Research Paper



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The Effects of Reactive Oxygen and Nitrogen Species (RONS) Produced by Surface Dielectric Barrier Discharge (SDBD) Non-Thermal Plasma with Treatment Time and Distance Variations to Kill *Escherichia coli*

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Abstract

Research on the inactivation of *Escherichia coli* causing diarrheal disease using non-thermal plasma SDBD has been carried out. SDBD is a new technique for non-thermal plasma generation with several advantages: low power generation, comprehensive treatment area coverage, and reducing the potential effects of burning and drying tissue. This study aimed to analyze the effect of treatment time variations, namely 0 as control, 60, 75, 90, 105, and 120 seconds and treatment distance variations of 3, 6, 9, 12, and 15 mm of non-thermal plasma treatment of SDBD on *E. coli*. The results of the non-thermal plasma SDBD treatment with variations in time and distance showed that the longer the treatment time, the more bacterial cells died. Colony counts decreased to 4.33×10^7 CFU/mL compared to the control, 409×10^7 CFU/mL, with a treatment time variation of 120 seconds, yielding the best treatment results. At the same time, the greater the bacterial death rate, with the best treatment results at a 3 mm treatment interval, with colony counts of 8×10^7 CFU/mL, compared to 409×10^7 CFU/mL in control. Based on these results, SDBD non-thermal plasma treatment can be used to inactivate or kill bacteria with effectiveness in killing bacteria depending on the length of treatment time and the distance of treatment.

Keywords

Non-thermal Plasma, SDBD, E. Coli, RONS, Treatment Time, Treatment Distance

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1. INTRODUCTION

Infectious diseases are one of significant health problems in almost all developing countries, including Indonesia. One of the most common infectious diseases is diarrhea. According to Ragil and Dyah (2017), diarrhea is one of the leading causes of illness and death in almost all geographic areas, and all age groups can be affected. In Indonesia, diarrheal disease is a potential endemic disease of Extraordinary Events (KLB), and it is frequently fatal. According to Kemenkes RI (2018), the number of sufferers of diarrheal disease based on the results of the diagnosis by health workers was 6.8%, while based on the symptoms experienced, 8%. The number of patients with diarrheal diseases classified according to age found that the age group of 1 to 4 years had the highest number of sufferers, namely 11.5%. In addition, the age group of 75 years and over has a relatively high number of sufferers, namely 7.2% (Prabhakara, 2010). Microorganisms that generally cause disease are called pathogens. Pathogens include bacteria, protozoa, viruses, prions, fungi, and worms. These pathogens can cause various symptoms and diseases, including diarrhea (Levy et al., 2018). Pathogens that cause diarrheal disease can come from viruses, for example, *Rotavirus* (40-60%), *Escherichia coli* (20-30%), *Shigella sp.* (1-2%), and the parasite *Entamoeba histolytica* (Ragil and Dyah, 2017).

Several conventional sterilization techniques have been developed to inactivate disease-causing microorganisms, such as sterilization using dry heat (oven), moist heat (autoclaving), and chemicals such as glutaraldehyde and the use of gamma irradiation (Moisan et al., 2001; Morent and De Geyter, 2011; Park et al., 2003). This conventional technique that has been used has several disadvantages, such as high processing temperatures, long sterilization times, and the use of toxic chemicals, resulting in changes to the material during sterilization, and this technique is quite expensive to use (Moisan et al., 2001; Morent and De Geyter, 2011). Based on the limitations of conventional methods, it is necessary to have a new and alternative

sterilization method, namely pretreatment with non-thermal plasma (plasma sterilization) (Hati et al., 2012).

Plasma is the fourth matter after solids, liquids, and gases. Plasma is defined as an ionized gas of free particles (Putra et al., 2021; Scholtz et al., 2021; Šimončicová et al., 2019). Based on the temperature, plasma is divided into thermal plasma and non-thermal plasma (Morent and De Geyter, 2011). Cold or non-thermal plasma is produced by applying an electric or electromagnetic field to a gas. Plasma contains excited states of molecules and atoms, cations and anions, free radicals, electrons, UV radiation, ozone, superoxide, hydroxyl radicals, single oxygen, atomic oxygen, nitrogen oxides, or nitrogen dioxide (Puligundla and Mok, 2017; Zheng et al., 2016). These species exhibit antimicrobial activity against various microorganisms, including bacteria, yeasts, and even bacterial and fungal spores (López et al., 2019).

Surface dielectric barrier discharge is a new technique for non-thermal plasma generation with several advantages: low power generation, comprehensive treatment area coverage, and reducing the potential effects of burning and drying tissue. Several non-thermal plasma sterilization techniques have been carried out, but further research is needed to optimize nonthermal plasma techniques. Therefore, this study aimed to analyze the effect of treatment time and distance of non-thermal SDBD plasma treatment on *E. coli*.

2. EXPERIMENTAL SECTION

2.1 Materials

The non-thermal plasma system used in this study is a surface dielectric barrier discharge. The non-thermal plasma of the surface dielectric barrier discharge is generated using a 20 V DC voltage source which is then transformed into a high voltage source to create the plasma. The scheme used in this study is shown in Figure 1. The plasma non-thermal discharge surface dielectric barrier uses two copper electrodes. A dielectric separates two copper electrodes. The two electrodes are connected to a voltage source, with one electrode connected to the ground and the other connected to a high-voltage source. The sample used in this study was *E. coli* obtained from the microbiology laboratory, Faculty of Medicine, Brawijaya University. In addition, some materials, namely nutrient agar (NA), are used as media for bacterial growth and sterile physiological NaCl for dilution.

2.2 Methods

2.2.1 *E. coli* Sample Preparation and SDBD Non-thermal Plasma Treatment

E. coli isolates that had been incubated for 24 hours were diluted with serial dilutions up to 10^{-6} with one loop of bacterial isolates homogenized with 9 mL of sterile physiological NaCl $(10^{-1}$ dilution).1 mL of the 10^{-1} dilution of bacterial suspension was homogenized with 9 mL of sterile physiological NaCl $(10^{-2}$ dilution). This step was carried out until a dilution of 10^{-6} . The results of the dilution were then treated using nonthermal plasma with the surface dielectric barrier discharge



Figure 1. Schematic of SDBD Non-thermal Plasma

with variations in treatment time (0 as control, 60, 75, 90, 105, 120 seconds) and treatment distance (3 mm, 6 mm, 9 mm, 12 mm, 15 mm). The results of the treatment were then spidered using a triangular rod. After that, it was incubated at 37°C for 24 hours by placing the petri dish in an inverted position. The next step was to count the number of colonies in each treatment. Each experiment was repeated three times.

2.2.2 Optical Emission Spectroscopy

Optical emission spectroscopy (OES) was used to characterize reactive plasma species and to analyze plasma composition, which can explain the relationship or mechanism between reactive spaces formed in plasma and the ability to inactivate bacteria (Wiegand et al., 2014). The optical emission spectrum was measured using Aurora 4000 at a wavelength of 200 to 900 nm with an integration time of 5000 ms and 3 repetitions of the spectrum capture and then averaged to obtain the optical emission spectrum from the plasma. The emission spectrum obtained was then analyzed qualitatively to determine the chemical species at each wavelength peak. The results of identifying the wavelength peaks were then analyzed using the National Institute of Standards and Technology atomic spectrum database and previous journal publications to identify chemically active species (Sarangapani et al., 2016).

2.2.3 Data Analysis

The data obtained were based on the effect of treatment time (0, 60, 75, 90, 105, 120 seconds) and treatment distance (3, 6, 9, 12, 15 mm) on the number of bacteria, tabulated and analyzed using ANOVA using SPSS software. 26. If p-value < 0.05 H_0 was accepted, then the length of treatment time and treatment distance affect the number of bacteria.

3. RESULT AND DISCUSSION

3.1 Optical Emission Spectroscopy of SDBD Non-thermal Plasma

An optical emission spectrum (OES) can measure and analyze reactive species produced by non-thermal plasma. The spectrum results obtained can be analyzed for reactive species by looking at the spectrum peaks. In the following section, Fig-



Figure 2. OES Spectrum of SDBD Non-thermal Plasma with Treatment Time Variation and Treatment Distance Variation

ure 2 depicts the spectrum obtained from a surface discharge dielectric barrier non-thermal plasma.

Spectrum results were obtained using OES for each variation of treatment time and distance. The time variation is taken every 60 seconds, 90 seconds, and 120 seconds, while the distance variation is taken every 3 mm. 6mm, and 9mm. The spectrum results with time variations show the increasing intensity with longer treatment time. In contrast, for variations in the distance, the intensity will be smaller if the distance from the plasma source is further away.

The resulting spectrum was measured using OES at a wavelength of 200 to 900 nm from the plasma source. The intensity emitted from the plasma source is recorded at each wavelength. In the formation of plasma, various chemical species are in an excited state. The chemical species produced in the gas phase were observed using OES during plasma release (Sarangapani et al., 2016). In the UV region, the emission spectrum shows that N_2 and N_2^+ excitation species' emission shows different peaks. A small peaks of OH appear at wavelengths 296,1 nm (Adhikari et al., 2021; Dhungana et al., 2020; Hosseini et al., 2018; Naz et al., 2021; Sarangapani et al., 2016). The low intensity of singlet oxygen is at a wavelength of 777.5 nm (Sarangapani et al., 2016). At the same time, the N_2 Second Positive System (SPS) has a prominent peak at a wavelength of 313-390 nm, N₂ first negative system (FNS) at a wavelength of 390-450 nm (Akter et al., 2020; Misra et al., 2015). From the reactive species produced in the gas phase plasma, long-lived or short-lived reactive species such as hydrogen peroxide (H₂O₂) and ozone (O_3) are formed as long-lived reactive species, and short-lived reactive species such as hydroxyl radicals (•OH), singlet oxygen $(^{1}O_{2})$, superoxide anion (O_{2}^{-}) , atomic oxygen (O), nitrite oxide (NO), and peroxynitrite (ONOO⁻), all of

2018).

Non-thermal plasma can decontaminate bacteria. The mechanism and level of decontamination capability vary depending on the length of treatment time, the distance of treatment, the amount of voltage used, and the source of the gas used. All play a role in how effective non-thermal plasma is at decontaminating bacteria (Amalda et al., 2020). In research on bacterial inactivation using SDBD non-thermal plasma treatment, variations in treatment time and treatment distances were used. For the treatment time, variations in treatment time were used, namely 60 s, 75 s, 90 s, 105 s, and 120 s. Various treatment distances were also used, namely 3 mm, 6 mm, 9 mm, 12 mm, and 15 mm. From the results of the treatment obtained, it can be seen in Table 1 and Table 2 that non-thermal plasma influences bacterial inactivation for variations in treatment time and variations in treatment distance. The longer the treatment time, the more bacteria are inactivated, or the number of inactivated bacteria is directly proportional to the length of treatment time. The effect of treatment time on colony number can be seen in Figure 3. Meanwhile, treatment results with distance variations and SBDB non-thermal plasma still affect the inactivation of bacteria. The smaller the distance of treatment used for treatment, the more bacteria will die, or the ability of the bacteria to live will be smaller. The effect of treatment distance on the number of colonies can be seen in Figure 5.

these reactive species exhibit antimicrobial activity (Xu et al.,

The results of the treatment showed that the treatment time of 120 seconds had a better ability to inactivate or kill bacteria than other treatments. The number of bacteria that grew after being treated for 120 seconds was an average of 4.3 x 10^7 CFU/mL. This number was much lower than the number of bacteria that grew without non-thermal plasma treatment, which was 409 x 10^7 CFU/mL. While for the shorter treatment time, the number of colonies that grew more and more was 105 s, 90 s, 75s, and 60 s, with the average number of colonies growing was 11 x 10^7 CFU/mL, 13 x 10^7 CFU/mL, 46.33 x 10^7 CFU/mL, 56 x 10^7 CFU/mL. Pictures of the number of colonies before (control) and after treatment with treatment time variations can be seen in Figure 4.

Meanwhile, from the results of the variation in treatment distance, 3 mm has a more extraordinary ability to inactivate bacteria than other treatment distances. The number of bacteria that grew after being treated at 3 mm averaged 8 x 10⁷ CFU/mL. This number is much decreased compared to the number of bacteria that grow when the treatment is carried out at a longer distance. At the furthest distance carried out in this study, which was 15 mm, the number of colonies growing on average was 253×10^7 CFU/mL. At the same time, the number of bacterial colonies in the control treatment was 409 x 10^7 CFU/mL. Images of colony count before (control) and after treatment with treatment distance variations can be seen in Figure 6.

Time (Second)		Distance (mm)	Treatment I	Number of Colonies (x 10 ⁷ CFU/mL) Treatment II	Treatment III
	Control		410	418	399
60		3	54	46	68
75		3	60	43	36
90		3	13	15	11
105		3	7	3	23
120		3	4	3	6

Table 1. Number of Colonies Before (Control) and After the Treatment with Treatment Time Variations (P < 0.05)



Figure 3. Graph of the Number of Colonies Before (Control) and After Treatment with Treatment Time Variations (60 s, 75 s, 90 s, 105 s, 120 s) ($\mathbb{R}^2 = 0.98188$)

The treatment of time variation and distance variation shows that there is an effect of non-thermal plasma treatment of SDBD. This effect is due to the reactive species formed during plasma generation. The RONS formed to have an important role in the inactivation of bacteria. RONS can disrupt the bonding of microbial cell structures with lipid peroxidation, damaging the RONS membrane as reactive free radicals (NO, •OH, and superoxide) or strong oxidizing agents (H₂O₂ and O₃) can penetrate microorganisms. Further chemical reactions can occur in the cytoplasm that can oxidize cellular proteins or microbial DNA (Klämpfl et al., 2012).

One of the molecules that are the main agent of bacterial inactivation is NO (Nitrogen Oxide). NO can destroy cells by dimerizing thymine bases on DNA strands, disrupting DNA replication (Amalda et al., 2020; Tian et al., 2010). Besides NO, the reactive species formed during plasma formation is H_2O_2 which has the potential to cause oxidative damage. Besides being able to cause oxidative damage, H_2O_2 also functions as a more potent hydroxyl radical (•OH) precursor. The •OH radical is a reactive oxygen species with excellent reactive abil-



Figure 4. Picture of the Number of Colonies Before (Control) and After Treatment with Treatment Time Variations

Time (Second)		Distance (mm)	Treatment I	Number of Colonies (x 10 ⁷ CFU/mL) Treatment II	Treatment III
	Control		410	418	399
120		3	10	6	8
120		6	12	13	14
120		9	49	70	85
120		12	137	109	224
120		15	284	242	233

Table 2. Number of Colonies Before (Control) and After the Treatment with Treatment Distance Variations (P < 0.05)



Figure 5. Graph of the Number of Colonies Before (Control) and After Treatment with Treatment Distance Variations (3 mm, 6 mm, 9 mm, 12 mm, 15 mm) ($R^2 = 0.99727$)

ity and can produce oxidative damage to cell components (Pai et al., 2018). The •OH radical is a strong oxidant that can cause a decrease in ATP, thereby causing low energy in cells. The •OH radical can also break the phosphodiester bond of DNA molecules, which causes DNA fragmentation, and can become lipids in cell membranes, resulting in cells being unable to replicate or causing cell death (Feng and Wang, 2020). In addition, another strong oxidizing agent plays a role in the inactivation of bacteria, namely ozone (O₃). Ozone has a considerable oxidation potential, which can damage cell walls and bacterial cytoplasmic membranes, increasing membrane permeability. As a result, there is a decrease in surface tension which results in cell leakage. Furthermore, ozone and other reactive species easily enter the cell and damage bacterial nucleic acids, damaging the pyramidal rings and breaking the bonds between the pyramidal rings and the sugar groups in nucleic acids. This nucleic acid damage will result in cell death (Kristanti and Dessy, 2012).

The concentration of reactive species formed during nonthermal plasma treatment significantly affects the sterilization

Distance	Treatment	Treatment	Treatment
(mm)	Ι	II	III
Control	\bigcirc		
3	\bigcirc		
6			\bigcirc
9		\bigcirc	
12			\bigcirc
15	\bigcirc		\bigcirc

Figure 6. Picture of the Number of Colonies Before (Control) and After Treatment with Treatment Distance Variations

efficacy. The longer the treatment time, the more reactive the plasma species contains and the more effective it is in killing bacteria. Similarly, the variation in treatment distance showed a relationship between the ability of bacteria to survive and the number of reactive species produced during plasma treatment. The results of the OES spectrum in Figure 3. show that the farther the treatment distance, the smaller the intensity of the spectrum, which indicates that the fewer reactive species formed, the lower the ability to kill bacteria.

4. CONCLUSION

The results of research that have been carried out on the effect of RONS produced by SDBD non-thermal plasma to kill Escherichia coli show that the time and distance of treatment affect the number of colonies that live before and after treatment. For variations in treatment time, the longer the treatment time, the more bacteria are inactivated, or the more bacteria die where the number of colonies that grow when treated with a treatment time of 120 s, which is 4.3×10^7 CFU/mL, is much lower than the control, which is 409 x 107 CFU/mL. As for variations in treatment distance, the farther the treatment distance, the more the number of colonies that live, where the number of colonies that grow when treated with 15 mm, namely 253 x 10⁷ CFU/mL, significantly decreased when treated with 3 mm, namely 8 x 10⁷ CFU/mL. Based on these results, further research can be investigated more deeply by looking at the effect of non-thermal plasma treatment on DNA, lipids, proteins, and bacterial cell morphology to determine the mechanism of non-thermal plasma treatment that kills bacteria. Additionally, several variations of parameters, such as gas source and voltage, can be carried out in order to obtain the most effective non-thermal plasma composition.

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