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Ultraviolet Light-Emitting Diode (UV-LED) Sterilization of Citrus Bacterial Canker Disease Targeted for Effective Decontamination of *Citrus Sudachi* Fruit

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A kind of citrus fruit with special flavor, *Citrus sudachi* harvested in Japan, are exported to various countries. However, the *Citrus sudachi* needs to be sterilized using aqueous solution of sodium hypochlorite because there is a possibility of the adhesion of *citrus bacterial canker* (CBC) which is not found in Europe. Due to the sterilization with time-consuming work, a more effective decontamination technique is required. A decontamination method using ultraviolet (UV) light irradiation is thus anticipated. Especially, the use of light emitting diodes (LEDs) with UV light has many advantages in terms of energy consumption, lifetime, and compactness; although an appropriate method is yet to be established. In this study, we evaluate the fundamental effectiveness of UV-LED decontamination on the basis of the bactericidal ability on CBC in petri dishes, using six kinds of UV-LEDs (265, 280, 285, 300, 310, and 365 nm). For each irradiation, the resultant bactericidal abilities (BAs) were evaluated precisely taking into account the differences in their optical absorptions. In addition, BAs per unit photon number were also estimated, as a fundamental wavelength-dependence of BA. As a result, the effectiveness of UV-LED irradiation with relatively short wavelengths was demonstrated clearly.

Key words : Citrus bacterial canker / Ultraviolet light-emitting diode (UV-LED) / UV decontamination / Citrus sudachi fruit.

INTRODUCTION

Over the past decade, globalization in both information and logistics has accelerated worldwide, and this trend will continue to gain momentum in the past COVID-19 era. Under such social and physical environments, the assessment and inspection of contaminants and pathogens adhering to humans and goods will be important for our safety. Among these, the safety of harvested foods will be of paramount importance. Until recently, imports and exports of harvested products were under strict control in each country. The transfer of unintentionally adhesive pathogens is a severe problem.

Citrus bacterial canker (CBC) is a serious disease affecting the production of almost all citrus fruits. *Xanthomonas citri* subsp. *citri* (Xcc), the etiologic agent of CBC, is a quarantine pathogen in the European Union (EU) (Golmohammadia et al., 2012; Grahm et al., 2004). Therefore, many kinds of citrus fruits must be decontaminated before entry into the EU marketing zone. *Sudachi* is one such citrus fruit that is generally decontaminated by sodium hypochlorite (Rabbee et al., 2019; Redondo et al., 2015) in the export to EU region. However, this decontamination process is difficult and labor-intensive because the fruit must be individually wiped in this technique, and the growth of the export quantity has then been slack. Therefore, the realization of a more effective decontamination method is strongly required.

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A decontamination method using the irradiation of ultraviolet light with relatively short wavelengths, such as deep ultraviolet (DUV, < 300 nm) or UV-C (< ~280 nm), is one of the most candidates to facilitate the decontamination of postharvest agriculture products (Esua et al., 2020), because the inactivation of various pathogenic micro-organisms can be induced by DUV or UV-C. The effective damage caused by the irradiation to the DNA of micro-organisms results in their inactivation. (Hijnen et al., 2006), in which a dimerization between two activated thymine moieties of the nucleic acids is generated and the photo-activation of thymine is enhanced by DUV or UV-C due to an absorbance peak of DNA places around 260 nm (Kaneko et al., 1979; Nagpal et al., 2021). Therefore, we anticipate the use of a decontamination method based on the inactivation of pathogenic micro-organisms using DUV light for the effective decontamination of large amounts of *sudachi* fruits.

LEDs with DUV (DUV-LEDs) are expected as an alternative light source for the UV decontamination technique soon. At present, mercury lamps, having a distinct and strong emission line with a wavelength of 254 nm, are the main light source for DUV irradiation. However, it is well known that the mercury lamps have some intrinsic problems in terms of lifetime, power consumption, and the usage of mercury itself. Compared with such conventional UV lamps, LED has some advantages, including various available wavelengths, no mercury, a compact and robust design, faster startup time, and lower voltages (Hirayama et al., 2014; Kneissl et al., 2019; Nagamatsu et al., 2019). Among these advantages, pathogen inactivation can be optimized due to the different wavelength outputs and compact reactor design. Accordingly, DUV-LEDs have been studied by many researchers as a decontamination technique in water (Blatchley et al., 1997; Oguma et al., 2013; Rattanakul et al., 2018; Song et al., 2016). The typical bacteria in water are *Escherichia coli*, reported to be effectively decontaminated at a 265 nm light emission. However, DUV-LEDs for decontamination are usually used at a peak wavelength of 280 nm because LEDs with a wavelength of 280 nm light emission efficiency are twice as high compared with those of 265 nm (Hirayama et al., 2014; Kneissl et al., 2019; Nagamatsu et al., 2019). Additionally, light attenuation by the generation of ozone is a problem in the UV-C wavelength region (Umar et al., 2015; Wijakhum et al., 2017), potentially limiting its use for several applications. On the other hand, UVB-LEDs with a wavelength of 280-285 nm not only have high light-emitting efficiency compared with that of UVC-LEDs but also low light attention in the air. Therefore, it is important that the effectiveness of UVB-LEDs is also carefully evalu-

ated from the point of view of practical uses.

In this study, we propose using a decontamination method with UV-LEDs for the sterilization of CBC targeted for the effective decontamination of the surface of *sudachi* fruits slotted for export. Additionally, we clarify the effective wavelength of LEDs and their decontamination effect. Recently, DUV-LED irradiation have been demonstrated to have a virucidal effect on SARS-CoV-2, generating intense discussion around the potential and feasibility of its practical uses (Inagaki et al., 2020; Nogueira et al., 2020; Torres et al., 2020). Based on related studies, the advancement and expansion of DUV-LED treatments in various fields are expected to promote human health and safety.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The CBC strain (*Xanthomonas campestris* pv. *citri*, KC20RR, MAFF673034), a standard pathogenic strain, was from the National Agriculture and Food Research Organization. First, the CBC strain with skim milk medium (10% skim milk, 1% monosodium glutamate) was defrosted in a water bath at 37°C. Second, 200µL of thawed CBC strain was coated on an adequate medium (XCSM medium) and kept at 27°C for 72 h. XCSM medium was suggested by Shiotani and Ozaki (1999) (Ozaki et al., 1999). This medium was already reported to be useful for analyzing the CBC strain by Shiotani et al (Shiotani et al., 2000, 2007, and 2008). This technique was used to control the CBC strain number in this study. Third, colony scraped from the medium was suspended in physiological saline (NaCl 0.9%) to an absorbance ratio of 0.1 at a wavelength of 600 nm. For determination of viable cell count, the CBC bacterial concentration in this stock solution was serially diluted from 10⁻¹ to 10⁻⁸ /cm³ with physiological saline, and 0.1 mL of each dilution was spread on the surface of the XCSM medium. After this preparation, each sample was irradiated by UV light, immediately. For each UV irradiation condition, three samples were prepared and evaluated.

UV irradiation study and devices

UVB- and UVC-LEDs were purchased from Nikkiso Co., Ltd. as a form of mounted device. The peak wavelengths of these LEDs were 265, 280, 285, 300, and 310 nm. The full width at half maximum values of these LEDs was approximately 15 nm. UVA-LEDs with a peak wavelength of 365 nm were produced by OptoSupply, Ltd. Each LED module was equipped with one chip. Light emission went through a flat quartz crystal window of 2.3 mm² which was used as a protection component. These emission spectra (Fig. 1) were measured

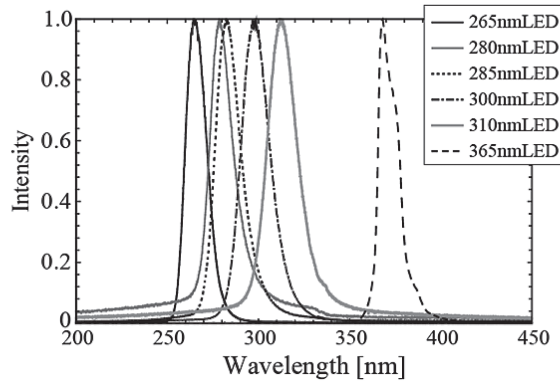


FIG. 1. The emission spectra of each UV-LED in this study. The relative intensity was normalized to each peak emission.

using a spectrometer (Ocean Optics, Inc.).

These LEDs were fixed in irradiation machines and mounted on an aluminum substrate, as shown in Fig. 2. The distance D between the LED and sample in a petri dish was 30 mm, determined by the height of the sample stage. The quantitative UV light power density was measured by a photodetector (UV-035EQ, OSI Optoelectronics) sensitive in the UV wavelength region at the sample plane. The irradiation power was controlled by a constant current circuit.

Evaluation of bactericidal ability

The suspended solution in petri dish shown above was irradiated with an arbitrary radiation amount from the measured value and irradiation time. The amount of radiation dose was thus determined as below:

$$\text{Dose amount [mJ/cm}^2\text{]} =$$

$$\text{Light intensity [mW/cm}^2\text{]} \times \text{Exposure time [s].}$$

The value of the bactericidal ability at 10^{-7} was the detection limit for this quantification method. For comparison, a non-irradiated sample was prepared as the typical control sample. After irradiation, the sample was cultivated for 72 h at 27°C. Colony-forming units (CFU) were counted in the sample, and the bactericidal ability (BA) was calculated using the following equation:

$$\text{BA} = \log_{10}\left(\frac{S}{C}\right),$$

where S and C are the CFU/mL in the irradiation sample and typical control sample, respectively.

Absorption spectrum measurement of CBC suspension

The net absorption spectrum of CBC was measured using a UV-VIS spectrometer (UV-1280, Shimadzu Corp.). A spoonful of CBC strain was carefully scooped from the surface of the cultivated skim milk media, thereby avoiding the peripheral skim milk portion. The CBC strain was dispersed in a water solution of physio-

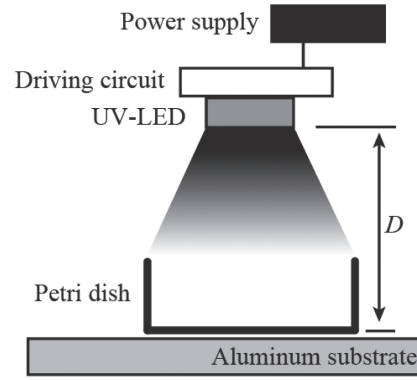


FIG. 2. Experimental set up for UV irradiation.

logical saline (NaCl 0.9%). The bacterial suspension was then poured into a quartz cell, with an optical path length of 10 mm, to measure the absorption spectrum. Simultaneously, the net NaCl water solution and skim milk medium (described in above) dissolved in distilled water at 1 vol% were prepared to measure their absorption spectra. These were used to verify the measurement either essential CBC absorption spectrum could be obtained or the CBC spectrum was influenced by the absorption of the other substances (NaCl, Skim milk, etc.).

RESULTS AND DISCUSSION

Evaluation of bactericidal effect of UV-LED irradiation

To verify the BA of the UV-LED irradiations, photographs of the cultivated samples irradiated by each UV-LED in petri dishes are shown in Fig. 3. Here, each sample was exposed to UV light with the wavelengths of 265, 280, 285, 300, 310, and 365 nm, at a dose amount of 28 mJ/cm². We can see that the number of ascertainable CBC-colony units becomes larger as the wavelength of UV-LED increases. Therefore, the effectiveness of UV irradiation can be quantitatively confirmed at relatively shorter wavelengths.

In the next stage, the effectiveness of UV irradiation was quantitatively evaluated based on its BA, as a function of irradiation dose amount, and the results were plotted in Fig. 4. The bactericidal characteristics can be categorized into three groups. The first was the relatively low ability group ($\text{BA} > -2$ at ~ 400 mJ/cm²) composed of two kinds of UV-LED with wavelengths of 310 and 365 nm. The Second was the moderate ability group ($-2 > \text{BA} > -4$ at ~ 100 mJ/cm²) composed of a UV-LED with a wavelength of 300 nm. The third was the relatively high ability group ($-4 > \text{BA} > -7$ at ~ 10 mJ/cm²) composed of three UV-LEDs with wavelengths of 265, 280, and 285 nm. In the section below, we discuss this interesting categorization into three groups. These

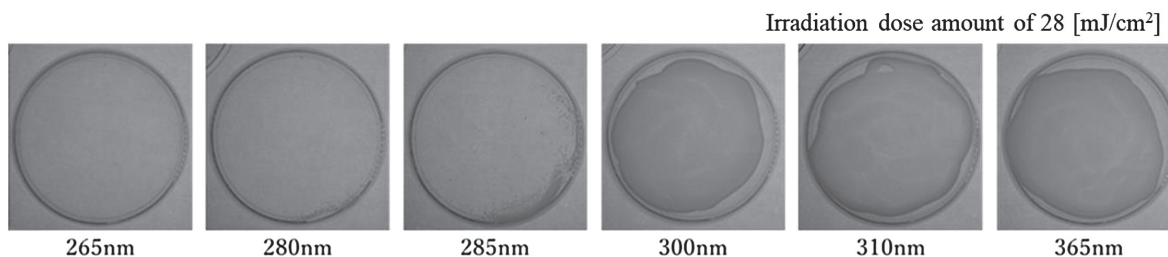


FIG. 3. Photographs of colony-forming observations of culture without dilution with physiological saline after UV irradiation of each wavelength. The diameter of the petri dish is 90 mm.

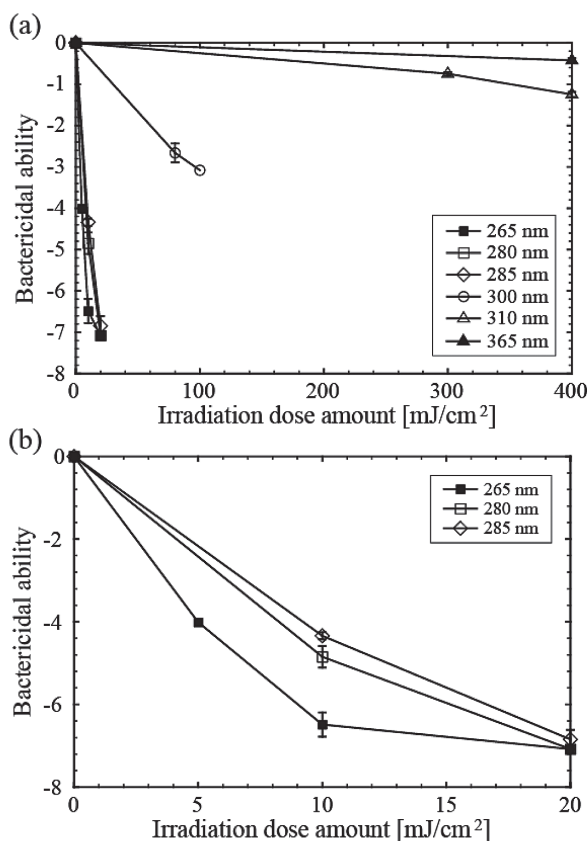


FIG. 4. Bactericidal ability as a function of the irradiation dose amount for each UV-LED. (b) Magnified graph for the bactericidal abilities of DUV-LEDs.

BA characteristics are ordered by the wavelength of the UV-LED, as shown in Fig. 4. The DUV-LED with a wavelength of 265 nm, which is the shortest wavelength in the present study, was the most effective irradiation in the UV-LED sterilization for CBC. Simultaneously, similar BA was also obtained using UV-LEDs with wavelengths of 280 and 285 nm. This result is encouraging because UV-LEDs with shorter wavelengths are more expensive, with an accompanying lower efficiency and lower supply volume in the market, even in the case of commercial products.

Figure 5 (a) shows a comparison result of the typical data for BA extracted from our experimental results in Fig. 4. In Fig. 5 (a), for UV-LEDs with wavelengths of 265, 280, and 285 nm, the dose amount was 10 mJ/cm². For UV-LED with wavelengths of 300 nm, the dose amount was 100 mJ/cm². For UV-LEDs with wavelengths of 310 and 365 nm, the dose amount was 400 mJ/cm². The dashed line indicates that the BA of NaOCl treatment, which is cited from a previous report by Zamuner et al. (2020), as a reference, with a BA value of up to 4-log. By comparing our results with the BA of an authorized approach (NaOCl treatments), we regard DUV-LED irradiation as a promising approach for CBC sterilization. Especially, the use of a DUV-LED with a wavelength of 265 nm significantly improved the BA for CBC.

Wavelength dependence in bactericidal effect

To consider the wavelength dependence of the BA in more detail, we attempted to correct the BA values in Fig. 5 (a). First, to correct the differences in irradiation dose amounts, the BA values were corrected as

$$BA' = \frac{BA}{\text{dose amount [10 mJ/cm}^2\text{]}},$$

and then graphed them, as shown in Fig. 5 (b). This correction emphasized the BAs of DUV-LEDs well. (Here, it must be noted that the BA was represented originally by the exponent of the rate of colony number after UV irradiation to that of the control. Therefore, the corrected BAs indicate the only relative magnitude relationship among their corrected BA values.) When UV light was irradiated to the sample, the substances absorbed the optical energy according to their absorption coefficients. The absorption and transmission spectra of a CBC (KC20RR) suspension were measured and plotted in Fig. 6 (a) and 6 (b), respectively, including the absorption spectra of the NaCl solution itself and of the culture medium (skim milk). Because NaCl solution has no distinct peak in the wavelength region from 210 to 400 nm, its absorption does not affect to the CBC absorption spectrum.

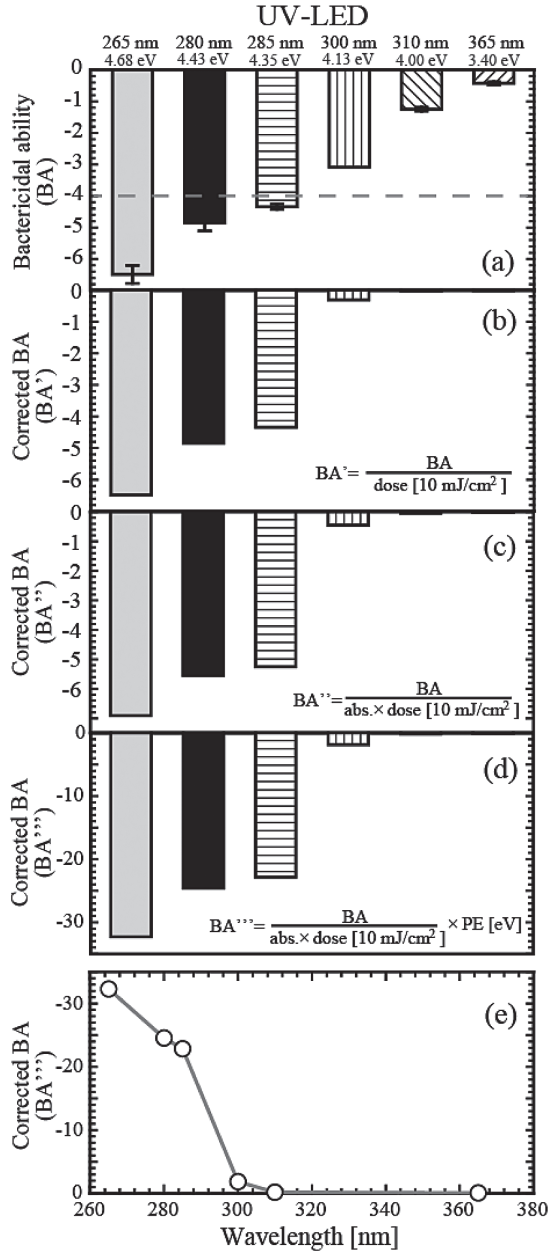


FIG. 5. (a) Comparison of the bactericidal abilities (BAs) in CBC strains irradiated by UV-LEDs. The dashed line indicates that the BA of NaOCl treatment, which is cited from a previous report by Zamuner et al. (2020), as a reference. (b), (c), and (d) Corrected BAs using dose amount, absorption, and unit photon number, respectively. (e) Spectrum of the corrected BA in (d).

Similarly, though the absorption spectrum of skim milk exhibits two distinct absorption ends around 230 and 280 nm, their influence cannot be obviously confirmed in the CBC spectrum. The CBC spectrum thus indicates substantial absorption characteristics. The absorption rate (abs.) for each UV-LED wavelength was obtained using the corresponding transmission T as

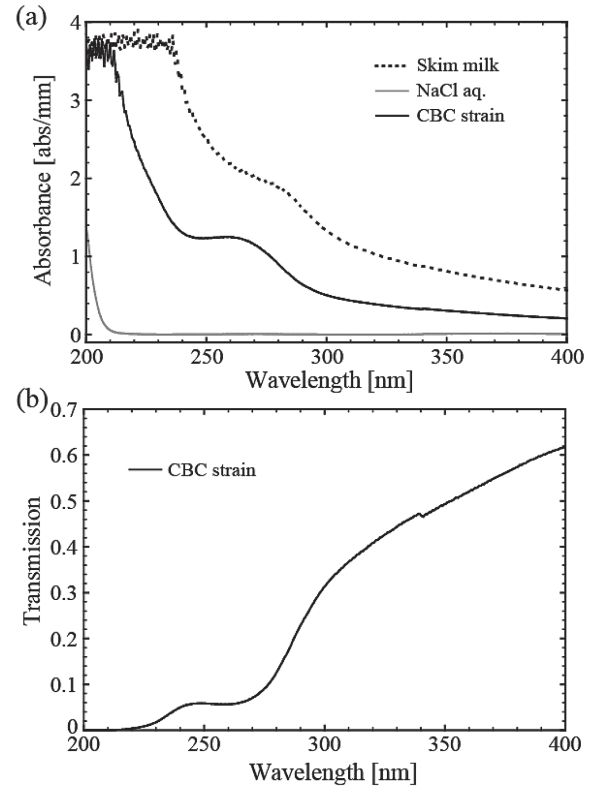


FIG. 6. (a) Absorption and (b) transmission spectra of CBC strain suspended in NaCl solution. Absorption spectra of NaCl solution itself and skim milk solution are also simultaneously plotted in (a).

$$\text{abs.} = 1 - T.$$

The absorption rate was then introduced into the equation BA' as follow:

$$BA'' = \frac{BA}{\text{abs.} \times \text{dose amount [10 mJ/cm}^2\text{]}}$$

and the calculated BA'' was graphed in Fig. 5 (c). It is confirmed that the bipolarization between the BAs of DUV-LEDs and those of other UV-LEDs was increased by a correction taking into account the optical absorption. Moreover, we attempted to correct the BAs using photon energy (PE) corresponding each UV-LED wavelength. Substantially absorbed optical energy was defined as

$$\text{Absorbed Optical Energy [J]} =$$

$$\text{abs.} \times \text{dose amount} = e \times \text{PE [eV]} \times N,$$

where e is the elementary charge (1.6×10^{-19} [C]), and N represents the incident photon number in the irradiation duration. Finally, the BA was corrected as follow:

$$BA''' = \frac{BA}{\text{abs.} \times \text{dose amount [10mJ/cm}^2\text{]}} \times \text{PE [eV]}.$$

The calculated BA''' was graphed in Fig. 5 (d). This characteristic thus correspond BAs per unit photon number, as a principle wavelength-dependence of BA. The BA''' was then plotted as a function of the corresponding wavelength in the spectrum, as we show in Fig. 5 (e). The corrected BA (BA''') spectrum increased rapidly toward shorter wavelength region at around 300 nm. This spectrum therefore has three regions. The first is the relatively small BA''' region in the wavelength region from 310 to 365 nm. The Second is the transitional wavelength region at around 300 nm. The third is the relatively high BA''' region corresponding to the DUV region. This categorization into three regions coincides with that of the BA characteristics of the irradiation dose amounts in Fig. 4, mentioned above. These results correspond two authorized bactericidal mechanisms at work in UV sterilization. As well known, thymine dimerization in DNA is induced by DUV irradiation because thymine has an absorption peak of around 260 nm (Setlow et al., 1963; Li et al., 2019; Nagpal et al., 2021). The chemical photoreaction (thymine dimerization) occurring between two activated thymines results in the inactivation of micro-organisms. The BA''' spectrum shape increasing at around 300 nm is significantly similar to that of thymine absorption (Bucher et al., 2016 ; Nagpal et al., 2021). This fact strongly supports the inference that the resultant high BAs resulting from the use of DUV-LEDs is attributable to thymine dimerization, even for this CBC sterilization study. On the other hand, reports elucidate another inactivation mechanism based on UV-A irradiation (Oppezzo et al., 2001 ; Hamamoto et al., 2007), that is oxidation inactivation. Therefore, two intrinsic UV inactivation mechanisms are present in the DUV and UV-A region, respectively, and the transitional wavelength (λ_{tr}) region is present at around 300 nm. Interestingly, the distinguishing moderate BA arose in the very narrow wavelength region of $285 < \lambda_{tr} < 310$ nm. This might be suggesting the presence of any synergetic effects between the two inactivation mechanisms.

As a result, the effectiveness of UV sterilization using DUV-LEDs was demonstrated clearly, and the wavelength dependence was verified through the corrective treatments of BAs, considering the experimental conditions, including the inactivation mechanisms.

DUV-LED developments for practical use

DUV-LED technologies are in development to optimize cost, efficiency, and cooling. Specifically, a cooling water system is necessary for heat management in LEDs, LED chip prices are still high in the DUV regions, and the UVC-LEDs have <10% external quantum efficiency (Hirayama et al., 2014; Kneissl et al., 2019;

Nagamatsu et al., 2019). However, these problems may soon be resolved, as many researchers are rapidly improving UV-LEDs. Even so, the UV-LED system is preferred for decontamination applications now.

CONCLUSION

From this study, DUV-LED clearly demonstrated the ability to decontaminate pathogenic bacteria. The decontamination system using LEDs also has many potential advantages and commercial applications. The export of citrus fruit to meet EU standards could be achieved by a system using UV-LEDs, based on the decontamination effect using UV radiation is higher than that of Sodium Hypochlorite exhibited in Fig. 5. Additionally, it is possible to treat many fruits at the same time, and the LED irradiation system has a high advantage for minimal human effort and cost. Furthermore, because the UV-LED radiation system can be used in an assembly-line-like manner, its productivity is high.

For the etiologic agent of CBC, we demonstrated the UV-LED decontamination of and confirmed that the effective wavelength for decontamination is between 265 and 285 nm. Simultaneously, it is suggested that the decontamination mechanism is based on the DNA damage and subsequent inactivation, generated from a photochemical reaction by DUV irradiation. The high decontamination effect of wavelengths shorter than 285 nm indicates the potential to decontaminate not only citrus fruits such as *sudachi* but also the surfaces of any number of foods for a wide range of commercial and public health applications.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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