Printed ISSN 1330–0008
Online ISSN 1333–9125
CD ISSN 1333–8390
CODEN FIZAE4

DIRECT AND INDIRECT PLASMA YEAST STERILIZATION

NASSER N. MORGAN[a], ASEM METAWA[a] and ABDOU A. GARAMON[b]

[a]*Physics Department, Faculty of Science (male), Al-Azhar University, Nasr City, Cairo, Egypt*

[b]*Center of Plasma Technology, Al-Azhar University, Nasr City, Cairo, Egypt*

Received 23 November 2009; Revised manuscript received 21 March 2010
Accepted 2 June 2010 Online 12 July 2010

Atmospheric-pressure parallel-plate dielectric barrier discharge has been designed for sterilization of Saccharomyces Cerevisiae and Candida yeasts. Oxygen has been used as the input working gas. The output gas after discharge operation was a mixture of both ozone and oxygen, with concentration that depends on the applied voltage between the electrodes, gap space and gas flow rate. Sterilization process has been done in two ways, by direct exposure to oxygen plasma inside the discharge cell and by indirect exposure to the plasma. Survivor curves, and scanning and transmission electron microscope were used to study the inactivation kinetics and morphology of the yeast surface before and after sterilization. It has been found that the indirect sterilization causes yeast inactivation in a short time, less than three minutes, while the direct sterilization took place in a longer time, more than ten minutes.

PACS numbers: 52.80.Dy, 52.75.Rx UDC 537.523, 533.924, 535.682

Keywords: atmospheric pressure discharge in oxygen, sterilization of yeast, exposure inside the discharge cell, indirect exposure, survivor curves, inactivation kinetics

1. Introduction

Recently atmospheric pressure dielectric barrier discharge (DBD) became one of the most important types of plasma used in chemical, industrial, biological and medical applications [1 – 5]. Ozone synthesis, hydrogen production, pollution control, removal of nitrogen oxides and control of automotive exhaust gases and conversion of green house gases are the most important chemical applications of DBD. Surface treatment of polymers, textiles, and wool are some of industrial applications of DBD. Sterilization of bacteria, fungis, virus and yeasts are some of the biological applications [6 – 8].

DBD is one of the non-equilibrium types of cold plasma which provides electron energies much higher than that of the ions and the neutral species. DBD reactor is one of the non-equilibrium cold plasma techniques. These plasmas exhibit electron energies much higher than those of the ions and the neutral species. The energetic electrons collide with the background gas, causing enhanced level of dissociation, excitation, and ionization. The ions and the neutrals inside the plasma remain relatively cold, so the plasma does not cause any thermal damage to surfaces it comes in contact with.

Dielectric barrier discharge is produced between two parallel electrodes of metal with at least one electrode covered with a dielectric material, e.g. glass, mica or ceramic materials. When a high ac voltage is applied between the electrodes, the discharge process will take place in the gap between the electrodes. DBD is a highly transient, low-temperature non-equilibrium discharge, operating under a broad range of pressures, from low pressure up to more than atmospheric pressure. DBD provides high-energy electrons (1 – 10 eV), able to produce ions, radicals and excited species in the working gas between the electrodes.

Dielectric barrier discharges at elevated pressure can be characterized by a large number of short-lived micro-discharges, that have been investigated experimentally as well as theoretically by many authors [9 – 15].

Recently, substantial amounts of research have been focused on sterilization using DBD plasma. During the DBD treatment, photons, electrons, ions and active chemical species from the plasma reach the surface of a biologically contaminated object and can eventually lead to its sterilization [6,8,16 – 20]

Plasma sterilization process was done in two ways:

1) Direct exposure to oxygen plasma inside the discharge cell. The samples were suspended on sterilized cover slides and inserted inside the discharge zone, subjected to all possible agents generated by the plasma including charged particles [20, 21], reactive species [22] and electromagnetic radiation (such as ultraviolet photons) [22 – 24].

2) Indirect exposure, in which the sample does not come in direct contact with the plasma. In this method, the yeasts are placed outside the discharge region and the charged particles do not affect the sample under treatment as they recombine before reaching it. In addition, the heat flux and the possible radiation flux are greatly reduced. This leaves mainly the long-lived radicals like ozone to directly interact with the biological sample [25].

The aim of this work is to compare the effect of direct and indirect plasma sterilization techniques on Saccharomyces Cerevisiae and Candida yeasts with oxygen as working gas. Survivor curves for the treated yeast and plots of the number of colony-forming units (CFUs) per unit volume versus treatment time are plotted on a semi-logarithmic scale with the CFUs on the logarithmic vertical scale and time on the linear horizontal scale. Scanning electron microscope (JSM-5600LV) and transmission electron microscope (JEOL 1010) have been used to study the

surface morphology and cell damage mechanism for the exposed and unexposed sample that help in understanding the mechanism of inactivation.

2. Experimental setup

A parallel plate DBD reactor has been designed with two rectangular stainless steel plates, one of them covered with a Pyrex glass plate 1.2 mm thick, that was cohesively pasted to the electrode (see Fig. 1). The area of each electrode was 20 cm × 10 cm and the gap space between them was varied from 2, 2.5 to 3 mm. The upper electrode was connected to the high-voltage terminal step-up transformer (1 – 30 kV) operated at 50 Hz. The system has been described in details elsewhere [26].

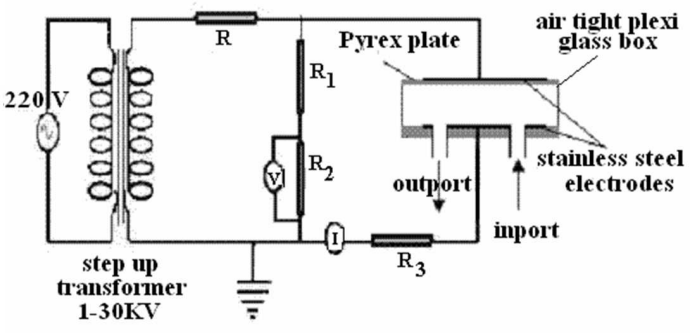

Fig. 1. Experimental setup used for the sterilization process.

The working gas (oxygen: commercial grade of purity of 99%) was injected through the inlet port of the plexi-glass box through a hole of the lower electrode, with flow rate of 0 – 1 l/min, and the exhaust gas was getting out from the exit port of the box through another hole in the lower electrode. Ozone concentration was measured using ozone detector (Model H1-AFX-Instrumentation, USA), that was connected directly to the outlet port.

3. Results and discussion

3.1. Direct sterilization

Saccharomyces Cerevisiae and Candida were isolated and cultivated in yeast mold broth medium. Sterilized cover slides were immersed in the solution to make a thin layer of the yeast. Slides were inserted inside the discharge zone of the DBD discharge cell and were exposed to oxygen plasmas for different exposure times under different discharge current at constant flow rate. The treated cover-slides were put in yeast mold agar medium poured in sterilized Petri dishes followed by adding of 0.1 ml of saline solution (6 g/l) to distribute the treated yeast cells in the dishes. Total viable count method was used to compare between control samples

and the plasma treated samples. Figures 2a and 2b illustrate the survival curves for the exposed yeast for different exposure times at discharge currents of 1 mA, 0.8 mA and 0.6 mA and the gap space of 2 mm.

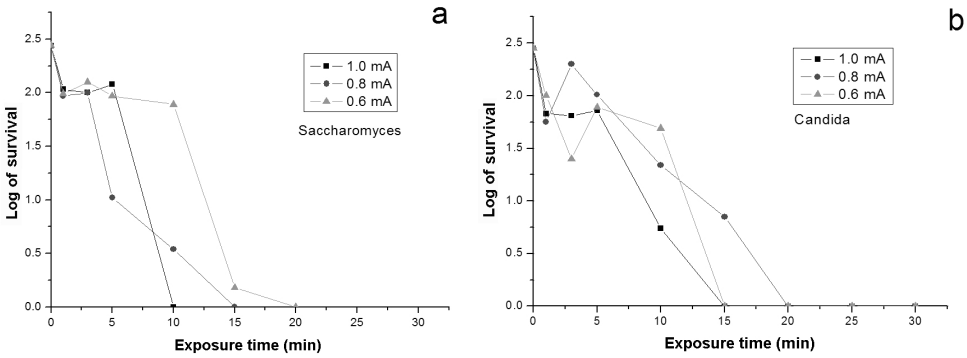

Fig. 2. Survival curve for (a) Saccharomyces cerevesiae (b) Candida in oxygen plasma at different exposure time.

There are two main hypotheses for the mechanisms of the cell death caused by gas discharge, both involving lethal damage to the cell membrane structures and ultimately leading to leakage of cyptoplasmic contents or lyses.

1) Electrostatic disruption mechanism suggests that the total electric force caused by accumulation of surface charge could exceed the total tensile force on the membrane [19].

2) Damage of the cell membrane or cellular components are suggested to be caused by energetic ions, radicals and reactive species generated inside the discharge reactor [19, 20].

The survivor curves shown in Figs. 2a and 2b show two phases of the inactivation process, the first proceeds slowly in which the active species react with the outer membrane of the cells causing wall damaging and the second phase proceeds quickly causing cell death.

The inactivation mechanism in oxygen DBD plasma might be attributed to:

1) Inactivation by active species contained in oxygen plasma, including atomic oxygen, and ozone those may cause oxidation of DNA and protein.

2) Inactivation by ion-etching (O^+,O^{+2}).

Scanning electron microscope (JSM-5600LV) and transmission electron microscope (JEOL 1010) were used to investigate the effect of oxygen plasma on the internal and external structure of the S. Cerevesiae yeast as shown in Figs. 3a, b, c and d. SEM in Fig. 3a for the control sample shows small elliptical cells that can appear round or ovoid in shape. The exposed yeast, with exposure time 10 min

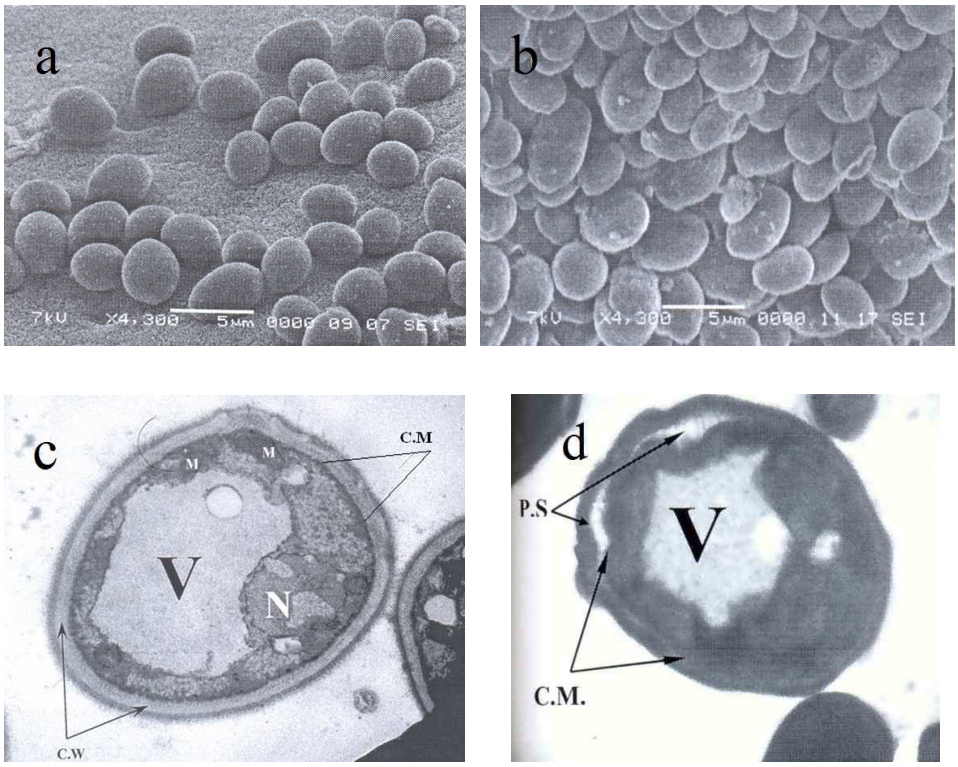

Fig. 3. SEM and TEM of S. Cerevisiae yeast in oxygen plasma. (a) SEM cionrole (b) SEM 10 min. *exposure (c) TEM control (d) TEM* 10 min. *exposure C.W: cell wall, C.M: cell membrane, N: nucleus, M: mitochondria, P.S preplasmic space, V: vacuole.*

inside the discharge zone, shows little morphological changes on the yeast surface after plasma exposure. Some cells become enlarged, few cells have been ruptured. TEM in Fig. 3c for the control sample shows the cell internal structure with double-layered cell wall (C.W), nucleus (N), contains the DNA and an inner organelle. The cell also contains a large storage vacuole. Other visible structures are mitochondria and the endoplasmic reticulum. The cell wall, composed of proteins and polysaccharides, gives the yeast cell its shape, and acts as a physical and chemical barrier. The TEM of the exposed yeast shown in Fig. 3d shows irregular cell membrane (C.M.) that seems to be dissociated from the cell wall (C.W.), resulting in damage of the cellular components caused by energetic ions, radicals and reactive species generated inside the discharge reactor. The mitochondria were affected by the discharge component and become darker than the control sample, resulting from some types of chemical reactions that took place inside the cell, leading to formation of organic residue or salts.

The effect of oxygen plasma on the internal and external structure of the Can-

dida yeast is shown in Figs. 4a, b, c and d. The cells have been exposed for 15 minutes inside the discharge zone. SEM of the exposed cells in Fig. 4b shows large morphological changes on the yeast surface, and the erosion and rupturing of the cell wall are clearly observed, while some cells lost all of their content. The charged particles inside the plasma play a very significant role in the rupturing of the outer membrane of the cell. The electrostatic force caused by charge accumulation on the outer surface of the cell membrane could overcome the tensile strength of the membrane and cause rupturing. Another possibility of rupturing may be attributed to the reactive species inside the plasma like oxygen atoms and hydroxyl groups. TEM of the exposed Candida yeast presented in Fig. 4d shows a complete separation between the cell wall and cell membrane. The cell wall was seriously damaged; it appears lighter than of the non-exposed samples, lost its rigidity, causing cells to lose their contents.

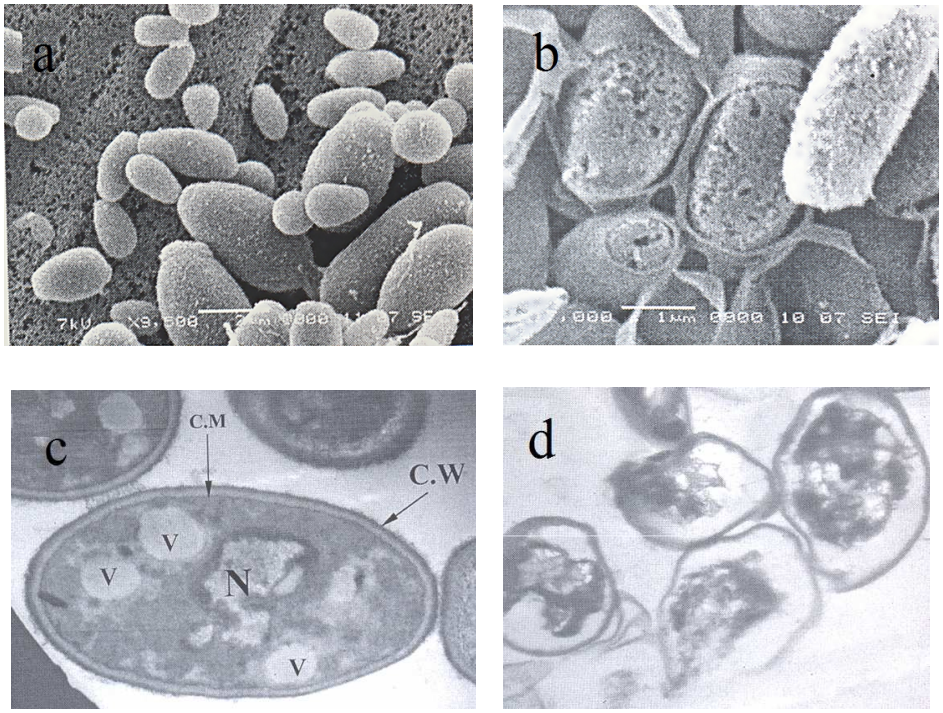

Fig. 4. SEM and TEM of Candida yeast in oxygen plasma before and after treatment (a) SEM control (b) SEM 15 min. *exposure (c) TEM control (d) TEM* 15 min. *exposure C.W: cell wall, C.M: cell membrane, N: nucleus.*

3.2. Indirect sterilization

Ozone and other gases resulting from the discharge process were transferred out of the discharge zone by a 50 cm plastic pipeline to a test tube containing saline

solution of the yeast. Ozone concentration was measured by ozone detector (model H1-AFX-Instrumentation, USA). It has been found that ozone concentration depends significantly on several parameters, including applied voltage, gas flow rate and the gap space between the electrodes.

Figure 5 shows the general behaviour of ozone concentration as a function of the applied voltage at different gas flow rates with the gap space of 3 mm.

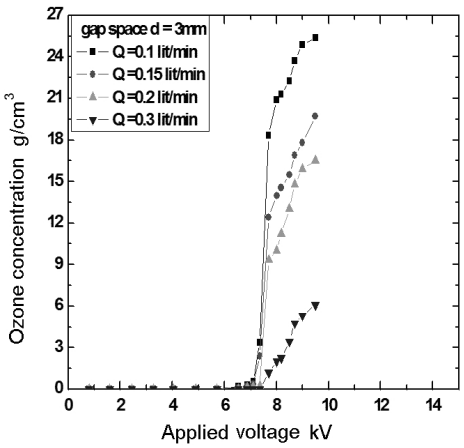

Fig. 5. Ozone concentration as a function of applied voltage at different gas flow rates.

Direct injection of ozone with different concentrations (15 g/m^3 and 10 g/m^3) inside a test tube that contained a saline solution of the yeast has been done for different times (0.5 – 4 min.). The inactivation took place in less than three minutes for both types of yeast for ozone concentration of 15 g/m^3, as shown in Figs. 6a and b.

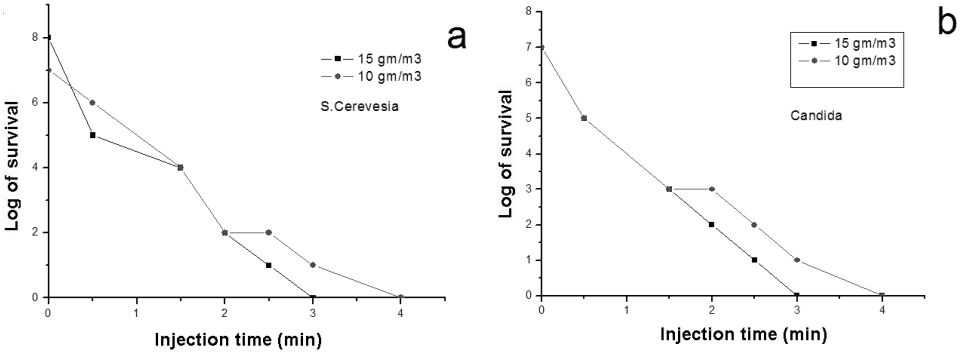

Fig. 6. Survival curve for (a) S. Cerevisiae (b) Candida yeast at ozone concentrations of 15 and 10 g/m^3 *for different injection times.* Only one phase can be noticed in the survivor curve of both types of yeast, as shown in Figs. 6a and b. SEM and TEM of the ozone treated S. Cerevisiae yeast

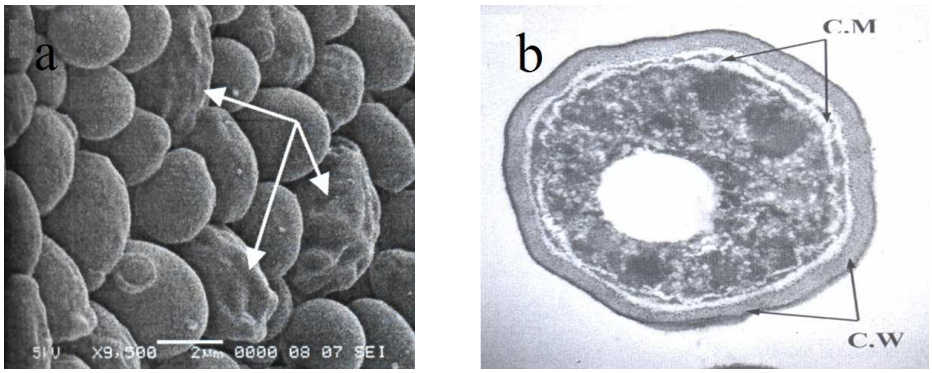

Fig. 7. (a) SEM and (b) TEM of S. Cerevisiae treated by ozone gas.

for three minutes are shown in Figs. 7a and b. SEM in Fig. 7a shows deformation in some cells. Wrinkled cell surfaces with much shrinkage in the cell dimensions are observed. TEM of the ozone treated cells shown in Figs. 7b shows wavy cell wall (more elastic) and irregular in shape. The cell membrane was seen to be separated from the cell wall what was not seen for the non-exposed cells.

Figures 8a and b show the SEM and TEM of Candida yeast treated with ozone for three minutes. Dramatic effect appears on all cell's morphology and ultra structure was greatly affected, while the cytoplasm lost it's homogeneity.

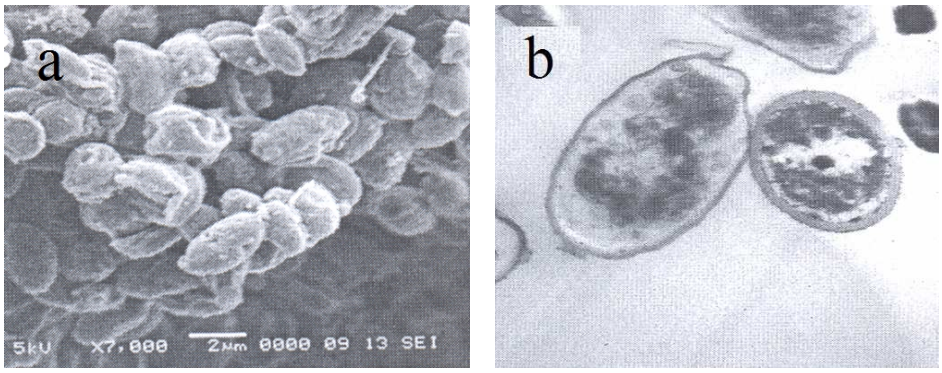

Fig. 8. (a) SEM and (b) TEM of Candida treated by ozone gas.

The different modes of action of ozone on a living organism involve the production of peroxides. The peroxides are responsible for the remarkable bactericidal and fungicidal effects of ozone. The inactivation of both types of yeast by ozone may be attributed to the oxidation of DNA and proteins or due to their interference with cellular respiration.

4. Conclusion

A low-cost atmospheric-pressure electric barrier discharge has been successfully used for yeast inactivation. The inactivation process was done using two methods, the direct and the indirect method. The direct method, offering the insertion of slides covered with saline solution of the treated yeast in the discharge cell and its exposure to various types of reactive species, including atomic oxygen, oxygen ions and ozone. Damaging of the cell membrane or cellular components is suggested to be caused by energetic ions, radicals and other reactive species generated inside the discharge zone. In the indirect method, ozone generated in the discharge cell was directed via a plastic pipeline to a test tube that contained the saline solution of the yeast. The different modes of action of ozone on a living organism involve the production of peroxides. The peroxides are responsible for the remarkable bactericidal and fungicidal effects of ozone. The inactivation of both types of yeast by ozone may be attributed to the oxidation of DNA and proteins or due to its interference with cellular respiration. The indirect method was found to be more efficient than the direct one since complete inactivation took place in three minutes while the direct sterilization needs fifteen minutes. The survivor curve for the yeast under direct inactivation shows two phases of the inactivation process, while the yeast treated by the indirect method shows only one phase.

References

[1] U. Kogelschatz, B. Eliasson and W. Egli, Pure Appl. Chem. **71** (10) (1999) 1819.

[2] U. Kogelschatz, Chemistry and Plasma Processing **23** (1) (2003) PAGE?

[3] B. Eliasson, M. Hirth and U. Kogelschatz, J. Phys. D: Appl. Phys. **20** (1987) 1421.

[4] B. Eliasson and U. Kogelschatz, IEEE Trans. Plasma Sci. **19** (1991) 309.

[5] Z. Falkenstein, Proc. 12th Int. Conf. on High-Power Particle Beams **1** (1998) 117.

[6] M. Laroussi, IEEE Trans. Plasma Sci. **30** (4) (2002) 1409.

[7] E. Forde, I. Guiney, K. Arshak, C. Adley, C. Barry and K. Jordan, IEEE Trans. Plasma Sci. **36** (2008) 2805.

[8] G. Fridman, A. D. Brooks, M. Balasubramanian, A. Fridman, A. Gutsol, V. N. Vasilets, H. Ayan and G. Fridman, Plasma Proc. Polymers **4** (2007) 370.

[9] U. Kogelschatz, B. Eliasson and W. Egli, Pure Appl. Chem., **71** (10) (1999) 1819.

[10] U. Kogelschatz, *Dielectric-Barrier Discharges: Their History, Discharge Physics and Industrial Applications*, Plasma Chemistry and Plasma Processing Vol. 23, No.1.

[11] B. Eliasson, M. Hirth and U. Kogelschatz, J. Phys. D: Appl. Phys. **20** (1987) 1421.

[12] B. Eliasson and U. Kogelschatz, IEEE Trans. Plasma Sci. **19** (1991) 309.

[13] D. Braun, U. Küchler and G. Pietsch, J. Phys. D: Appl. Phys. **24** (1991) 564.

[14] D. Braun, V. Gibalov and G. Pietsch, Plasma Sources Sci. Technol. **1** (1992) 166.

[15] F. Massines, P. Segur, N. Gherardi, C. Khamphan and A. Richard, Surf. Coat. Technol. **174 – 175** (2003) 8.

[16] X. M. Shi, G. J. Zhang, Y. K. Yuan, Y. Ma, G. M. Xu and Y. Yang, IEEE Trans. Plasma Sci. **36** (2008) 498.

[17] Y. Ma, G. J. Zhang, X. M. Shi, G. M. Xu and Y. Yang, IEEE Trans. Plasma Sci. **36** (2008) 1615.

[18] E. Forde, I. Guiney, K. Arshak, C. Adley, C. Barry and K. Jordan, IEEE Trans. Plasma Sci. **36** (2008) 2805.

[19] E. Stoffels, Y. Sakiyama and D. B. Graves, IEEE Trans. Plasma Sci. **36** (2008) 1441.

[20] M. Laroussi, A. Mendis and M. Rosenberg, New J. Phys. **5** (2003) p. 41.1.

[21] D. A. Mendis, M. Rosenberg, and F. Azam, IEEE Trans. Plasma Sci. **28** (4) (2000) 1304.

[22] M. Laroussi and F. Leipold, Int. J. Mass Spectrom. **233** (13) (2004) 81.

[23] M. Moisan, J. Barbeau, S. Moreau, J. Pelletier, M. Tabrizian and L. H. Yahia, Int. J. Pharm. **226** (1/2) (2001) 1.

[24] N. Philip, B. Saoudi, M. C. Crevier, M. Moisan, J. Barbeau and J. Pelletier, IEEE Trans. Plasma Sci. **30** (4) (2002) 1429.

[25] N. N. Morgan, M. A. Elsabbagh, S. Desoky and A. A. Garamoon, Eur. Phys. J. : Appl. Phys. **46** (2009) 31001.

[26] O. Minayeva and M. Laroussi, IEEE Int. Conf. Plasma Sci., Baltimore, MD (2004) p.12.

IZRAVNA I NEIZRAVNA STERILIZACIJA MIKROBA PLAZMOM

Sagradili smo posudu s usporednim pločama i dielektričnom pregradom za postizanje izboja na atmosferskom tlaku, namijenjenu za proučavanje sterilizacije mikroba Saccharomyces Cerevisiae i Candida. Radni plin bio je kisik. Izlazni plin iz posude bio je smjesa ozona i kisika, čija je koncentracija ovisna o vaponu izboja među elektrodama, procijepu i brzini protjecanja plina. Sterilizacija se provodila na dva načina, izravnim izlaganjem u izbojnoj posudi i neizravnim izlaganjem plazmi. Za proučavanje kinetike prekida aktivnosti stanične plazme i oblika mikrobne površine rabili smo krivulje preživjelih stanica prije i poslije sterilizacije. Ustanovili smo da neizravna sterilizacija dovodi do prekida aktivnosti mikroba u kratkom vremenu, manje od tri minute, dok je izravna sterilizacija trajala dulje, više od deset minuta.