Disinfection of methicillin-resistant *Staphylococcus Aureus* on flat surface by 460 nm light and hydrogen peroxide combination

Truong Nguyen Thuan Thien[1], Ngo Nguyen Vu[2], Tran Trung Tin[3], Vu Van Van[2], Nguyen Thi Thu Hoai[1], Vu Minh Thiet[2*]

[1]International University, Vietnam National University - HCMC, Vietnam
[2]Nguyen Tat Thanh University, Ho Chi Minh City, Vietnam
[3]Ho Chi Minh City University of Technology, Vietnam National University - HCMC, Vietnam
[*]Corresponding author: vmthiet@ntt.edu.vn

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ABSTRACT

Staphyloxanthin (STX) is a carotenoid pigment produced by *Staphylococcus aureus* to protect the bacteria from oxidation stress. Eliminating Staphyloxanthin from *S. aureus* cell membrane by inducing the pigment photolysis using 460nm light, then killing weakened bacteria with an H_2O_2 solution could be a new approach to develop anti - *Staphylococcus aureus* procedure. A model to prove the feasibility of this combination to kill Methicillin-Resistant S*taphylococcus Aureus* (MRSA) bacteria on a flat surface was tested. Material and method: Staphyloxanthin pigment extracted from MRSA bacteria was treated with 460nm light at different light intensities to evaluate the photolysis potential of 460nm light. The change in MRSA shape after 460nm light treatment was also investigated by Scanning electron microscopy. Using glass cover-slips as an emulated model for contact surface in public, the combination of different 460nm light intensities and H_2O_2 0.75% was tested on the surface loaded with MRSA living cells, and the number of MRSA cells survived after treatment was enumerated. Result: Higher intensity and longer light treatment yielded a higher photolysis effect, as 100 and 200 mW/cm^2 light intensity could degrade 77.50% to 83.45% of STX pigment after 20 minutes of irradiation. Also, MRSA cells had significant changes as more wrinkles and bumps appeared under high-intensity 460nm light. When tested on the flat surface of the coverslip, the strongest MRSA eradication effect can be observed in the combination of 200 mW/cm^2 light treatment with 0.75% H_2O_2 solution, as 100% MRSA cells were completely killed after 20 minutes of treatment.

1. Introduction

Staphylococcus aureus is a pathogenic bacterium commonly found to be transmitted from person to person by various routes (Kozajda, Jeżak, & Kapsa, 2019). In addition to the common direct human-to-human transmission route, *S. aureus* bacteria can also be spread in the community through surfaces and equipment in public places, especially in healthcare facilities (Ho & Dinh, 2020; Shi et al., 2015). The transmission of *S. aureus* through surface contact is a major public health concern, especially with dangerous strain as methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA can persist on the touching sites of many different surfaces, such as door handles, bathroom sinks, hand knobs, or toilets (Fritz et al., 2014). Many studies proved difficulties in

current MRSA disinfection strategies, especially in many public and household areas with daily cleaning procedures without specialized disinfectant agents and equipment (Lei, Jones, & Li, 2017). Therefore, an effective MRSA eradication process using simple chemicals to kill the bacteria colonized on public surfaces is needed to be developed.

One common characteristic of many strains of *S. aureus*, including MRSA strains, is the ability to produce a golden pigment called staphyloxanthin (Clauditz, Resch, Wieland, Peschel, & Götz, 2006). This carotenoid pigment contributes to the stability of *S. aureus* cell membrane, and increases the survival chance of the bacterium when attacked by oxidation agents from the environment or the immune system of the host. Elimination of staphyloxanthin on *S. aureus* membrane is a new approach to eradicate *S. aureus* as the bacterium is weaker when exposed to oxidation stress without the protection of the pigment (Clauditz et al., 2006; Xue et al., 2019). Staphyloxanthin can be photobleached by blue light at 460nm wavelength due to the light exciting and breaking down the double bonds C = C in the molecular structure in a process called photolysis (Dong et al., 2019). A combination of blue light at 460nm wavelength and hydrogen peroxide (H_2O_2), an oxidizing agent, has been proven to have a lethal effect on MRSA in planktonic culture (Dong et al., 2019).

Killing *S. aureus* by utilizing blue light at 460nm wavelength in combination with H_2O_2 has a wide range of potential applications, such as skin infection treatment, heal care equipment cleansing, or public surface disinfection, etc. While therapy treatment using the mentioned combination is being studied and yielding promising results (Xue et al., 2019), surface disinfection of *S. aureus* using 460nm blue light in synergy with H_2O_2 is currently not fully investigated. For this reason, in this study, we performed a model of disinfection strategy on a flat surface using 460nm light from a high-energy LED system in conjunction with a low concentration of H_2O_2 solution. The result of this study aims to set a foundation for the method using this approach to clean and eradicate MRSA bacteria on public surface areas.

2. Material and method

The blue light of 460nm wavelength is generated by the LED system provided by Ho Chi Minh City University of Technology, Vietnam. The blue LED system uses a COB LED LE B P2W chip (Osram Licht AG Company, Germany) which is calibrated to produce light at 460nm wavelength with a narrow spectral peak and low heat emission (Figure 1). The strain of *Staphylococcus aureus* used in the study was MRSA strain ATCC® 33592™ (American Type Culture Collection - USA). The experiments were performed in Microbiology laboratory, at NTT Hi-Tech Institute, Vietnam.

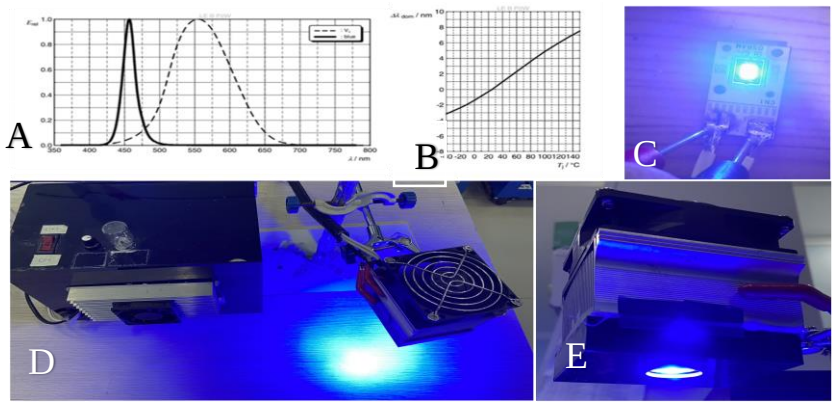

Figure 1. The 460nm LED light system was used in this study. (A): Spectral peak of COB LED LE B P2W chip showing the narrow spectral peak at 460nm. (B): Spectra peak shifts graph of COB LED LE B P2W chip showing the stable spectrum of 460nm light when the LED chip heated over time. (C): COB LED LE B P2W chip emitting blue 460nm light during circuit test

(D and E): The complete 460nm LED system

2.1. Evaluate the photolysis of Staphyloxanthin by 460nm light

Staphyloxanthin (STX) crude pigment was extracted from MRSA colonies growth on Tryptic Soy Agar (Merck, Germany) for 48 hours at 37°C. Pigment extraction was performed following the protocol by Sullivan and Rice (2021). STX crude pigment contained in glass vials was then exposed under 460nm light at 50 mW/cm^2, 100 mW/cm^2, and 200 mW/cm^2 intensity for 5, 10, and 20 minutes (Figure 2). After the exposure ended, the light-treated pigment's absorbance was measured immediately at 470nm to calculate the Percentage of photolysis caused by 460nm blue light.

The percentage of photolysis of STX was calculated by the formula (Valliammai et al., 2021):

$$\%P = \frac{OD470\ C - OD470\ S}{OD460\ C} X\ 100 \quad (1)$$

OD_{470} C is the absorbance at 470nm of STX extract at the beginning. OD_{470} S is the absorbance at 470nm of the STX extract after 460nm light treatment. %P is the percentage of photolysis of STX extract caused by 460nm light. The higher the value of Percentage of photolysis (P%), the stronger the photolysis caused by 460nm blue light.

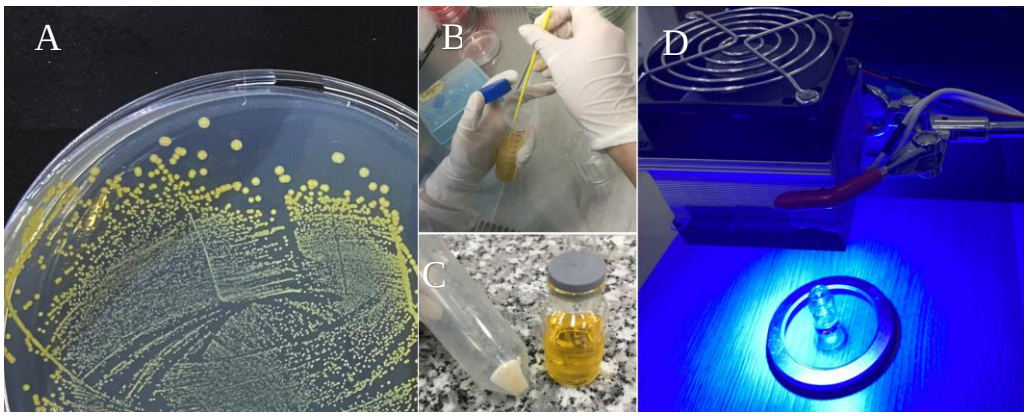

Figure 2. Evaluate the photolysis of extracted STX pigment from MRSA bacteria cells by 460nm light. (A): MRSA bacteria producing STX having golden color. (B): Harvesting the MRSA biomass from colonies. (C): The crude extract of STX having golden color and the biomass of MRSA after all STX extracted from the cell wall. (D): STX crude extract was treated with 460nm light

2.2. Screening the changes in MRSA cells by Scanning electron microscopy

To examine the effect of 460nm light on MRSA membrane, observing the shape of bacteria cells after 460nm light treatment was archived by Scanning Electron Microscopy (SEM). MRSA colonies after 24 hours cultured on TSA agar were resuspended in Phosphate-buffered saline (PBS) solution and diluted to the concentration of 10^6 CFU/ml. The bacterial solution was treated with 03 different light intensity of 460nm light (50, 100, and 200 mW/cm^2) for 20 minutes. 460nm light-treated MRSA sample was immediately prepared for fixation procedure (Watson, McKee, & Merrell, 1980). The surface image of light-treated MRSA cells was taken using S-4800 Field Emission Scanning Electron Microscope (FE-SEM).

2.3. Eradication of MRSA on a flat surface by a combination of 460nm light and H_2O_2

To demonstrate the MRSA eradication effect of 460nm light and H_2O_2 combination, an

experimental model of MRSA disinfection was established on the surface of Microscope cover slip. MRSA bacteria colonies were resuspended in PBS solution and diluted to 10^6 CFU/ml density. 5µl of bacteria solution was spread on an area of 20 x 20mm and let dried naturally by air flow in Class II Biological Safety Cabinet (ESCO Class II Type A2). The cover slips spread with MRSA was then exposed under 460nm light at 50, 100, and 200 mW/cm^2 at 5, 10 and 20 minutes. After 460nm light treatment, the MRSA spot on cover slip was covered with 10µl H_2O_2 0.75% solution. The bacterial surface was let covered with H_2O_2 solution for 5 minutes, then the cover slips were picked up by forceps and pressed on TSA plate to let the surface of bacteria contact with the agar surface. The cover slip was removed after 2 - 5 minutes and the TSA plates were incubated at 37ºC for 24 hours to enumerate the survived bacteria cells after surface treatment (Figure 3).

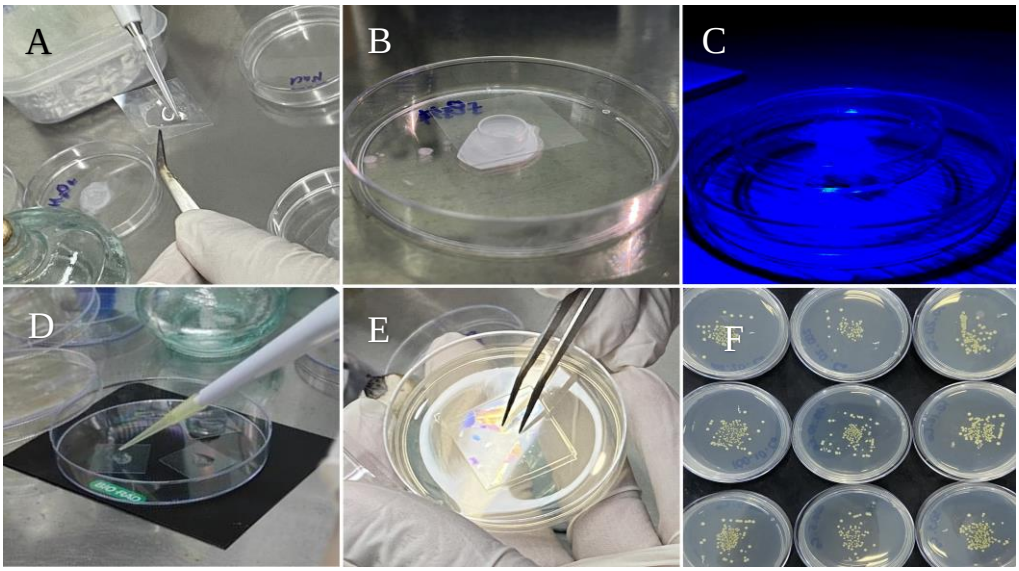

Figure 3. Procedure of testing the MRSA disinfection model on flat surface using glass cover slip. (A): Spreading MRSA on cover slip surface. (B): Air dried to let bacteria cells fixed on the surface. (C): Treating the bacterial surface using 460nm light. (D): Cover the MRSA bacteria with H_2O_2 75% solution. (E): Applied the cover slip to the agar to transfer the MRSA cells to agar medium. (F): Incubate the agar plates and enumerate survival bacterial cells

3. Result

3.1. The photobleaching efficiency of 460nm light on STX pigment extracted from MRSA cells

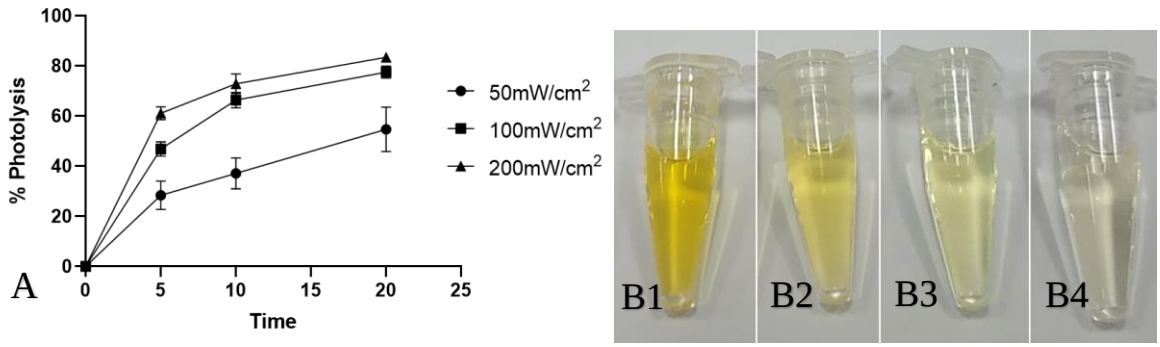

Figure 4. Photolysis effect of different 460nm light intensity over time. (A): The %P value of STX extract treated with 460nm light at 50 mW/cm^2, 100 mW/cm^2, and 200 mW/cm^2 intensity over 20 minutes. (B) The chang in the color of STX extracted treated with 200 mW/cm^2 intensity

460nm light over time. B1- Original color, B2- After 5 minutes treatment, B3 - After 10 minutes treatment, B4- After 20 minutes treatment

This experiment proved the photolysis efficiency of STX pigment by 460nm light depending on light intensity and time of exposure. The strength of 460nm to degrade STX was expressed by the value of %P, as the higher %P value during a period of time, the more STX pigment degraded by 460nm light, representing the stronger photolysis effect. More specifically, STX pigment degradation under all 3 levels of intensity (50 mW/cm^2, 100 mW/cm^2, and 200 mW/cm^2) gradually increased over time, with the efficiency of photolysis was highest in the first 5 minutes of treatment and slowed down after 10 minutes. Photolysis efficiency of 50 mW/cm^2 was the lowest compared to the other light intensities, with an average %P value of 28.36% after 5 minutes of irradiation, and maxed out at 54.67% after 20 minutes of irradiation. The photolysis of 100 and 200 mW/cm^2 light intensity was superior, as 77.50% to 83.45 % of STX pigment can be degraded after 20 minutes of irradiation. It is noteworthy that the %P value of STX after being treated with 100 and 200 mW/cm^2 light intensity tends to equal to each other's, especially after 10 to 20 minutes of treatment (Figure 4). This result shows that the photolysis of STX supposed to have a limit at higher light energy, as increasing the light intensity over a certain level will not yield significant greater efficiency.

3.2. MRSA morphology changes under 460nm light treatment

STX is the pigment located in *S. aureus* cell membrane, and was proved to enhance the stability of the bacterial cell (Clauditz et al., 2006). The sudden photolysis of the pigment directly on *S. aureus* membrane can produce negative effect on *S. aureus* membrane. By this hypothesis, an aggressive dose of 460nm light can produce observable changes in the size and shape of bacterial cells. Under the influence of 460nm light at moderate level (50 mW/cm^2, Figure 5), MRSA cells started to have abnormal signs, starting with wrinkles and bumps on surface of several cells. At higher light intensity (100 and 200 mW/cm^2, Figure 5B) the changes in MRSA cell morphology were more aggressive, as well as the increased number of abnormal cells in the bacterial cells cluster. The changes in the shape of MRSA cells can even be observed by Gram Staining, together with varied levels of stained cell colors (Figure 5A). As previous studies on 460nm effect on *S. aureus*, the 460nm light is not enough to completely eradicate the bacteria in planktonic environment (at least at 10^6 CFU/ml density) (Dong et al., 2019). However, 460nm light may affect the behavior or metabolism pathway of MRSA as a way to cope with the stress of losing STX on the cell membrane. This could lead to the observable strange shapes of MRSA cell as presented in this study. The exact mechanism of this phenomenon is currently being investigated.

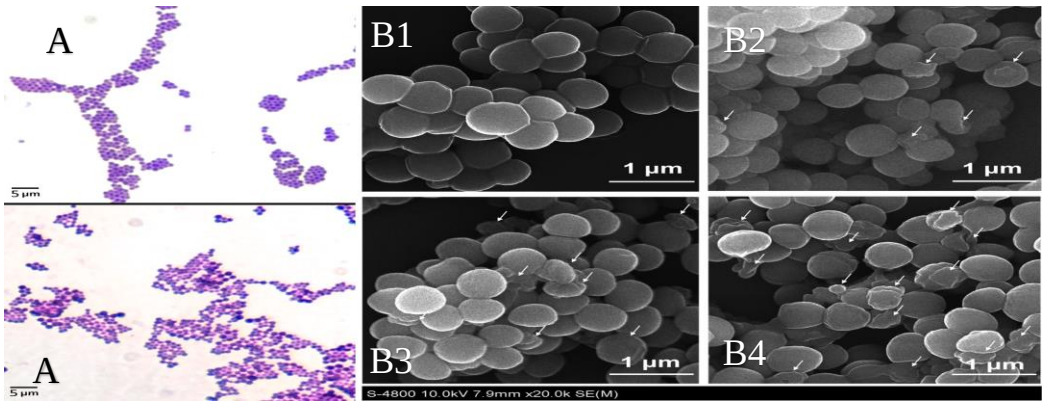

Figure 5. Changes in MRSA morphology after treated with 460nm light (A): Gram staining of MRSA cells treated with 460nm light 200 mW/cm^2 for 20 minutes. A1 - Before light treatment, A2 - After light treatment. Magnification: 100x; bars = 5µm. (B): Abnormal MRSA cells

(arrows) observed under Scanning Electron Microscope. B1 – Normal MRSA cells. B2 - Treated with 50 mW/cm^2 for 20 minutes. B3 - Treated with 100 mW/cm^2 for 20 minutes. B4 - Treated with 200 mW/cm^2 for 20 minutes. Magnification: 20000x; bars = 1µm

3.3. Effect of MRSA surface disinfection by 460nm light and H_2O_2 combination

As a result of the 460nm light treatment, MRSA cells were more vulnerable to H_2O_2 solution, presenting lower MRSA cells surviving on the surface of the glass cover slip. Similar to the trend of photolysis of STX, higher 460nm light intensity and longer illuminating duration result in a stronger disinfection effect. After 50 mW/cm^2 light intensity treatment, 0.75% H_2O_2 solution can only kill 25 - 30% density of MRSA cells on the glass surface. Increasing 460nm light intensity to 100 mW/cm^2 significantly increased the anti-MRSA effect of the H_2O_2 solution as only 10 minutes of treatment can lead to 40 - 50% of MRSA killed (Table 1). The strongest eradication effect can be observed in this study is the combination of 200 mW/cm^2 light treatment at 20 minutes with 0.75% H_2O_2 solution, as 100% MRSA cells were completely killed (Figure 6).

Table 1

The density of MRSA cells survived (CFU/cm^2) on the surface after H_2O_2 and 460nm light combination treatment. Each data is the mean of three replications

	No treatment (Negative control)	**H_2O_2 0.75% treatment only**	**H_2O_2 0.75% with 5 mins light treatment**	**H_2O_2 0.75% with 10 mins light treatment**	**H_2O_2 0.75% with 20 mins light treatment**
50 mW/cm^2	112 ± 7.7	88 ± 8.9	89 ± 1.2	69 ± 2.1	70 ± 3.2
100 mW/cm^2	115 ± 7.1	88 ± 7.7	51 ± 7.3	39 ± 11.4	17 ± 3.8
200 mW/cm^2	122 ± 7.6	97 ± 5.9	21 ± 7.8	4 ± 2.6	0

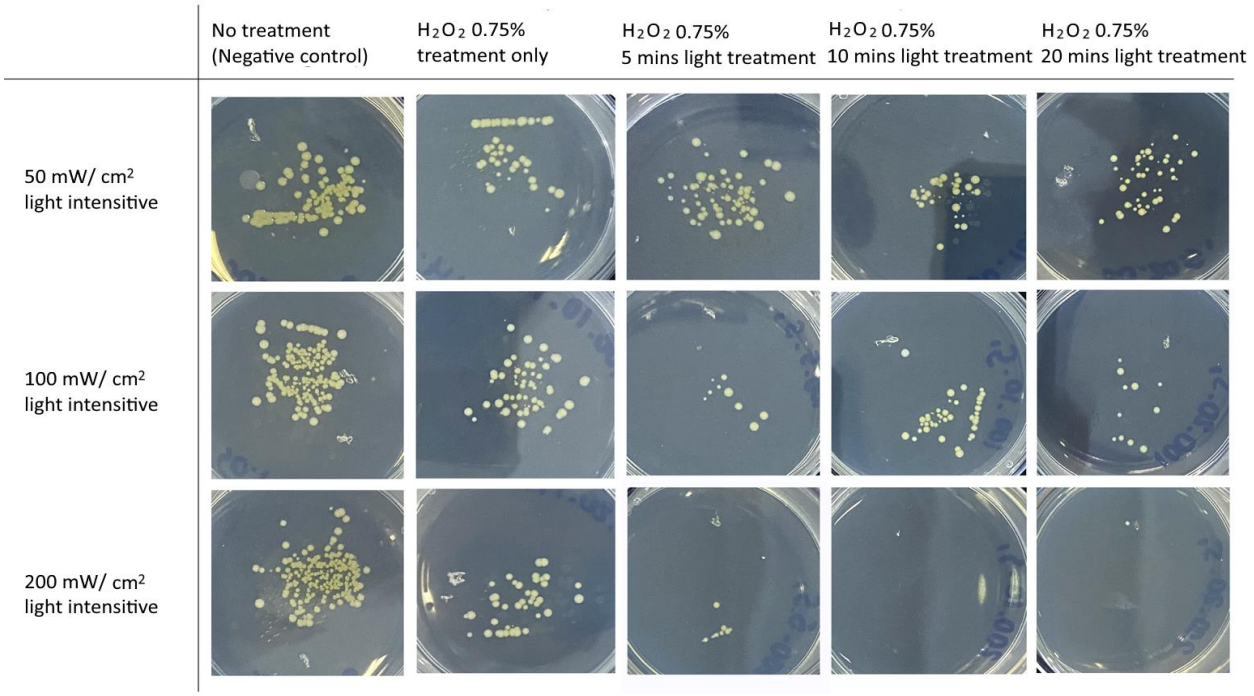

Figure 6. Survived MRSA cells on coverslip surface after H_2O_2 and 460nm light combination treatment

This result showed a similar trend with the experiment performed by Dong (Dong et al., 2019) in using the combination of 460nm light and H_2O_2 to kill MRSA. In the experiment, 10^7

CFU/ml of MRSA bacteria in planktonic culture were treated with 200 mW/cm^2 light for 20 minutes and were completely killed off by H_2O_2 0.0375% after the blue light treatment. The observable anti-MRSA effect increased with higher light intensity and time duration. In this study, although we used a higher concentration of H_2O_2 solution (0.75%), not all MRSA cells on the glass surface were killed (especially after 20 minutes of 100 mW/cm^2 light treatment). The eradication efficiency of MRSA on the surface may be lower than the planktonic cell treatment due to the arrangement of MRSA cell layers when air-fixed, as upper layers of cells may cover and reduce the light effect on lower MRSA cell layers. However, this issue can be overcome by increasing the light intensity and H_2O_2 concentration.

The result shows the promising potential of 460nm light in synergy with H_2O_2 solution to disinfect surfaces contaminated with MRSA, the effect can be enhanced by increasing light intensity and time duration of treatment.

4. Conclusion and suggestion

H_2O_2 solution is an anti-bacterial oxidizer widely used to treat wounds and cleanse medical equipment or instruments. Using H_2O_2 as a disinfectant on MRSA-infected surfaces has the advantage that H_2O_2 is low cost, easy to obtain and prepared, and does not cause harm to human health at low concentrations (below 3%). However, MRSA bacteria and *S. aureus,* in general, have several mechanisms to resist the attack of H_2O_2, such as the production of catalase enzyme or the presence of Staphyloxanthin pigment - an anti-oxidizer - on the cell membrane. Increasing the concentration of H_2O_2 may result in greater effectiveness, but also poses more risks from other damage caused by H_2O_2 oxidation activity. By utilizing 460nm light at suitable intensity, the tolerance of this bacterium to H_2O_2 can be greatly decreased and easier to be killed. This combination of 460nm light and H_2O_2 can be developed into a reliable method aimed to kill MRSA on surfaces in healthcare facilities.

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References

Clauditz, A., Resch, A., Wieland, K. P., Peschel, A., & Götz, F. (2006). Staphyloxanthin plays a role in the fitness of staphylococcus aureus and its ability to cope with oxidative stress. *Infection and Immunity, 74*(8), 4950-4953.

Dong, P. T., Mohammad, H., Hui, J., Leanse, L. G., Li, J., Liang, L., ... Cheng, J. X. (2019). Photolysis of staphyloxanthin in methicillin- resistant staphylococcus aureus potentiates killing by reactive oxygen species. *Advanced Science, 6*(11), Article 1900030.

Fritz, S. A., Hogan, P. G., Singh, L. N., Thompson, R. M., Wallace, M. A., Whitney, K., ... Fraser, V. J. (2014). Staphylococcus aureus contamination of environmental surfaces in households with children infected with methicillin-resistant S. aureus. *JAMA Pediatrics, 168*(11), 1030-1038.

Kozajda, A., Jeżak, K., & Kapsa, A. (2019). Airborne Staphylococcus aureus in different environments - a review. *Environmental Science and Pollution Research, 26*(34), 34741-34753.

Lei, H., Jones, R. M., & Li, Y. (2017). Exploring surface cleaning strategies in hospital to prevent contact transmission of methicillin-resistant Staphylococcus aureus. *BMC Infectious Diseases, 17*(1), 85-94.

Shi, L. S., Xu, C. J., Jia, H. B., Chen, W., Zhou, X. F., & Li, X. H. (2015). Spread of Staphylococcus aureus between medical staff and high-frequency contact surfaces in a large metropolitan hospital. *International Journal of Nursing Sciences, 2*(4), 366-370.

Ho, T. V., Dinh, H. T. N. (2020). Survey on the causative agent of wound infection and evaluate the antibiotic resistance of *Staphylococcus aureus* bacteria at Military Hospital 175. *Tạp chí Khoa học Công nghệ và Thực phẩm, 20*(2) 112-112.

Sullivan, L. E., & Rice, K. C. (2021). Measurement of staphylococcus aureus pigmentation by methanol extraction. *Methods in Molecular Biology, 2341*, 1-7.

Valliammai, A., Selvaraj, A., Muthuramalingam, P., Priya, A., Ramesh, M., & Pandian, S. K. (2021). Staphyloxanthin inhibitory potential of thymol impairs antioxidant fitness, enhances neutrophil mediated killing and alters membrane fluidity of methicillin resistant Staphylococcus aureus. *Biomedicine & Pharmacotherapy, 141*, Article 11933.

Watson, L. P., McKee A. E., & Merrell B. R. (1980). Preparation of microbiological specimens for scanning electron microscopy. *Scanning Electron Microscopy, (Pt 2)*, 45-56.

Xue, L., Chen, Y. Y., Yan, Z., Lu, W., Wan, D., & Zhu, H. (2019). Staphyloxanthin: A potential target for anti-virulence therapy. *Infection and Drug Resistance, 12*(2019), 2151-2160.