

Bacterial Sterilization Using Non-Thermal Plasma Method Surface Dielectric Barrier Discharge (SDBD): Effect of Treatment Duration on Colony Count, DNA, Protease Enzymes, and Cell Morphology

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Abstract

The ability of *Escherichia coli* to become increasingly resistant to sterilization has resulted in high cases of this bacterial infection. To overcome this problem, a new method is needed. The circuit design uses 2 parallel plates of copper material. The first plate is HV electrode and second plate is ground electrode which is separated by a dielectric layer. Non-thermal plasma with surface dielectric barrier discharge is generated using a 20 VDC voltage source and then transformed into a high voltage source to generate plasma. Distance between plasma source and fixed sample is 3 mm. OES is used to observe reactive species produced in plasma indicated by magnitude of intensity at certain wavelengths. SDBD non-thermal plasma could be used to inactivate bacteria depending on treatment time. The longer treatment time, greater inactivation ability. For the number of colonies after being treated for 120 s, namely 4.33×10^7 CFU/mL, it was much lower than control, which was 409×10^7 CFU/ml. For DNA after being treated for 120 s, results of genome from *Escherichia coli* were no longer visible or faded, marked by a DNA concentration of 8.18 ng/ul, far lower than the control DNA concentration of 124.44 ng/ul. For the activity of the protease enzyme, the time variation of 105 s had the smallest activity value of the protease enzyme, namely 35.375 U/mL compared to control, which was 52.307 U/mL whereas for cell morphology after 120 s treatment showed increasingly severe cell damage observed using SEM. Non-thermal plasma SDBD configurations can be used to inactivate or kill bacteria. Effectiveness or capability of non-thermal plasma also depends on the treatment time. SDBD nonthermal plasma ability increases with the longer treatment time.

Keywords: SDBD non-thermal plasma, Colony number, DNA, Protease enzymes, Cell morphology

Introduction

Microorganisms are the cause of disease and other health problems which are always a challenge in the world of health. Microorganisms such as bacteria, fungi and viruses can produce toxic by-products that can normal physiological balance as well as attack human host [1]. For this reason, proper handling of these microorganisms is needed, one of which is by sterilizing them. Effective and economical methods of sterilization are needed due to increased health awareness. Sterilization currently being developed is by using plasma. Some of advantages obtained by using plasma sterilization are low cost required, design of plasma generator is simple, and there is no organic residue after using the treatment so that its use is safe and comfortable [2].

Escherichia coli is a Gram-negative, facultative anaerobic, member of Enterobacteriaceae with a cell size of 2.0 - 6.0 um in length and 1.1 - 1.5 um in diameter, straight rod-shaped, single, in pairs or in short chains [3]. *Escherichia coli* is a normal microflora in the intestine, but can become a pathogen under certain conditions. *Escherichia coli* as a pathogenic bacterium is often found as a bacterium that causes urinary tract infections, gastrointestinal infections, and is involved in postoperative wound infections [4]. *Escherichia coli* bacteria can be found in the digestive tract of humans and animals, or materials that have been contaminated with human and animal feces. *Escherichia coli* bacteria reproduce by dividing [5]. Many gene manipulation systems have been developed using *Escherichia coli* as the host bacteria, producing countless enzymes and other industrial products [6]. Although *Escherichia coli* bacteria normally live in digestive tract, there are many cases of diarrhea caused by this bacteria [7].

Based on its virulence, *Escherichia coli* bacteria can be divided into several groups, and one of them is *Enterohaemorrhagic Escherichia coli* (EHEC) [8]. Many cases of EHEC are mainly caused by *Escherichia coli* O157:H7. This bacterium is one of the serotypes of *Escherichia coli* which is pathogenic and harmful to humans. EHEC O157:H7 causes haemorrhagic colitis characterized by bloody diarrhea and haemolytic uremic syndrome. Bloody diarrhea occurs due to verotoxin or shiga like toxin produced by EHEC O157:H7 [9]. This serotype is often called verotoxigenic *Escherichia coli* (VTEC) which is extracellular, neurotoxic, and immunogenic. In cases of haemorrhagic colitis, it is usually characterized by abdominal pain accompanied by cramps, fever or without fever, watery diarrhea, sometimes accompanied by bleeding, vomiting, nausea. Whereas in cases of haemolytic uremic syndrome it can lead to kidney failure [10].

Plasma consists of positively and negatively charged ions, free electrons, free radicals and intermediate reactive atoms, molecules and UV photons which are the result of ionized state of gas [11]. Plasma can be classified according to its temperature into 2 major groups, namely thermal plasma and non-thermal plasma [11,12]. Thermal plasma can reach temperatures of up to several thousand degrees Celsius and is used in applications where high temperatures are required, such as in foundry processes in the metallurgical industry or in chemical synthesis processes. Non-thermal plasma is a partially ionized gas that is not in thermal equilibrium because temperature of its electrons is much higher than that of the ions and neutrals. Non-thermal plasma is generated by application of an electric or electromagnetic field to a gas. Energy of field causes free electrons to accelerate and ionize gaseous atoms and molecules, which releases more free electrons which in turn triggers new ionizations [12,13].

The product produced by plasma has ability as an antimicrobial against microorganisms such as bacteria, fungi and viruses. These products include positive and negative ions, free radicals, electrons, UV radiation and also reactive oxygen species and reactive nitrogen species. For this reason, non-thermal plasma has potential to decontaminate or kill microorganisms [11,14].

Plasma technology has been used like low pressure plasma or atmospheric pressure plasma in terms of decontamination [2,15,16]. Non-thermal plasma can kill bacteria without using antibiotics, so it doesn't cause resistant effects and also antibiotic residues [15]. In addition, Since treatment with nonthermal plasma is administered at room temperature, damaging effect on biological tissues can be minimized while retaining ability to disinfect and sterilize [12]. Based on ability of plasma to inactivate or kill bacteria, it is necessary to know impact of plasma treatment on damage to *Escherichia coli* bacterial cells. In this study, an analysis of the effect of treatment on number of colonies, DNA, protease enzymes, and cell morphology was carried out using SDBD non-thermal plasma.

Materials and methods

Preparation of *E. coli* bacteria samples and non-thermal plasma treatment of surface dielectric barrier discharge

Escherichia coli isolates aged 24 h were diluted with serial dilutions up to 10^{-6} with 1 ml 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 is carried out until a dilution of 10^{-6} . The dilution results were then processed using non-thermal plasma with a surface dielectric barrier with variations in treatment time of 0 as control, 50, 75, 90, 105 and 120 s, with electrode distance from bacterial and a fixed gas source of 3 mm and free air. The results of treatment are then spidared using a triangular rod. After that, it was incubated at 37 °C for 24 h by placing the petri dish in an inverted position. The next step is to count number of colonies in each treatment. Each experiment was repeated 3 times.

Bacterial DNA ttest

Bacterial isolates that had been grown on nutrient agar media for 24 h were taken as many as 6 oses and then mixed into 200 µl of aquadest for all bacterial treatments. The resulting mixture was put into ZR BashingBead Lysis Tube and added 750 µl of Bashingbead Buffer, then vortexed for 7 min and centrifuged for 1 min at 10,000 rpm. The resulting supernatant was taken 400 µl and put into Zymo Spin III-F Filter then centrifuged again for 1 min at 8,000 rpm. After centrifugation, 1,200 µl of Genomic Lysis Buffer was added to collection tube and 800 µl of mixture was taken to be put into Zymo-spin IICR Column, which was attached to a collection tube below to be centrifuged again for 1 min at 10,000 rpm. The centrifugation results were added with 200 uL of DNA Pre-Wash Buffer then centrifuged again for 1 min at 10,000 rpm and added with 500 µL g-DNA Wash Buffer to be centrifuged for 1 min at 10,000 rpm. Next step was to install a new sterile microtube to add 60 µL of DNA Elution buffer to matrix column and centrifuged for

30 s at 10,000 rpm. Results of centrifugation were then added to the loading dye and electrophoresed for 30 min at 100 volts [17].

Protease enzyme activity test

One ose of *Escherichia coli* isolate was inoculated on tripton soy broth (TSB) medium and then incubated for 24 h at 37 °C. The incubation results were transferred to a sterile dish and then treated with non-thermal plasma with a surface dielectric barrier discharge. After completion of the treatment, as much as 2 mL of the sample was taken and then centrifuged. The supernatant of 1 mL from the centrifuge tube was taken and added to 5 mL of phosphate buffer containing 1 % casein and then incubated at 37 °C for 10 min. The incubation results were then added with 5 mL of 5 % TCA and centrifuged. The supernatant from the centrifuge was taken as much as 2 mL, then 5 mL of Na₂CO₃ and folin reagent were added. After mixing, the solution was then spectated at a wavelength of 660 nm, and from the Spectro results, the activity of the protease enzyme was calculated [18].

Cell morphology using a scanning electron microscope (SEM)

Scanning Electron Microscope (SEM) was used to observe changes in morphology of all bacterial cells without non-thermal plasma treatment (control) and bacteria with non-thermal plasma treatment. The first step before being observed using SEM all bacteria must be made into preparations first. All bacteria in each treatment were centrifuged at 5,000 rpm at 4 °C for 10 min. Then washed using physiological NaCl 2 times and centrifuged again at 5,000 rpm at 4 °C for 5 min. Centrifugation results were incubated with glutaraldehyde for 30 min and centrifuged again at 5,000 rpm at 4 °C for 10 min. The centrifugation results were washed with PBS pH 7 for 10 min and placed on a coverslip to be fixed using Bunsen. After the fixation process was complete, the samples were washed using graded ethanol, namely 30, 50, 70, 90 and 96 %. The last step of the sample is coated with gold and can be observed using a SEM.

Optical emission spectroscopy

Optical emission spectroscopy (OES) is 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. The optical emission spectra were measured using Aurora 4,000. The spectrometer was operated at a wavelength of 200 to 900 nm with an integration time of 5,000 ms and 3 repetitions of the spectrum capture and then averaged to obtain the optical emission spectrum from the plasma. The emission spectra were analyzed qualitatively to determine the chemical species at each wavelength peak and then analyzed using the atomic spectrum database of the National Institute of Standards and Technology and previous journal publications for the identification of chemically active species [19].

Data analysis

The data obtained were based on the effect of treatment time (0, 60, 75, 90, 105, 120 s) on the total bacteria, tabulated and analyzed using ANOVA using SPSS 26 software. If the *p*-value < 0.05 H₀ is accepted, then the length of treatment time affects the total number of bacteria.

Results and Discussion

Optical emission spectrum (OES) spectrum of surface dielectric barrier discharge nonthermal plasma

Obtained the spectrum were analyzed for reactive species by looking at the peaks [20]. Spectrum results obtained using OES are taken for each time variation, namely 60, 90 and 120 s with a fixed distance and source of gas, 3 mm, and air. The results of OES spectrum from discharge surface of non-thermal plasma dielectric barrier is a correlation to time variation where wave peaks can be seen in **Figure 2** which shows reactive species formed from the resulting non-thermal plasma [21].

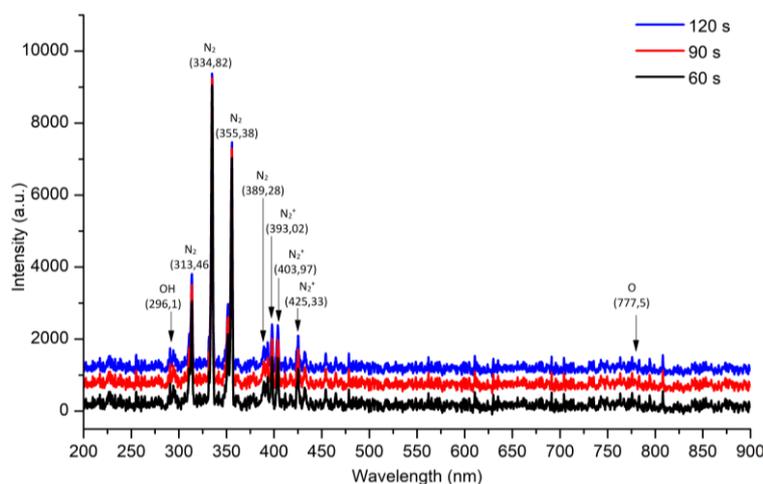


Figure 2 OES Spectrum of SDBD non-thermal plasma with treatment time variation.

From this spectrum, there is no significant difference in peak intensity concentration for each time variation, where the longer the treatment time, the intensity for each wavelength remains. This shows that the longer the treatment time, the more reactive species are formed and the greater the inactivation ability of the microorganisms. The resulting spectrum was measured using OES at a wavelength of 200 to 900 nm from the plasma source, and the intensity emitted from the plasma source was recorded at each of these wavelengths. In the non-thermal plasma formation resulting from SDBD used for bacterial decontamination, several reactive species were generated in the gas phase, which was observed using OES during plasma release [11]. The emission spectrum shows that the emission of N_2 and N_2^+ excitation species show different peaks in the UV region. Emission from SPS N_2 is the main result of electron collisions [20].

The treatment duration was longer but the intensity concentration did not change significantly because the treatment duration did not significantly affect the intensity of the plasma. The plasma intensity here is the product of the transition of electrons returning from the excited state to the ground state. The occurrence of excitation and de-excitation is directly influenced by the input energy given or in this case the voltage. So that the voltage is the main parameter that more significantly affects the value of the intensity not the duration of time given [22].

The N_2 Second Positive System (SPS) has a main peak at a wavelength of 313 - 390 nm, the N_2 first negative system (FNS) at a wavelength of 390 - 450 nm [23,24]. At the same time, a small peak of OH appears at a wavelength of 296.1 nm [21,25,26]. OH is generated from the dissociative excitation process of water particles [20]. The $3p5P \rightarrow 3s5S0$ transition has produced an oxygen band at a wavelength of 777.5 nm. The oxygen atom can be formed through the dissociation of the electron collision of the oxygen molecule or the penning ionization of the nitrogen molecule [11,20]. The reactive species produced in the next plasma release phase will produce other reactive oxygen species and reactive nitrogen species, which are effective in inactivating microorganisms [13].

Effect of nonthermal plasma treatment on colony number

Non-thermal plasma can decontaminate bacteria. The mechanism and level of decontamination ability vary depending on type of gas used, duration of treatment, treatment distance, amount of voltage used, and the gas source used. All play a role in how effective non-thermal plasma is at decontaminating bacteria [15]. Research on bacterial inactivation using non-thermal plasma treatment using time variations in the treatment process has been carried out with variations in the time used, namely 60, 75, 90, 105 and 120 s. The results of the treatment can be seen in **Figure 3**. Treatment time of 120 s has a greater ability to inactivate bacteria than other treatments. Number of bacterial colonies decreased as the duration of treatment increased. When compared to control sequentially the number of bacterial colonies became 13.7, 11.25, 3.2, 2.7 and 0.98 % for treatment durations of 60, 75, 90, 105 and 120 s.

SDBD non-thermal plasma treatment showed that non-thermal plasma affected inactivation or killing of bacteria. The longer time used for treatment, more bacteria that die. This effect occurs due to formation of Reactive Oxygen and Nitrogen Species (RONS) during plasma generation. RONS appears to have an important role in inactivating bacteria by interfering with the bonding of microbial cell structures through lipid peroxidation events and causing damage to bacterial cell membranes. RONS can damage various

components of bacterial cells due to reactive free radicals (NO, OH, and superoxide) as well as strong oxidizing agents (H₂O₂ and O₃). Another chain chemical reaction will occur in bacterial cytoplasm which will oxidize cellular protein or microbial DNA and result in cell death [27].

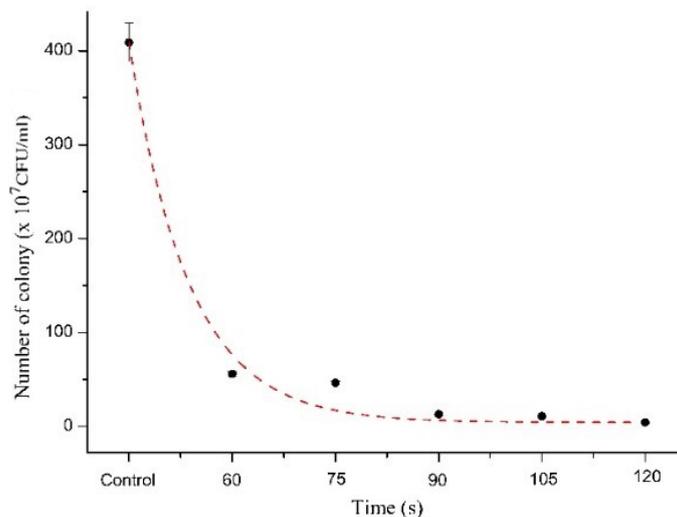


Figure 3 Correlation between duration of treatment and number of bacterial colony.

Effect of non-thermal plasma treatment on escherichia coli bacterial cell DNA

The effect of non-thermal plasma treatment on bacterial DNA was also observed using the agarose gel electrophoresis technique to observe *Escherichia coli* DNA fragmentation after non-thermal plasma treatment. The non-thermal plasma treatment used is by using variations in treatment time. From the results obtained, length of time of treatment greatly affects or results in degradation of the DNA of *Escherichia coli* genome. It can be seen from **Figure 4** the longer treatment time for *Escherichia coli* genomic DNA, more fading it was until at time of 120 s of treatment, genomic DNA of *Escherichia coli* was no longer visible. These results are supported by data on DNA concentration testing using nanodrop technique which can determine bacterial DNA concentrations when not given non-thermal plasma treatment and when bacterial DNA concentrations have been given non-thermal plasma treatment with different time variations.

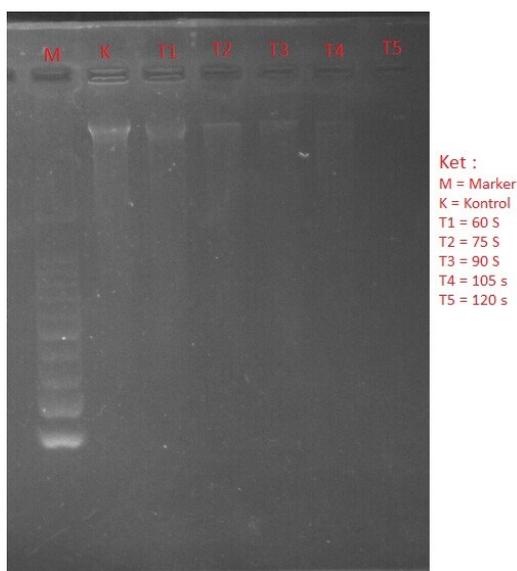


Figure 4 Band gap DNA of *Escherichia coli* bacterial cells.

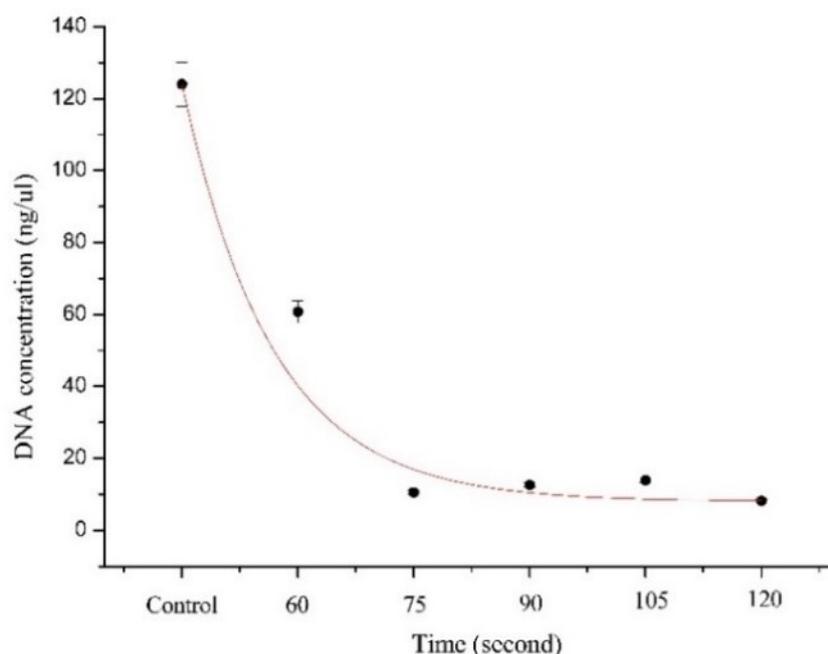


Figure 5 Correlation between duration of treatment time and DNA concentration.

The amount of DNA concentration decreased when compared to control treatment when time duration given was increased. From **Figure 5** it can be seen that the most optimal and efficient treatment is at 75 s which is equal to 8.45 % when compared to control concentration. The decrease that occurred in 60 s treatment was not too significant, namely 48.85 % when compared to the control, whereas in 90, 105 and 120 s treatment the curve was sloping and there was no significant change after going through 75 s treatment with a DNA concentration of 10. 1, 11.2 and 6.6 %. Non-thermal plasma SDBD treatment using time variation showed that one of cell death factors was caused by DNA damage due to non-thermal plasma treatment. The longer treatment time, the greater damage. Detecting DNA damage markers confirms the induction of DNA damage after nonthermal plasma treatment [28]. The yield of DNA fragmentation after nonthermal plasma treatment increased with increasing nonthermal plasma treatment time [29]. Other results showed increased genomic DNA damage with increasing treatment time. This was characterized by an increase in the concentration of reactive species produced by nonthermal plasma, which potentially increased sensitivity to oxidative stress generated by plasma [30]. As a result of changes and damage that occurs in DNA, it will cause the DNA strand to break. DNA damage that occurs causes transversion (purine substitution for pyrimidine and pyrimidine substitution for purine), thereby changing the bonds between DNA bases [31,32]. In addition, reactive species produced by plasma also react with deoxyribose carbon which causes breaking of N-glucosidic bond, resulting in apurinic or apyrimidine sites (base site). These changes cause errors in strand reading, mutagenesis, and cell death [13].

Effect of SDBD non-thermal plasma treatment on protease enzyme activity

The effect of non-thermal plasma treatment on protein can be analyzed by testing the activity of protease enzymes. *Protease* is an enzyme that hydrolyzes peptide bonds in proteins into oligopeptides and amino acids [33]. The resulting protein that has been hydrolyzed into simple forms or bonds will be easier to use in metabolism. Protein hydrolysis provides the necessary amino acids for the synthesis of new proteins. The best protein hydrolysis uses Enzymes. This enzyme is produced extracellularly and intracellularly and plays an important role in cellular metabolic processes and their regulation. If the enzyme activity is disturbed, the ability to develop from microorganisms or bacteria will be reduced [34].

The UV-Vis spectrophotometer technique was used in the protease enzyme activity test with a wavelength of 660 nm. The sample's absorbance before and after treatment was then calculated as protease enzyme's activity. The results of protease enzyme activity and effect of non-thermal plasma treatment time can be seen in **Figure 6**. The results obtained showed that the activity of the protease enzyme before treatment of 52 U/mL decreased after treatment with time variations of 60, 75, 90, 105 and 120 s, namely 37,420, 42,447, 35,375, 26,355 and 33,818 U/mL. From these results, the non-thermal plasma treatment with a time variation of 105 s had smallest protease enzyme activity value of 35.375 U/mL. The decrease

in protease enzyme activity after non-thermal plasma treatment is thought to be caused by the denaturation of the enzyme so that enzymatic reaction is disrupted. Research on protein breakdown showed a conformational change and a decrease in enzyme activity caused by exposure to DBD plasma. Oxidation can affect enzyme activity. The result of decreased enzyme activity may reflect normal level of bacterial function and the level of bacterial damage caused by non-thermal plasma exposure [35].

Oxidative stress caused by reactive species generated during the non-thermal plasma treatment process can change structure and function of enzymes. As a result, it will disrupt metabolic processes of cells by preventing the formation of proteins needed for enterobacterial [36]. Reactive species produced during the non-thermal plasma treatment process are also capable of breaking peptide bonds, oxidizing amino acid side chains, especially sulfur amino acids, such as methionine and cysteine, and amino acids that store aromatic rings (tryptophan, phenylalanine, and tyrosine), and generating cross-links in proteins where all these effects result in protein and enzyme modification [13]. Therefore, oxidation and oxidative processes can affect enzymatic system and cause extensive damage to bacterial proteins, leading to bacterial death [37].

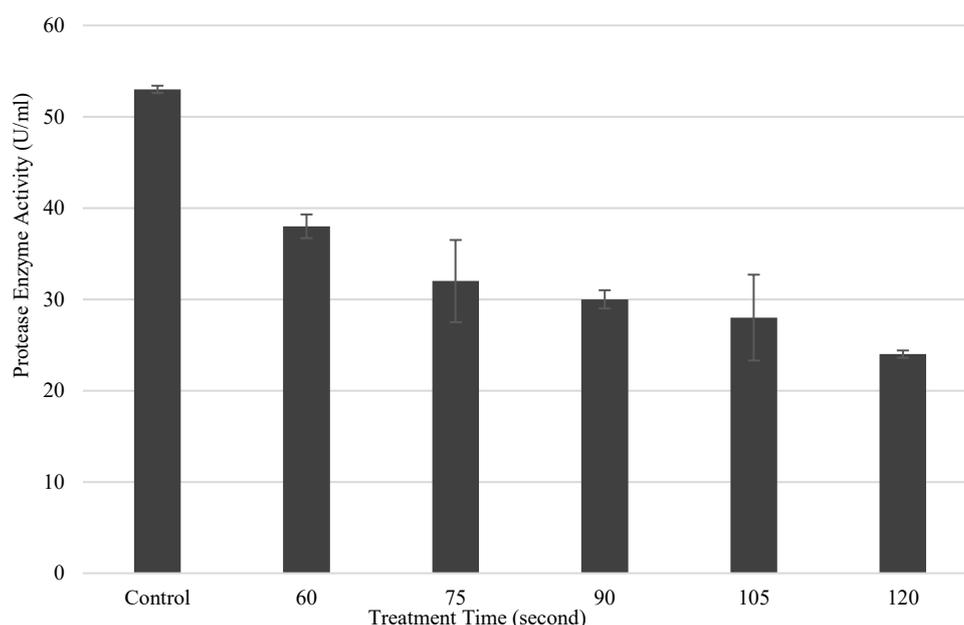


Figure 6 Correlation between duration of treatment time and protease enzyme activity.

Cell morphology results using scanning electron microscope (SEM)

From the results of observations made using SEM, there is cell damage and changes in cell shape after non-thermal plasma treatment. Some bacteria are no longer rod-shaped but rounded, become coccoid and lose their bacillus shape [38].

Cell damage is directly proportional to the increase in treatment duration. The longer the plasma non-thermal treatment time is given, the more bacterial cells are damaged and experience changes in their morphological form. The results of observations using SEM can be seen in **Figure 7**. Cell damage that appears is triggered by an oxidation process due to RONS produced by non-thermal plasma [39]. The main components of bacterial cell walls and membranes are organic species such as peptidoglycan, proteins, and lipids, which cause them to be susceptible to oxidation caused by reactive species produced by non-thermal plasma [36], oxidation of lipid membranes caused by RONS can weaken bacterial membranes [40]. Protein molecules that make up bacterial cell membranes are known to be susceptible to oxidation processes, so non-thermal plasma treatment on bacteria can cause erosion, resulting in the rupture of bacterial cell walls. In addition to causing cell wall rupture, the use of non-thermal plasma can also inhibit formation [15].

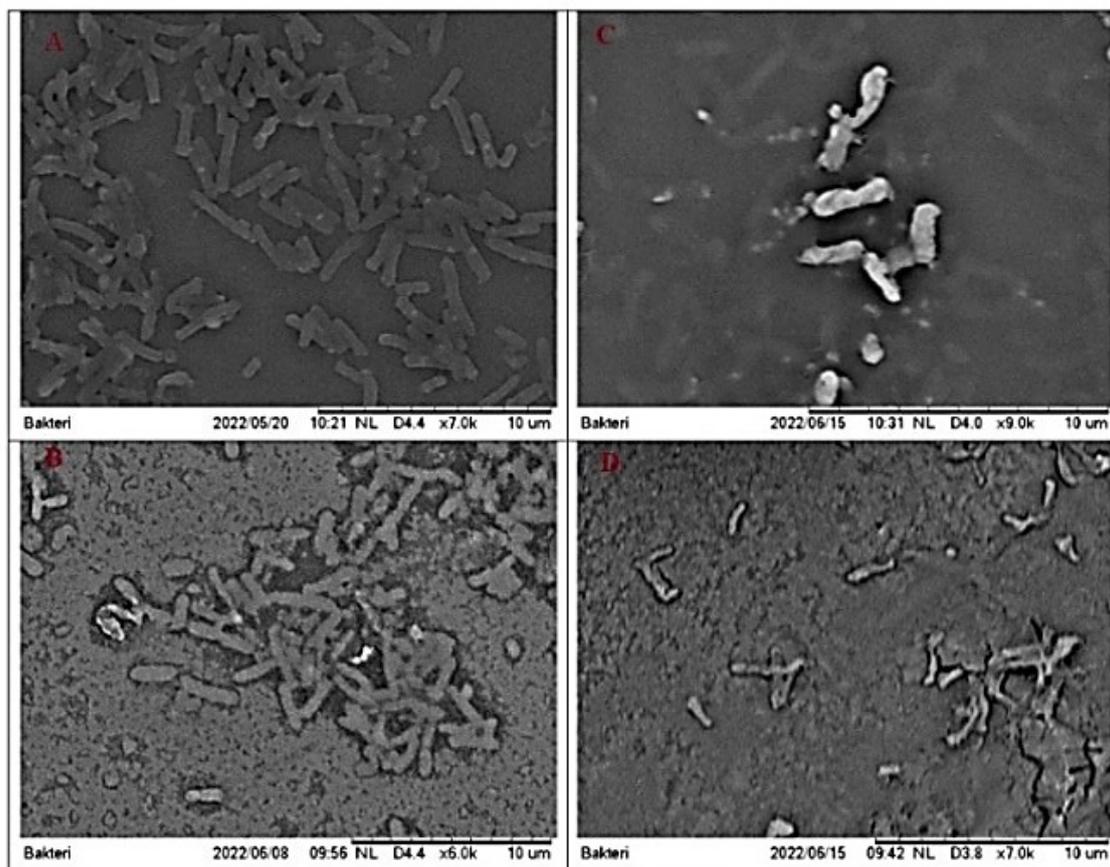


Figure 7 The morphology of *Escherichia coli* bacterial cells was observed using SEM (A. control, B. 60 s, C. 90 s, and D. 120 s).

Conclusions

The results of inactivation of *Escherichia coli* bacteria using non-thermal plasma SDBD showed that configuration of non-thermal plasma SDBD could be used to inactivate or kill bacteria. The effectiveness or capability of nonthermal plasma also depends on treatment time. SDBD nonthermal plasma ability increases with the longer treatment time. The results showed changes after SDBD nonthermal plasma treatment, namely number of colonies, DNA, protease enzyme activity, and bacterial cell morphology. The number of colonies after being treated for 120 s was 4.33×10^7 CFU/ml which was much lower than the control which was 409×10^7 CFU/mL. The results of the *Escherichia coli* genome showed DNA damage where the longer the treatment time. In the 120 s treatment, *Escherichia coli* genome has faded and is not even visible anymore. This result is supported by the results of DNA concentration where DNA concentration before treatment was 124.44 ng/ul, whereas after being treated for 120 s the concentration of DNA decreased by 8 ng/ul. The results of the protease enzyme activity test showed that treatment time of 105 s had the smallest activity value of the protease enzyme, which was 35.375 U/mL compared to the control, which was 52.307 U/mL. While the results of cell morphology observations using SEM showed that after being treated for 120 s, the bacterial cells underwent a change in shape and cell damage was getting worse compared to control.

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