast Strain

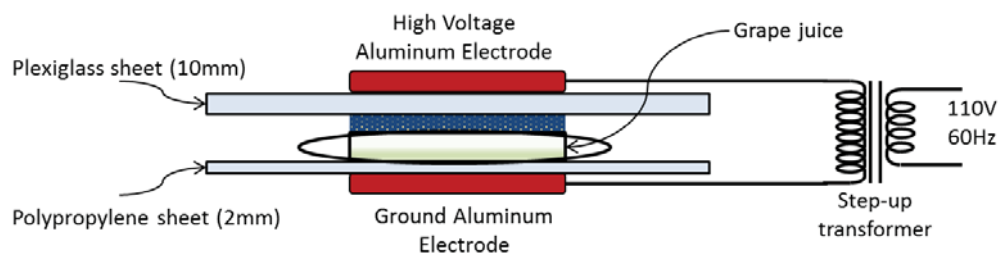
Ethanol Red yeast (*Saccharomyces cerevisiae*; LeSaffre Yeast Corp., Milwaukee, WI) was used throughout this investigation. The organism was maintained at 4°C on Sabouraud (SAB) dextrose agar slants. In preparation for trials with plasma, yeast were cultivated in Bacto SAB broth with either 2% glucose or 2% sucrose, as needed. Cells were grown overnight in a New Brunswick Scientific model G76 gyrotory water bath at 30°C and 180 rpm in 250-mL Erlenmeyer flasks containing 50 mL of broth. Cells were aseptically transferred to sterile 50-mL centrifuge tubes and harvested by centrifugation at 12,000g for 8 min at 4°C in a Sorvall Legend XTR refrigerated centrifuge equipped with a FIBERLite® F15-8 × 50C rotor. The supernatant was discarded. The yeast cells were washed by resuspension in sterile DI water and harvested again, as above. The washed cell pellet was resuspended again, as above. Cell density of the suspension was determined by counting under a microscope using a hemocytometer. The cells were then diluted with either sterile DI water or sterile grape juice to produce starting cell densities of  $\sim 1 \times 10^7$  cells/mL.

### C. Grape Juice Preparation

Seedless, green, table grapes were obtained from a local market and homogenized in a Hamilton Beach juice extractor. Pulp was removed from the homogenate by centrifugation at 5000 rpm for 10 min, and the supernatant was filtered to remove coarse particles. The filtrate was stored at -20°C until needed. Before use, the juice was thawed and sterilized by vacuum filtration through a Stericup with a 0.22-μm polyethersulfone membrane (EMD Millipore Corp., Billerica, MA).

## D. Plasma Treatment of Yeast

Yeast suspensions (5–20 mL, as needed) were aseptically pipetted into sterile Petri dishes (150 mm diameter) and inserted in 1-gal Ziploc freezer bags. The bags were purged for 60 s at 1260 mL/min with dry air or modified air (MA65; 65% O<sub>2</sub>, 30% CO<sub>2</sub>, and 5% N<sub>2</sub>) and heat-sealed with a FoodSaver GameSaver Deluxe Plus. Bags with yeast samples were positioned between aluminum electrodes (6 in diameter; Fig. 1) and exposed for 0–4 min to HVACP at 18–80 kV, generated with a high-voltage step-up transformer (Phenix Technologies, Accident, MD; model 6CP130-36-6.5X). Samples were refrigerated for 20–24 hr at 4°C–8°C, after which the cells were aseptically transferred to sterile screw-cap tubes, serially diluted with sterile DI water, plated on SAB agar with 4% glucose, and then incubated at 30°C. After 36–48 hr, yeast colonies were counted, and the resulting CFU data were used to determine cell survival.



**FIG. 1:** Schematic diagram of the apparatus used for HVACP treatment of yeast

## E. Microscopy

### 1. Vital Staining

Yeast cells were pelleted by centrifugation and resuspended in aqueous 0.4% Trypan blue, which is taken up by dead, but not live, cells, enabling visualization and distinction between live and dead yeast. Stained cells were examined by light microscopy.

### 2. Scanning Electron Microscopy

Yeast cells were pelleted by centrifugation. Pellets were fixed with 2% paraformaldehyde and 3% glutaraldehyde in a 100 mM cacodylate buffer (pH 7.2) for at least 24 hr at 4°C and then collected on 0.2-μm filters. The filters were rinsed three times (15 min/rinse) in 100 mM cacodylate buffer. Samples were postfixed in 1% osmium tetroxide in 100 mM cacodylate buffer for 1 hr, washed several times with DI water, and then dehydrated through graded ethanol solutions (25%, 50%, 70%, 85%, 95%, and 100%; two changes at each percentage for 15 min). Samples were infiltrated with hexamethyl-

disilazane (HMDS) and dried from pure HMDS. Dried filters were mounted on aluminum stubs with double-sided tape and colloidal silver paint and then sputter-coated with gold-palladium with a Denton Desk II Sputter Coater (Denton Vacuum, Inc., Moorestown, NJ). Images were captured using JEOL JSM-5800LV scanning electron microscopy (SEM) at 10 kV (Japan Electronic Optics Laboratory, Peabody, MA).

## F. Analytical

### 1. Protein Determination

Plasma-treated cell suspensions were syringe-filtered through Millex-GV PVDF filters (EMD Millipore; pore size 0.22  $\mu\text{m}$ ). Protein amounts in the filtrates were determined using the bicinchoninic acid method with 0.2% bovine serum albumin as a standard.<sup>8</sup>

### 2. Invertase Activity

Invertase was measured in intact yeast cells as described by Silveira et al.<sup>9</sup> The cells were suspended in 50 mM acetate buffer containing 50 mM NaF at pH 5.0 (buffer A). After addition of 200 mM sucrose to buffer A, the cells were incubated for 15 min at 30°C. Invert released by the enzyme was measured as reducing sugar using the method of Nelson.<sup>10</sup>

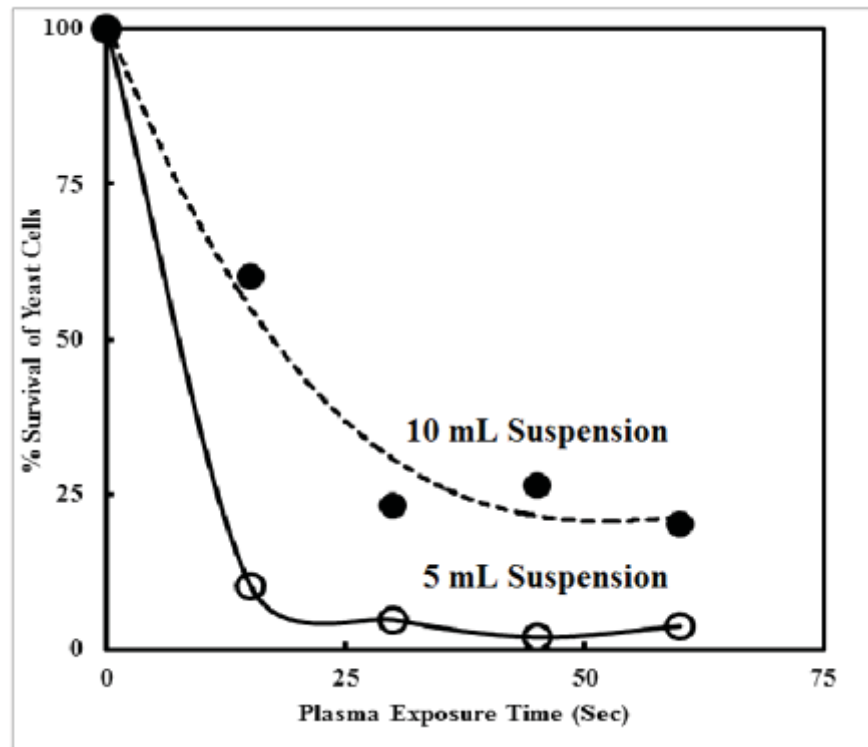
## III. RESULTS AND DISCUSSION

### A. Suspension Volume and Cell Densities

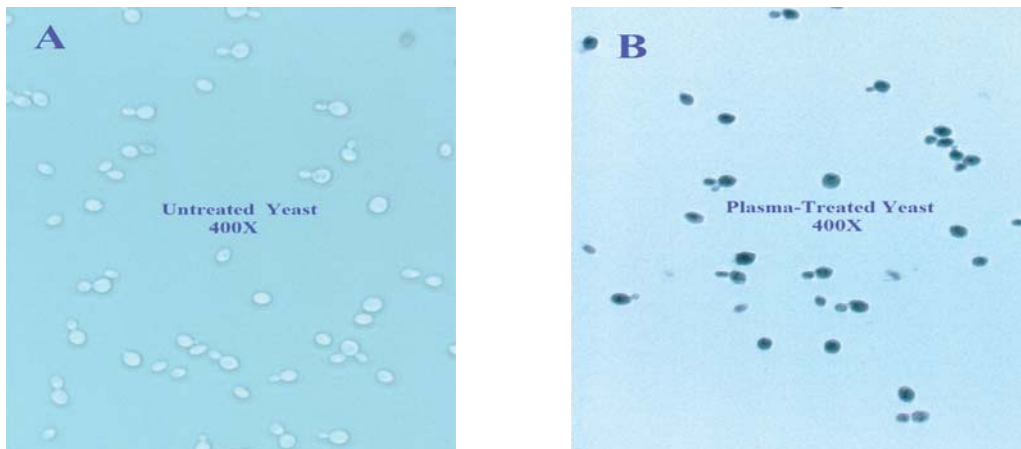
Yeast were directly exposed to plasma in dry air at different cell densities in suspensions of 5 and 10 mL for 0–60 sec. At cell densities of  $10^5/\text{mL}$ , yeast survival was 0% at all exposure times for a 5-mL suspension volume. In 10-mL suspensions, survival was ~3% after 15 s exposure. At exposure times > 15 s, no cells survived (data not shown). At cell densities of  $10^7/\text{mL}$ , surviving cells were found at all exposure times. Survival rates were higher in 10- compared to 5-mL suspension volumes and decreased with exposure times (Fig. 2). The ability of plasma to kill yeast cells is influenced by initial cell density and suspension volumes. The loss of viability due to plasma as determined by plate counts was independently confirmed by vital staining of untreated and plasma-treated yeast with Trypan blue, which stains dead, but not live, cells. Following plasma treatment, yeast cells were stained by the dye; in contrast, untreated (i.e., live) cells remained unstained (Fig. 3).

### B. Plasma Gas

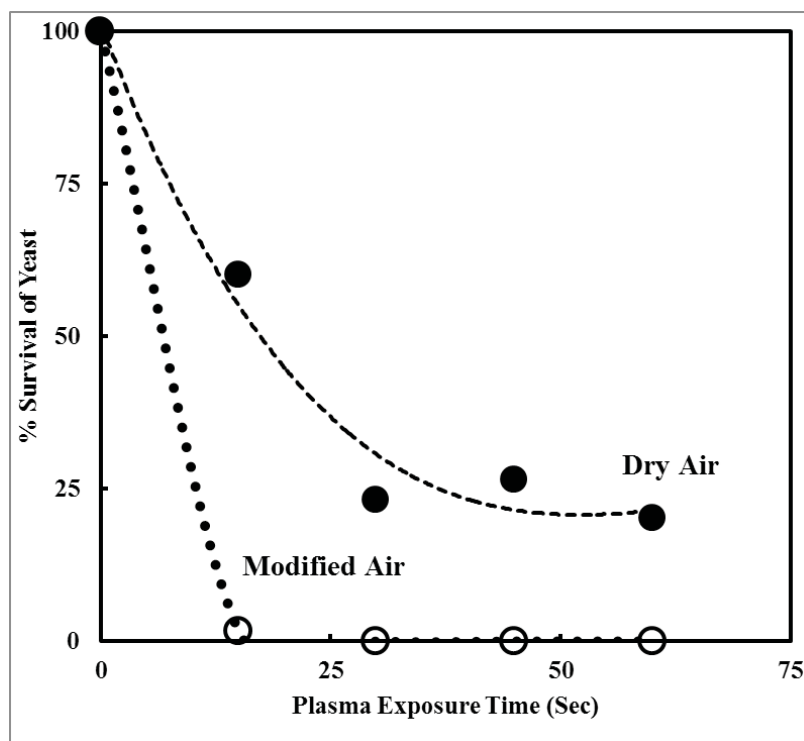
Figure 4 compares the effect of plasma generated in MA65 and dry air on yeast cells. The data show that plasma is more effective in killing yeast in MA65 than in dry air. The RGS responsible for killing cells includes ozone ( $\text{O}_3$ ), nitrous oxides ( $\text{NO}_x$ ),  $\text{H}_2\text{O}_2$ , etc.,



**FIG. 2:** Effect of suspension volume on viability of yeast exposed to HVACP. Plasma exposure was direct at 18 kV, and initial cell densities were  $8.8E + 06$  CFU/mL.



**FIG. 3:** Untreated (A) and plasma-treated (B) yeast cells stained with aqueous 0.4% Trypan Blue. Plasma treatment was direct for 30 s at 60 kV, initial cell density was  $8.50E + 06$ , and survival of the cells after plasma treatment was 0%.



**FIG. 4:** Viability of yeast exposed to HVACP in dry air and modified air (65% O<sub>2</sub>, 30% CO<sub>2</sub>, and 5% N<sub>2</sub>). Plasma exposure was direct at 18 kV, suspension volumes were 10 mL, and initial cell densities were 8.8E + 06 and 6.6E + 06 CFU/mL for yeast in dry air and modified air, respectively.

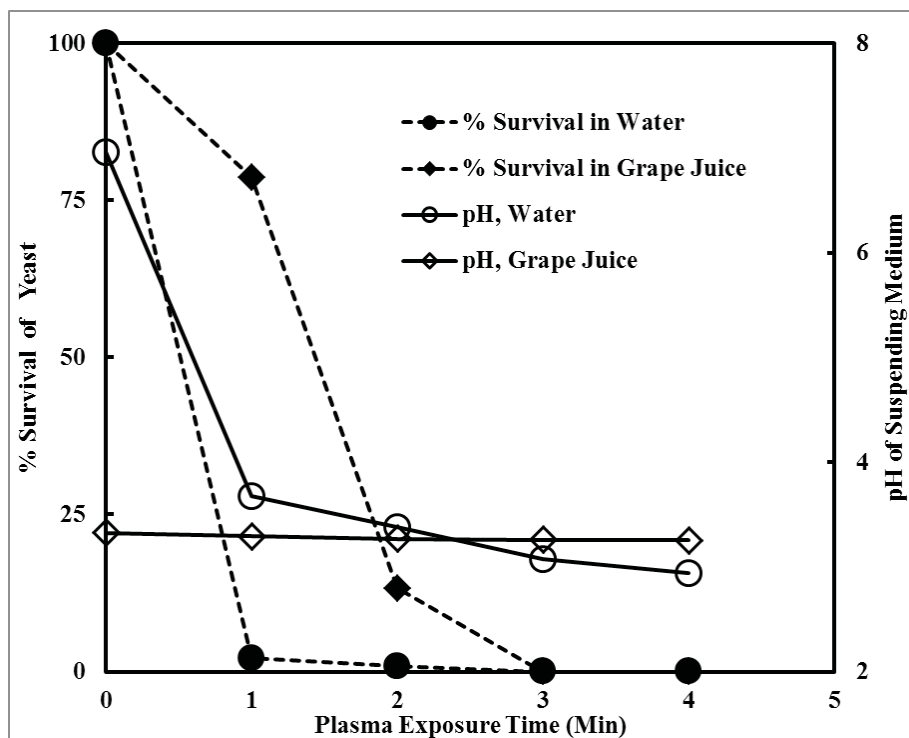
that can cause lipid peroxidation, enzyme inactivation, DNA cleavage, and cell leakage by electroporation, leading to cell death.<sup>11</sup> Because these species are more abundant in plasma from MA65 due to its high oxygen content (65%), the enhanced effect on yeast is to be expected.<sup>12,13</sup>

### C. Suspension Media

The effect of plasma was tested on yeast suspended in both water and grape juice. The latter contains numerous organic compounds, the most abundant being soluble sugar, which can be as high as 20%.<sup>14,15</sup> It was of interest to test the killing power of plasma in a suspending medium with a high organic “load” and to determine how effective plasma would be in eliminating yeast contaminants in fruit juices. These are preserved by pasteurization and the use of preservatives, and both can negatively impact juice color and organoleptic properties.<sup>6</sup> Removing yeast contaminants using nonthermal or chemical approaches such as HVACP would eliminate this problem. The use of atmospheric cold plasma for juice preservation has been tested by others.<sup>16,17</sup>

None of the yeast survived exposure to plasma at 80 kV, regardless of the suspending solution (Fig. 5). However, whereas all of the yeast were killed after ~1 min in water, exposure to plasma for 3 min was required to kill all of the yeast in juice suspensions. In both environments, killing is due to the damaging effects of RGS; however, a longer plasma exposure time was required to kill all of the yeast in grape juice.

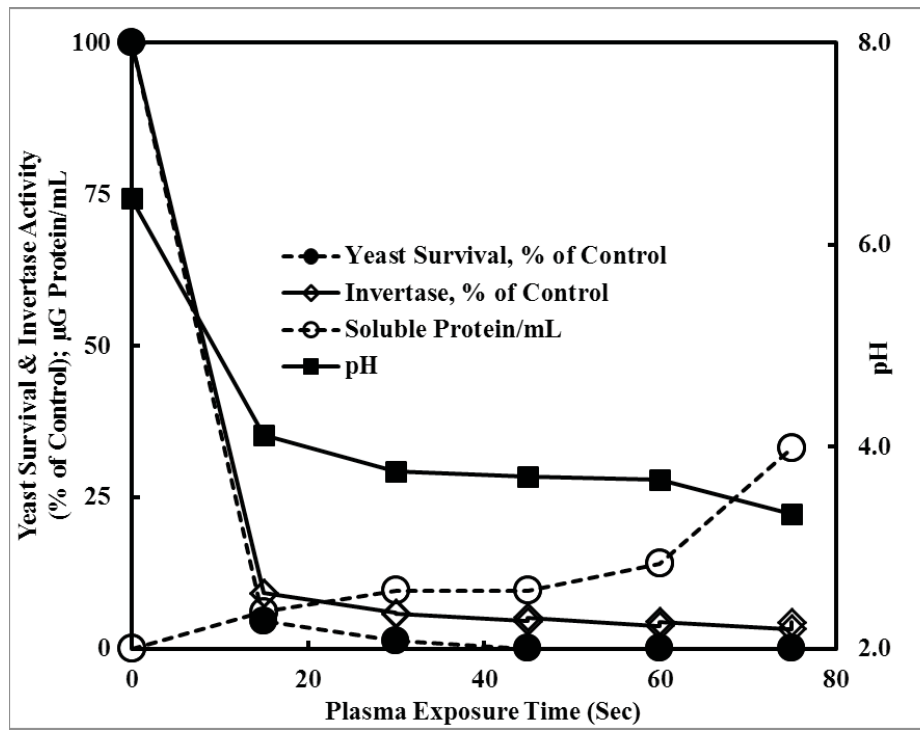
Figure 5 also shows that plasma treatment decreased the pH of the yeast-water suspension from 6.96 to 2.94, possibly due to formation of peroxyntitrous acid.<sup>18</sup> Acidification was also observed in other trials (Fig. 6) and may contribute to the lethality of plasma. In contrast, the normal pH of the juice suspension (~3.3) remained stable at all exposure times, possibly due to the buffering capacity of organic acids in grape juice.<sup>19,20</sup>



**FIG. 5:** Effect of HVACP on viability of yeast in water and white grape juice. Plasma exposure was direct at 80 kV in dry air, and initial cell densities were  $5.22 \times 10^6$  and  $6.30 \times 10^6$  CFU/mL for water and grape juice, respectively.

#### D. Yeast Cellular Enzymes and Cell Structure

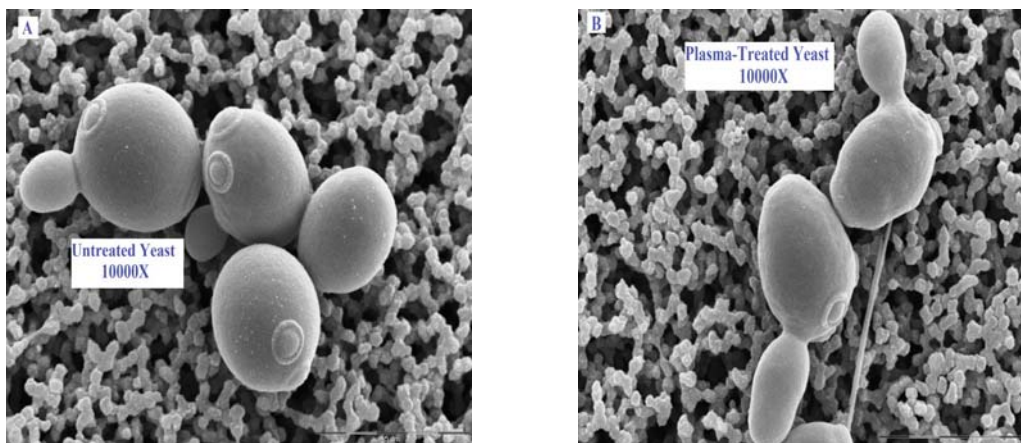
We examined the effect of plasma on yeast invertase. This enzyme is localized in the periplasmic space of the cell, that is, between the cell membrane and cell wall. Invertase is a glycoprotein consisting of ~50% mannan with a molecular weight of ~270 kDa.<sup>21,22</sup> Yeast



**FIG. 6:** Effect of HVACP on viability of yeast and activity of cell-wall-bound yeast invertase. Plasma exposure was direct at 60 kV in dry air, and initial cell density was  $6.58 \times 10^6$  CFU/mL.

were treated with plasma for varying exposure times and voltages, after which invertase activity in the intact cells was measured. Under the treatment conditions, essentially all of the yeast were killed within 30 s at 60 kV (Fig. 6). The plasma also reduced the pH of the yeast suspension and caused the release of soluble protein from the treated cells. In addition, cell-wall-bound invertase was also affected by plasma. After 15 s of exposure, > 90% of the invertase was inactivated; maximum loss of activity (> 96%) occurred after 75 s of exposure. In other trials, yeast viability and survival of invertase were higher with plasma at lower voltages (data not shown). These results show that plasma can denature enzymes in intact yeast cells. The ability of plasma to inactivate enzymes, which has been demonstrated by others,<sup>23,24</sup> can be attributed to the loss of protein's secondary structure by interaction with plasma RGS. It is a likely contributor to yeast cell death.

Figure 7 shows SEM of yeast cells. Untreated yeast cells have a characteristic smooth, elliptical shape. However, cells exposed to plasma are misshapen and have a slightly elongated and somewhat “wrinkled” appearance, suggestive of damage to the cell wall. The observation that plasma causes the release of soluble protein (Fig. 6) and DNA (data not shown) from yeast suggests that damage to cell membranes also contributes to loss of viability.



**FIG. 7:** SEM of untreated (A) and plasma-treated (B) yeast. Plasma treatment was direct for 75 s at 18 kV, initial cell density was  $7.13\text{E} + 06$  CFU/mL in 10 mL of DI water, and survival after plasma treatment was 19.2%.

Other investigators have demonstrated the ability of plasma to kill yeast.<sup>25,26</sup> Misra et al.<sup>26</sup> reported a greater than three-fold  $\log_{10}$  reduction of yeast-mold counts on plasma-treated strawberries. Plasma has been shown to kill the yeast *Candida albicans*, possibly by inhibiting the synthesis of ergosterol, a component of yeast cell membranes.<sup>27,28</sup> In the same organism, plasma was shown to damage the cell surface and disrupt membrane integrity, resulting in the leakage of protein and nucleic acid.<sup>29</sup> Consistent SEM data are presented in our observations of protein and DNA release from *S. cerevisiae*. Ma et al.<sup>30</sup> presented evidence that plasma causes the accumulation of reactive oxygen species and  $\text{Ca}^{++}$  in yeast, resulting in mitochondrial dysfunction and DNA fragmentation.

#### IV. CONCLUSIONS

The data presented here show that yeast cells are killed by exposure to HVACP. The effect of plasma is a function of applied voltage, treatment gas, and exposure time. Other parameters that affect viability during plasma treatment include yeast cell density and suspension volume. Cell-wall-bound invertase is inactivated by plasma, demonstrating the latter's ability to denature protein. Plasma was effective in eliminating yeast in grape juice suspensions, demonstrating that it can be used for extending the shelf life of beverages prone to spoilage by yeast.

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## REFERENCES

1. Niemira BA. Cold plasma decontamination of foods. *Ann Rev Food Sci Technol*. 2012;3(1):125–42.
2. Misra NN, Pankaj SK, Walsh T, O'Regan F, Bourke P, Cullen PJ. In-package nonthermal plasma degradation of pesticides on fresh produce. *J Haz Mat*. 2014;271:33–40.
3. Hoffman C, Berganza C, Zhang J. Cold atmospheric plasma: Methods of production and application in dentistry and oncology. *Med Gas Res*. 2013;3:21–35.
4. Pankaj SK, Bueno-Ferrer C, Misra NN, Milosavljević V, O'Donnell CP, Bourke P, Keener KM, Cullen PJ. Applications of cold plasma technology in food packaging. *Trends Food Sci Technol*. 2014a;35(1):5–17.
5. Pankaj SK, Bueno-Ferrer C, Misra NN, Bourke P, Cullen PJ. Zein film: Effects of dielectric barrier discharge atmospheric cold plasma. *J Appl Polymer Sci*. 2014;131:40803.
6. Gunes G, Blum LK, Hotchkiss, JH. Inactivation of yeast in grape juice using a continuous dense phase carbon dioxide processing system. *J Sci Food Agric*. 2005;85:2362–8.
7. Shinohara T, Kubodera S, Yanagida F. Distribution of phenolic yeasts and production of phenolic off-flavors in wine fermentation. *J Biosci Bioeng*. 2000;90(1):97–9.
8. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano EK, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem*. 1987;163(1):279.
9. Silveira MCF, Carvajal E, Bon EPS. Assay for in vivo yeast invertase activity using NaF. *Anal Biochem*. 1996;238:26–8.
10. Nelson N. A photometric adaption of the Somogyi method for determination of glucose. *J Biol Chem*. 1944;53:375–80.
11. Han L, Patil S, Boehm D, Milosavljević V, Cullen P, Bourke P. Mechanisms of inactivation by high-voltage atmospheric cold plasma differ for *Escherichia coli* and *Staphylococcus aureus*. *Appl Environ Microbiol*. 2016;82:450–8.
12. Lu H, Patil S, Keener KM, Cullen PJ, Bourke P. Bacterial inactivation by high-voltage atmospheric cold plasma: Influence of process parameters and effects on cell leakage and DNA. *J Appl Microbiol*. 2013;116:784–94.
13. Wan Z, Chen Y, Pankaj SK, Keener, KM. High voltage atmospheric cold plasma treatment of refrigerated chicken eggs for control of *Salmonella enteritidis* contamination on egg shell. *Food Sci Technol*. 2017;76:124–30.
14. Sani AM. Determination of grape juice concentrate composition. *Nutr Food Sci*. 2013;143(5):462–6.
15. Miele A, Rizzon LA, Nascimento SC, Gianello C. Physicochemical composition, minerals, and pesticide residues in organic grape juices. *Food Sci Technol (Campinas)*. 2015;35(1):120–6.
16. Almeida FDL, Cavalcante RS, Cullen PJ, Frias JM, Bourke P, Fernandes FAN, Rodrigues S. Effects of atmospheric cold plasma and ozone on prebiotic orange juice. *Innov Food Sci Emer Tech*. 2015;32:127–35.
17. Bursać Kovačević D, Putnik P, Dragović-Uzelac V, Pedišić S, Režek Jambrak A, Herceg Z. Effects of cold atmospheric gas phase plasma on anthocyanins and color in pomegranate juice. *Food Chem*. 2016;190:317–3.
18. Naitali M, Kamgang-Youbi G, Herry J-M, Bellon-Fontaine M-N, Brisset J-L. Combined effects of long-living chemical species during microbial inactivation using atmospheric plasma-treated water. *Appl Environ Microbiol*. 2010;76(22):7662–4.
19. Mato I, Suarez-Luque S, Huidobo JF. A review of the analytical methods to determine organic acids in grape juices and wines. *Food Res Int*. 2005;29:1175–88.

20. Mato I, Suarez-Luque S, Huidobo JF. Simple determination of main organic acids in grape juice and wine by using capillary zone electrophoresis with direct UV detection. *Food Chem.* 2007;102:104–12.
21. Gascon S, Neumann NP, Lampen JO. Comparative study of the properties of the purified internal and external invertases from yeast. *J Biol Chem.* 1968;243:1573–7.
22. Chu FK, Waterik W, Maley F. Factors affecting the oligomeric structure of yeast external invertase. *Arch Biochem Biophys.* 1983;223:543–55.
23. Pankaj SK, Misra NN, Cullen PJ. Kinetics of tomato peroxidase inactivation by atmospheric pressure cold plasma based on dielectric barrier discharge. *Innov Food Sci Emer Tech.* 2013;19:153–7.
24. Misra NN, Pankaj SK, Segat A, Ishikawa K. Cold plasma interactions with enzymes in foods and model systems. *Trends Food Sci Technol.* 2016;55:39–47.
25. Misra NN, Patil S, Moiseev T, Bourke P, Mosnier JP, Keener KM, Cullen PJ. In-package atmospheric pressure cold plasma treatment of strawberries. *J Food Eng.* 2014;125:131–8.
26. Misra NN, Moiseev T, Patil S, Pankaj SK, Bourke P, Mosnier JP, Keener KM, Cullen PJ. Cold plasma in modified atmospheres for post-harvest treatment of strawberries. *Food Bioproc Technol.* 2014;7:3045–54.
27. Klämpfl TG, Isbary G, Shimizu T, Li Y-F, Zimmermann JL, Stolz W, Schlegel J, Morfill GE, Schmidt H-U. Cold atmospheric air plasma sterilization against spores and other microorganisms of clinical interest. *Appl Environ Microbiol.* 2012;78:5077–82.
28. Rahimi-Verki N, Shapoorzadeh A, Razzaghi-Abyaneh M, Atyabi S-M, Shams-Ghahfarokhi M, Jahanshahi Z, Gholami-Shabani M. Cold atmospheric plasma inhibits the growth of *Candida albicans* by affecting ergosterol biosynthesis and suppresses the fungal virulence factors in vitro. *Photodiag Photodyn Ther.* 2016;13:66–72.
29. Kvam E, Davis B, Mondello F, Garner AL. Nonthermal atmospheric plasma rapidly disinfects multidrug-resistant microbes by inducing cell surface damage. *Antimicrob Agent Chemother.* 2012;56:2028–36.
30. Ma RN, Feng HQ, Liang YD, Zhang Q, Tian Y, Su B, Zhang J, Fang J. An atmospheric-pressure cold plasma leads to apoptosis in *Saccharomyces cerevisiae* by accumulating intracellular reactive oxygen species and calcium. *J Phys D: Appl Phys.* 2013;46:1–8.