ORIGINAL ARTICLES

EFFECTIVENESS OF PURPLE LED FOR INACTIVATION OF BACILLUS SUBTILIS AND ESCHERICHIA COLI BACTERIA IN IN VITRO STERILIZERS

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Abstract

Bacteria are inactivated using a technique called photodynamic inactivation, which combines light with a photosensitizer with the right spectrum. The objective of this study is to ascertain the efficiency of purple LEDs for photoinactivating *Bacillus subtilis* and *Escherichia coli* bacteria as well as the ideal purple LED exposure energy density. This study technique involves exposing bacteria to purple LED radiation. Two elements of variation are used during irradiation. The first variation is the illumination variation at distances of 3 cm, 6 cm, 9 cm, and 12 cm. The second variation involves changing the amount of radiation for 30, 60, 90, and 120 minutes. The Total Plate Count (TPC) method was used to count the number of colonies. Statistical tests were utilized in data analysis, namely the One Way Anova test (analysis of variance). The results of this study indicated that 395 nm purple LED irradiation caused a decrease in Log CFU/mL of *Bacillus subtilis* and *Escherichia coli* bacteria. Inactivation of *Bacillus subtilis* bacteria showed a higher mortality percentage than *Escherichia coli* bacteria. Changes in other irradiation distances also showed a higher percentage of death for *Bacillus subtilis* bacteria than *Escherichia coli* bacteria. The highest percentage of death was 98.5% for Bacillus subtilis bacteria and 94.3% for *Escherichia coli* bacteria at position C with an irradiation distance of 3 cm and an energy density of 524 J/cm^2 with an LED exposure time of 120 minutes. This shows that the percentage of death of bacteria *Bacillus subtilis* and *Escherichia coli* increased with increasing doses of LED energy with the greatest percentage of death in Gram-positive bacteria *Bacillus subtilis*.

Key words: health security, photodynamic inactivation, purple LED, *Bacillus subtilis, Escherichia coli.*

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ЭФФЕКТИВНОСТЬ IN VITRO ИНАКТИВАЦИИ БАКТЕРИЙ BACILLUS SUBTILIS И ESCHERICHIA COLI В СТЕРИЛИЗАТОРАХ С ИСПОЛЬЗОВАНИЕМ ОБЛУЧЕНИЯ В ФИОЛЕТОВОЙ ОБЛАСТИ

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Резюме

Инактивация бактерий может быть выполнена с использованием метода, называемого фотодинамической инактивацией, в основе которого лежит активация фотосенсибилизатора светом определенного спектра. Целью данного исследования является определение эффективности светодиодов с излучением в фиолетовой области спектра для фотоинактивации бактерий Bacillus subtilis и Escherichia coli, а также определение оптимальной плотности энергии воздействия. При облучении были использованы два изменяемых параметра. Первый параметр — это расстояние от источника облучения до облучаемой поверхности (3 см, 6 см, 9 см и 12 см). Второй параметр – время облучения (30, 60, 90 и 120 мин). Для подсчета количества колоний использовали метод общего подсчета чашек (Total Plate Count). При анализе данных использовали статистические тесты, а именно тест One Way Anova (дисперсионный анализ). Результаты этого исследования показали, что светодиодное излучение в фиолетовой области спектра с длиной волны 395 нм вызывало снижение log КОЕ/мл бактерий *Bacillus subtilis* и *Escherichia coli*. Воздействие на бактерии *Bacillus subtilis* показало более высокий процент смертности, чем для бактерий *Escherichia coli*. Лучшие результаты были получены при расстоянии до источника облучения 3 см, плотности энергии 524 Дж/см^2, и времени воздействия светодиода 120 мин. В этом режиме было инактивировано 98,5% бактерий *Bacillus subtilis* и 94,3% бактерий *Escherichia coli*.

Ключевые слова: безопасность, фотодинамическая инактивация, фиолетовый светодиод, *Bacillus subtilis, Escherichia coli*.

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Introduction

The goal of sterilizing health services is to achieve the best/highest level of individual/community health by effort, work, or health-related activities. In hospitals and other healthcare facilities in Indonesia, infection and sepsis continue to be among the leading causes of mortality and morbidity. The spread of infections and diseases can have a negative impact on the health of employees, patients, and the medical procedures performed at healthcare facilities. The presence of contaminating bacteria as an infection source has a significant impact in settings that should be kept sterile, including operating rooms, laboratories, and existing medical equipment. *Bacillus subtilis* is a common contamination microorganism detected in medical equipment. *Escherichia coli* is the bacteria that is frequently discovered in food. These bacteria's spores are also agents that contribute to food spoiling, operate as disease vectors, and can alter food quality, which makes them a serious problem for the food industry [1].

Additionally, the cleanliness of the food we eat has an impact on our health. The application of cleanliness, safety, comfort, and regularity which decreases or even avoids the possibility of contamination is the primary principle of food sanitation. using food sanitation to regulate the usage of raw materials, processing locations, and auxiliary equipment [2]. It must be ensured that the tools used are clean before they may be used to prepare and serve food.

All biological forms are eliminated during sterilization. From a microbiological perspective, a thing is free of living microorganisms if it is sterile. Among all living things, bacterial spores are the most resistant to sterilization. The quantity and kind of microorganisms, the degree and type of contamination by other substances, and whether or not microorganisms are present on the device all affect how effectively a device is sterilized [3]. Dental instruments and other items that come into contact with blood or bodily tissue must be sterilized. Dry heat sterilization is one of the most popular sterilization processes. The drawbacks of the dry heat sterilization approach include the lengthier sterilization time, the slow and uneven material penetration, the need for an oven and a constant power source, the inability to disinfect plastic and rubber devices, and the high cost of the sterilizing equipment. In order to effectively inactivate contaminating bacteria, a different approach including photodynamic inactivation is therefore required. Previous studies have been conducted to see the effectiveness of light for inactivating bacteria and fungi [4, 5].

Photoinactivation is the process of preventing cellular metabolism due to cytoplasmic membrane damage brought on by reactive oxygen in lipids and proteins [6]. The membrane transport system in the bacterial cell is either inactive or undergoes cell lysis as a result of reactive oxygen. The light source, the photosensitizer as a substance, and the free radicals that lead to cell inactivation are the three primary causes of photodynamic' success. Endogenous porphyrins, which some bacteria naturally create and which are photosensitizers and light-absorbing compounds (sensitive to light). Every porphyrin molecule has the capacity to absorb light of a specific wavelength [7]. Bacterial cells will be photo inactivated when the right combination of light and photosensitizer is used [8]. A photosensitization mechanism, such as the absorption of light by porphyrins, triggers reactions in the substrate to begin the photoinactivation process. External photosensitizers from various materials such as chemicals [9], drugs [10], organic/natural materials [11] and metals [12] can be added to increase the effectiveness of photoinactivation. The type and number of

photosensitizers, which act as light-absorbing molecules, determine this photosensitization.

The Light-emitting diode (LED), a complicated semiconductor that can convert electrical energy into light, has the advantage of only releasing a little amount of heat in the light it generates. It is one of the light sources that has a porphyrin absorption spectrum range of the photosensitizer type. The porphyrin absorption spectral region of the photosensitizer type is present in LED light sources. Additionally, because LEDs only generate a minimal amount of heat in the light they provide, they are superior to conventional light sources for the phototherapy process [13]. Previous studies demonstrated the ability of LEDs to inactivate bacteria [5, 14, 15]

The result study previously showed that a purple LED with a wavelength of 408.6 nm, energy of 61.2 joules, and a photoinactivation effect of 42.11% was the best light source for inactivating *S. mutans* [14]. In another investigation the visible light with a wavelength of 405 nm used to test the susceptibility of *Bacillus* and *Clostridium endospores* [16]. Another study with light 405 nm showed endospores can greatly be affected by light's bactericidal effects [17]. In contrast, the inactivation of the *Listeria monocytogenes bacteria* utilizing high-intensity light with a 405 nm wavelength [18]. The findings demonstrated that optimum inactivation is induced by exposure to the 400–450 nm wavelength range at a rather high dose level (750 J/cm^2). Longer than 450 nm exposure does not result in substantial inactivation. The most efficient wavelength for inactivating *L. monocytogenes* is 405 nm of light, according to an analysis with a 10 nm bandwidth between 400 and 450 nm. These findings informed the choice of a shorter wavelength, higher intensity purple LED light source for this research. In this study, *Bacillus subtilis* and *Escherichia coli* bacteria will be photo inactivated in vitro using the best purple LED exposure energy density and effectiveness.

Materials and methods

Bacterial Culture

The bacterial strain, *Bacillus subtilis* ATCC 9466 and *Escherichia coli* ATCC 25922 was inoculated from Tryptone Soy Agar (Oxoid, UK) and taken on Tryptone Soy Broth sterile (Oxoid, UK). The culture of bacteria was incubated at 37°C until bacterial colonies reached ~108 CFU/mL or 1.0 McFarland Standard.

Purple LED Exposure

Purple LEDs with a peak wavelength of 395 nm were exposed to *Bacillus subtilis* and *Escherichia coli* bacteria. The instruments used in this study were purple LEDs 395 nm arranged on 10x10 pieces of PCB and assembled in an acrylic box with a volume of 15x15x15 cm^3 and controlled by a microcontroller. The instrument is equipped with a display of time (minutes), PWM (%), and irradiation temperature (°C). The process of irradiating the LEDs on the samples was carried out in various positions, namely positions A, B, C, D, and E according to Fig. 1. Treatment of bacteria was carried out at various distances, namely at a distance of 3 cm, 6 cm, 9 cm, and 12 cm and time variations exposure for 30 minutes, 60 minutes, 90 minutes and 120 minutes. Table 1 shows the average values of the measurement data for the intensity of a 395 nm purple LED at a distance of 3 cm, 6 cm, 9 cm and 12 cm.

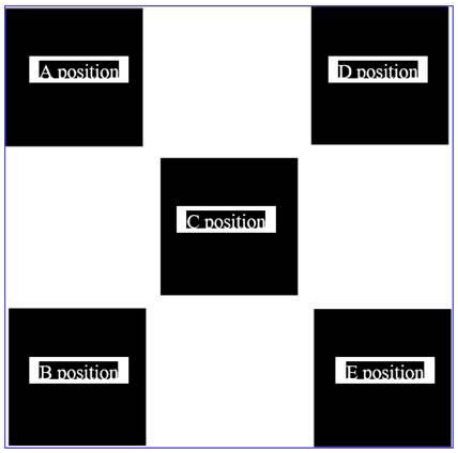

Рис. 1. Положение образца.
Fig. 1. Position of sample.

Table 1
The average values of the measurement data for the intensity of a 395 nm purple LED at a distance of 3 cm, 6 cm, 9 cm and 12 cm
Таблица 1
Средние значения данных измерений интенсивности излучения фиолетового светодиода с длиной волны 395 нм на расстоянии 3 см, 6 см, 9 см и 12 см

Distance, cm	A Position, $\frac{mW}{cm^2}$	B Position, $\frac{mW}{cm^2}$	C Position, $\frac{mW}{cm^2}$	D Position, $\frac{mW}{cm^2}$	E Position, $\frac{mW}{cm^2}$
3	49.4	59.6	89.0	54.1	54.5
6	39.2	41.6	67.6	41.2	40.3
9	27.0	27.0	55.1	24.9	26.9
12	15.6	20.5	35.0	23.0	12.9

Data analysis

Using the total plate count approach, the number of bacterial colonies that were growing was counted. The following formula is used to determine the percentage decrease in the number of bacterial colonies:

$$\%death = \left| \frac{\sum treatments - \sum control}{\sum control} \right| x\,100\,\%$$

The One-Way ANOVA test was used as the statistical analysis (analysis of variance). The purpose of this test is to identify any variations in the outcomes of each treatment group.

Results and discussion

Bacillus subtilis and *Escherichia coli* bacteria were exposed to purple LEDs at varying distances of 3, 6, and 9 cm for periods of 30, 60, 90, and 120 minutes with a power width modulation (PWM) value of 100%. Fig.1 and 2 showed *Bacillus subtilis* and *Escherichia coli* viability after LED exposure to (a) position A, (b) position B, (c) position C, (d) position D, (e) position E.

Based on research data, power density and energy density values are obtained at each position. The position that has the greatest power density is at position C so that the energy density value has the greatest value as well. The power density at positions A, B, D, E have almost the same

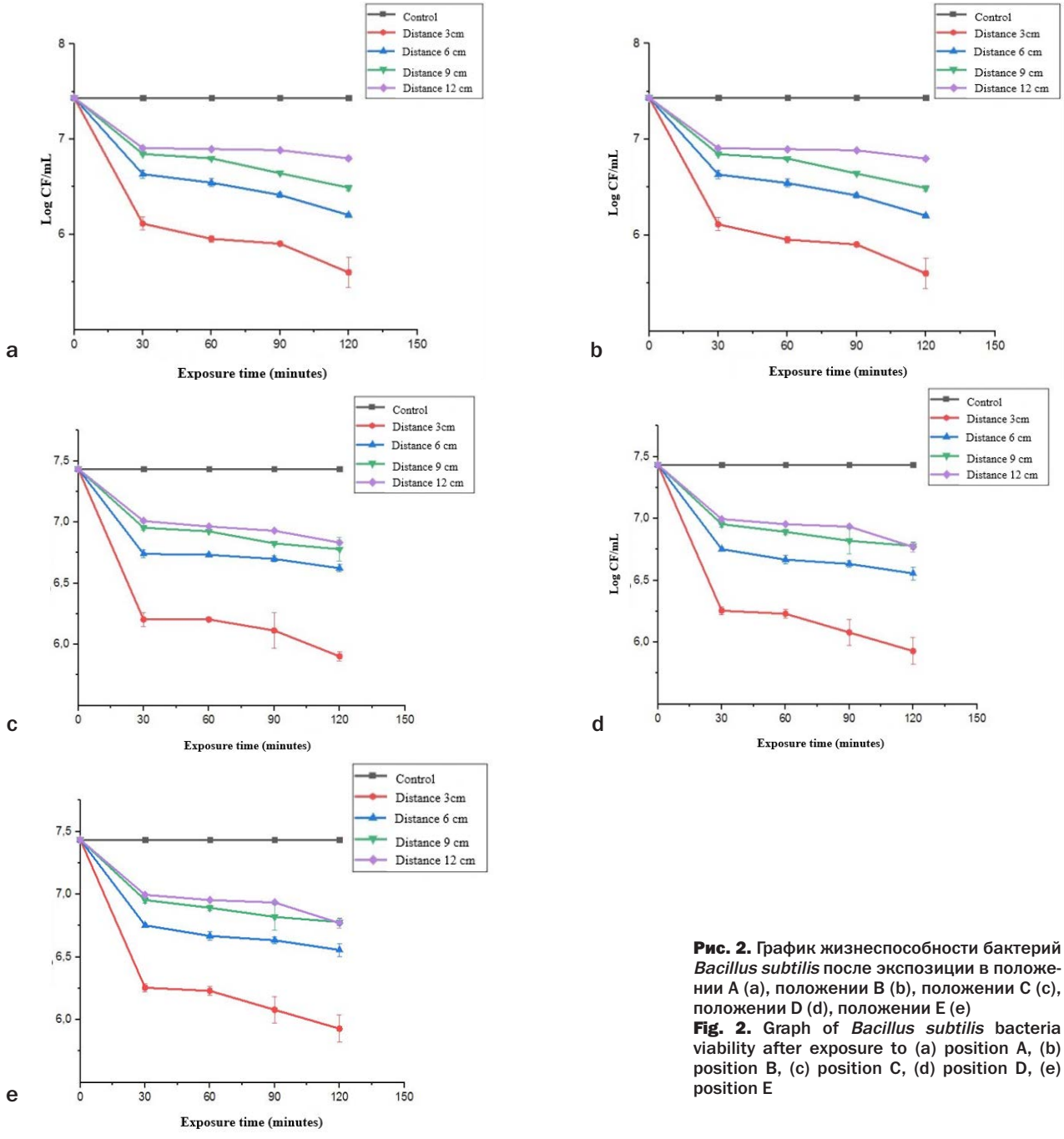

Рис. 2. График жизнеспособности бактерий *Bacillus subtilis* после экспозиции в положении A (a), положении B (b), положении C (c), положении D (d), положении E (e)

Fig. 2. Graph of *Bacillus subtilis* bacteria viability after exposure to (a) position A, (b) position B, (c) position C, (d) position D, (e) position E

values so that the energy density or dose at positions A, B, D, and E also have almost the same values. Therefore, the value of the percentage of deaths in these positions has almost the same value. One Way Anova test findings with a significance or probability (p) value of 0.00 indicate that there is a significant difference between the treatments in this study.

The results of this study indicated that 395 nm purple LED irradiation caused a decrease in Log CFU/mL of *Bacillus subtilis* and *Escherichia coli* bacteria. Inactivation of *Bacillus subtilis* bacteria showed a higher mortality percentage than *Escherichia coli* bacteria. Changes in other irradiation distances also showed a higher percentage of death for *Bacillus subtilis* bacteria than *Escherichia coli* bacteria. The highest percentage of death was 98.5% for *Bacillus subtilis* bacteria and 94.3% for *Escherichia coli* bacteria at position C with an irradiation distance of 3 cm and an energy density of 524 J/cm^2 with an LED exposure time of 120 minutes. This shows that the percentage of death of bacteria *Bacillus subtilis* and *Escherichia coli* increased with increasing doses of LED energy with the greatest percentage of death in Gram-positive bacteria *Bacillus subtilis.*

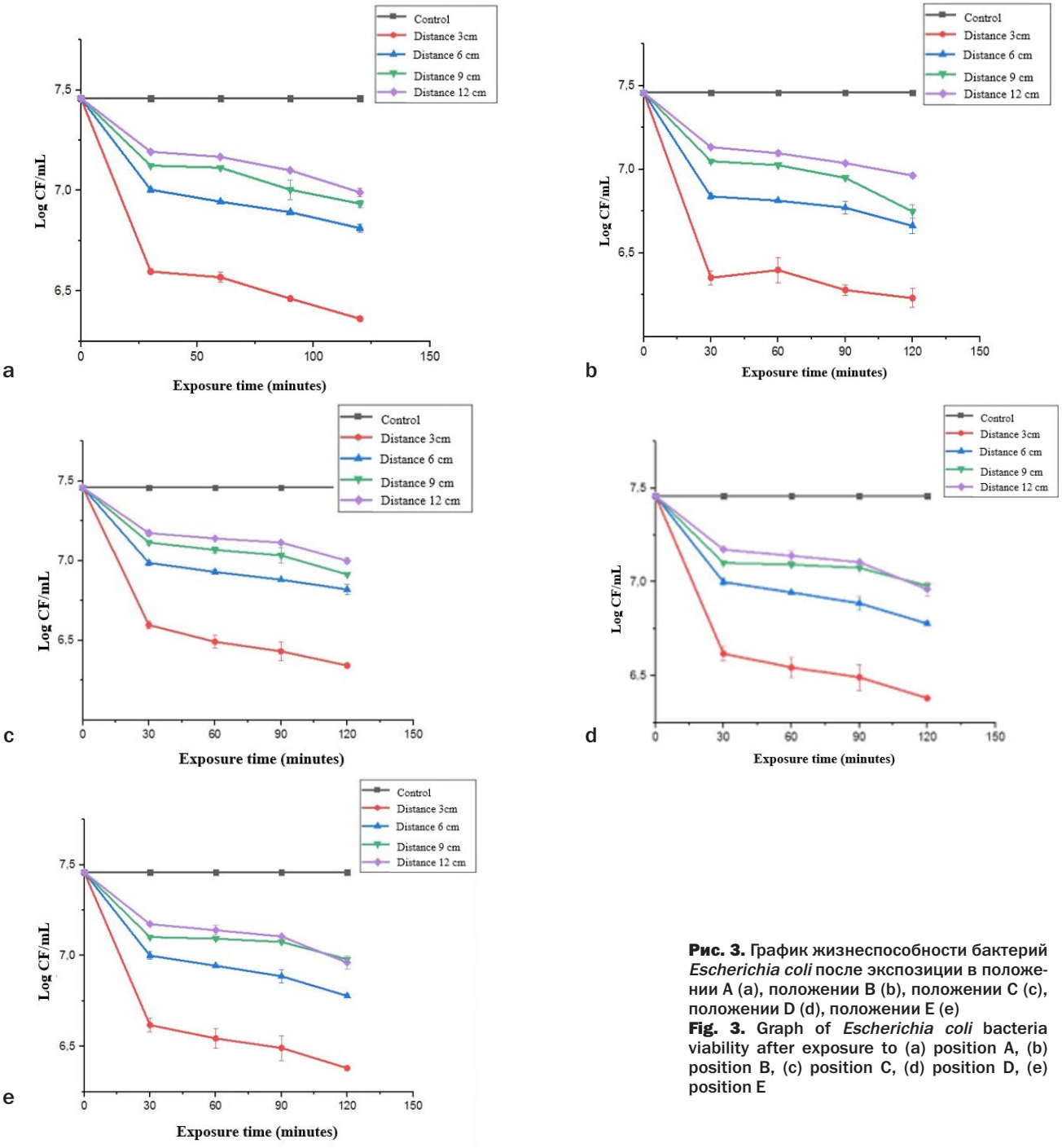

Рис. 3. График жизнеспособности бактерий *Escherichia coli* после экспозиции в положении A (a), положении B (b), положении C (c), положении D (d), положении E (e)
Fig. 3. Graph of *Escherichia coli* bacteria viability after exposure to (a) position A, (b) position B, (c) position C, (d) position D, (e) position E

Bacteria are photo inactivated by the process of photodynamic inactivation (PDI), which is regulated by oxygen, photosensitizer material, and light source. Photoinactivation is the process of preventing cellular metabolism due to cytoplasmic membrane damage brought on by reactive oxygen in lipids and proteins. The membrane transport system in the bacterial cell is either inactive or undergoes cell lysis as a result of reactive oxygen. A 395 nm-wavelength purple LED light source was employed in the study. *Bacillus subtilis* and *Escherichia coli* bacteria's absorption spectrum range is taken into account while adjusting the wavelength.

Power density and energy density numbers are determined for each place based on research data. Position C has the highest power density, which also means that this position also has the highest energy density rating. Because the power density at points A, B, D, and E is almost identical, the energy density or dose at these locations is almost identical as well. As a result, the percentage of fatalities in these positions is practically the same.

A photophysical mechanism underlies the photoinactivation process that happens in *Bacillus subtilis* and *Escherichia coli*. *Bacterial cells* will be photo inactivated when the right combination of light and photosensitizer is used. A photosensitization mechanism, such as light absorption by porphyrins, triggers reactions in the substrate to produce radical oxygen species (ROS) [19]. First step is absorption of photon energy by endogenous porphyrin (10^{-15} s) followed by porphyrin molecule excitation. The excitation is generally achieved via a one photon transition between the ground state and a singlet excited state. Intersystem crossing generates the sensitizer triplet state. The lifetime of this state is longer (ms) so it will react with biology subtract in types I and II photochemistry mechanisms. A Type I mechanism involves hydrogen-atom abstraction or electron-transfer between the excited porphyrin and a substrate, yielding free radicals [20]. These radicals can react with oxygen to form an active oxygen species such as the superoxide radical anion. In a Type II mechanism, singlet oxygen is generated via an energy transfer process during a collision of the excited porphyrin with triplet oxygen. Effect of ROS is damage to the cytoplasmic membrane, allowing leakage of cellular contents or inactivation of membrane transport systems and enzymes that caused peroxidation in lipid and membrane proteins and cell lysis [21, 22].

Bacillus subtilis and *Escherichia coli* are Gram positive and Gram-negative bacteria. Differences of PDT inactivation effect on Gram-positive and Gram-negative lies in the structure of the cell wall [23]. On the outside wall of Gram-positive bacteria with a thickness of 15-80 nm consisting of 100 layers peptidoglycan associated with lipoprotein that binds to the outer membrane and the peptidoglycan teichuronic acid negatively charged relatively porous. The outer membrane of Gram-negative bacteria consisting of lipopolysaccharide, phospholipids, and lipoproteins. outer membrane serves as a barrier against the damaging effects of the outside of the cell and has a permeability to certain molecules [24]. The outer membrane forms an effective barrier permeability. Photochemical reactions type I operative for Gram (+) and reaction type II operative to Gram (-). 90% of singlet oxygen reacts to the cell lipid bilayer and proteins associated with the membrane transport system, lipid and protein peroxidation occurs causing damage to the cytoplasmic membrane and protein denaturation resulting in inactivation of the membrane transport system, interference with cell wall synthesis and the emergence of a multilamellar structure on the side of the cell divider. which cleaves and leaks potassium ions and then cell lysis occurs [23].

Bacillus subtilis and *Escherichia coli* undergo photoinactivation by a photophysical mechanism. When the correct amount of light and photosensitizer are utilized, bacterial cells will be photo inactivated. The photoinactivation process starts when processes in the substrate are triggered by a photosensitization mechanism, such as light absorption by porphyrins. Reactive oxygen is produced during this process, which causes the bacterial cell to lyse or disables the membrane transport system. While others blame radicals like HO for the destruction, many authors mistakenly assume that O_2 is the sole species that matters when it comes to bacterial PDI. According to a theory, Gram-positive bacteria are more sensitive to O_2, whereas Gram-negative bacteria are more sensitive to HO. Variations in the amount that PS binds to the bacteria's microenvironment or the amount of NaN3 that enters the bacterial cell walls may also contribute to variations in NaN_3 inhibition. By employing S. verbascifolium as the PS, we found that the PDI reaction was oxygen-dependent, considerably inhibited by sodium azide, and only marginally inhibited by mannitol. Through the use of type II and type I reactions, respectively, this demonstrated the dependency on singlet oxygen and, to a lesser extent, hydroxyl radicals.

Conclusion

The results of this study indicated that 395 nm purple LED irradiation caused a decrease in Log CFU/mL of *Bacillus subtilis* and *Escherichia coli* bacteria. Inactivation of *Bacillus subtilis* bacteria showed a higher mortality percentage than *Escherichia coli* bacteria. Changes in other irradiation distances also showed a higher percentage of death for *Bacillus subtilis* bacteria than *Escherichia coli* bacteria. The highest percentage of death was 98.5% for *Bacillus subtilis* bacteria and 94.3% for *Escherichia coli* bacteria at position C with an irradiation distance of 3 cm and an energy density of 524 J/cm^2 with an LED exposure time of 120 minutes. This shows that the percentage of death of bacteria *Bacillus subtilis* and *Escherichia coli* increased with increasing doses of LED energy with the greatest percentage of death in Gram-positive bacteria *Bacillus subtilis*.

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