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Blue light induced free radicals from riboflavin in degradation of crystal violet by microbial viability evaluation

Ji-Yuan Liang[a], Jeu-Ming P. Yuann[a], Zong-Jhe Hsie[a], Shiuh-Tsuen Huang[b,*], Chiing-Chang Chen[b,*]

[a] Department of Biotechnology, Ming-Chuan University, Taoyuan 33343, Taiwan
[b] Department of Science Education and Application, National Taichung University of Education, Taichung 40306, Taiwan

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ABSTRACT

Crystal violet (CV) is applied in daily use mainly as a commercial dye and antimicrobial agent. Waste water containing CV may affect aquatic ecosystems. Riboflavin, also known as vitamin B_2, is non-toxic and an essential vitamin required for the functions of the human body. Riboflavin is photosensitive to UV and visible light in terms of generating reactive oxygen species. This study investigated the potential application of blue light on riboflavin, so as to come up with an effective way of degrading CV during its treatment. Photosensitivity of CV leading to degradation in the presence of riboflavin was investigated by light intensity, exposure time, and irradiation dosage. The degradation of CV during riboflavin photolysis treatment was studied by a UV/vis spectrometry and chromatography. The effects of CV degradation on microbial viability are relevant when considering the influences on the ecosystem. This study proved that riboflavin photochemical treatment with blue light degrades CV dye by ROS formation. The riboflavin photolysis-treated CV solution appeared to be transparent during conformational transformations of the CV that was rearranged by free radical species generated from riboflavin photolysis. After riboflavin photolysis, colony-forming units (CFUs) were determined for each CV solution. CFU preservation was 85.2% for the CV dissolved riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 120 min. Degradation of CV by riboflavin photochemical procedures can greatly reduce antimicrobial ability and serve as an environmental friendly waste water treatment method. Our results presented here concerning riboflavin photolysis in degradation of CV provide a novel technique, and a simple and safe practice for environmental decontamination processes.

1. Introduction

Crystal violet (CV), a triphenylmethane dye, has been extensively used as a histological stain, antibacterial agent, textile industry dye, dermatological agent, targetable sensitizer [1], and veterinary medicine [2]. The World Bank estimates that 17–20% of industrial water pollution comes from textile industry dye, leading to major impact on the quality of water resources. CV is a steady dye that persists in the environment for a long period of time and has toxic effects on aquatic ecosystems. It has been reported that that CV acts as a mitotic poison, potent carcinogen, and potent clastogene in addition to promoting tumor growth in some species of fish [3]. CV is easily absorbed into fish tissues by water exposure. It has been reported that CV has carcinogenic and mutagenic effects in rodents and can induce renal, hepatic and lung tumor in mice [3].

CV is not only a commercial textile dye, but also an antimicrobial agent. CV is highly effective against Gram-positive bacteria due to its ability to penetrate the cell wall and covalently bond to proteins [4]. Waste water containing CV possesses a serious threat to aquatic ecosystems. Hence, the removal of CV from wastewater of different industries is essential not only to protect the human health, but to the protection of soil and water ecosystems as well.

Reactive oxygen species (ROS) are generally reactive molecules or radical species, including hydrogen peroxide (H_2O_2), the hydroxyl radical (·OH), the superoxide anion radical ($O_2^{\cdot -}$), and the peroxyl radical (ROO·) [5]. Photolysis treatment has been applied to the degradation of CV in photoreaction systems that generate ·OH. The catalyst of photochemical degradation has usually been composed of heavy metals and the light source being mostly UV radiation, such as BiO_xI_y/g-C_3N_4 [6], BiO_xI_y/GO [7], BiO_xCl_y/BiO_mBr_n [8], $SrTiO_3$ [2], Pt-TiO_2 [9], ZnO [10,11], Fe_2O_3 [12], and Fe^{2+} or Fe^{3+}/H_2O_2 [1,13]. Heavy metals and UV radiation are hazardous. Nevertheless, formaldehyde was formed

* Corresponding authors.
E-mail addresses: hstsuen@mail.ntcu.edu.tw (S.-T. Huang), ccchen@mail.ntcu.edu.tw (C.-C. Chen).

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from the photodegradation mechanism of CV dye through the photocatalytic pathway [2]. Formaldehyde is a particularly hazardous substance. The researches concerning CV degradation intermediates on ecosystems were few. It is important to avoid any influences of CV photo-degradation intermediates on the ecosystems.

The micronutrient riboflavin (vitamin B_2) is required by the human body to undergo cellular processes, such as the TCA cycle, for energy production. It is also required by flavoproteins, such as the cofactors flavin adenine dinucleotide (FAD) and riboflavin-5′-phosphate (FMN), to metabolize fats, ketone bodies, carbohydrates and proteins [14]. Riboflavin is generally recognized as safe when used in accordance with good manufacture or feeding practice [6]. The micronutrient riboflavin is non-toxic and is an essential vitamin required for the functions of the human body.

Riboflavin is sensitive to light [15]. It has been illuminated under UV [14–18] and blue light [19,20] in order to reach a photo-excited state. After being light photo-energized, riboflavin is converted into triplet excited-state riboflavin. $O_2^{\cdot -}$ or singlet oxygen is produced through the reaction of the triplet excited-state riboflavin [15,21]. Our previous study reported that riboflavin photolysis with blue light treatment has been applied to the inactivation of *E. coli,* and *Staphylococcus aureus* strains, including a methicillin-resistant strain (MRSA), by damaging nucleic acids and DNA cleavages caused by $O_2^{\cdot -}$ [19,20,22].

$O_2^{\cdot -}$ generated from light-excited riboflavin can be utilized to examine the effect of luminance on light reactions of nitro blue tetrazolium (NBT) [23]. NBT is used as an indicating scavenger that is reduced by $O_2^{\cdot -}$ and in turn can be used to determine the production of $O_2^{\cdot -}$ [23,24].

Light quality and intensity have been shown to be the major factors that correlate with the $O_2^{\cdot -}$ formed from riboflavin photolysis [23,25]. Previous studies have investigated irradiation of riboflavin with blue [19,20] and ultra-violet light (UV) [16–18]. The wavelength of blue light is longer than that of UV. Lights with shorter wavelengths such as UV that have high energy may cause damage to cells. UV or even high-intensity radiation can be considered as a highly risky practice. The cytotoxicity of UV and high-intensity radiation, however, needs to be carefully evaluated. The micronutrient riboflavin is non-toxic and is an essential vitamin required for the functions of the human body. Riboflavin is sensitive to light and can be illuminated by blue light [19,20]. The blue light wavelength is longer than UV light, while the irradiation dose of blue light is less than UV light.

$O_2^{\cdot -}$, an oxidant, can be formed from ·OH and hydroperoxide compounds, causing cell damage, inflammation, atherosclerosis and aging of cells [23,26]. Our previous study reported that riboflavin photolysis with blue light treatment has been applied to inactivation of *E. coli* by damaging nucleic acids and DNA cleavages caused by $O_2^{\cdot -}$ [19,20]. By scavenging $O_2^{\cdot -}$, the oxidation of lipid membranes can be prevented [27]. It would be of interest to further investigate if the $O_2^{\cdot -}$ generated from riboflavin photochemical treatments via visible light enhances the efficacy of CV degradation.

CV is used as an industry dye and as an antimicrobial agent. It has been reported that CV is very effective against *Candida*, *Streptococcus* and *Staphylococcus* species, and is moderately effective against Gram-negative bacteria [4]. Wastewater containing CV affects microbial ecosystems. Therefore, the microbial viability is an essential clue for estimating the efficiency of CV contamination. It requires the development of efficient, and environmentally friendly waste water treatment processes without sterilization. *S. aureus* is commonly found on the skin and in the nasal mucous membranes of humans and animals. *S. aureus* is often used as an indicator organism for determining the quality of hygiene standards. Therefore, if the degradation of CV via riboflavin photolysis can be shown to decrease the efficacy of CV antimicrobial ability, an environmentally friendly waste water treatment protocol can be established.

Riboflavin mediated photolysis with blue light could potentially degrade and eradicate environmental CV dye. The aim of the current study was to develop an effective method of CV degradation by applying blue light to riboflavin in photochemical reactions. This study compares the effects of riboflavin photolysis on CV degradation and inactivation of *S. aureus*, based on light intensity, irradiation time, and irradiation dosage. The effects of light quality on the production of $O_2^{\bullet -}$ from light-excited riboflavin were investigated by NBT reduction assay. The degradation of the microbial viability of CV was used as an indicator of the effect on the environment and as a clue to estimate the efficiency of the novel technique.

2. Materials and Methods

2.1. Chemicals

L-Methionine, riboflavin, mono-potassium phosphate and potassium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO). CV hydrate was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Nitro blue tetrazolium chloride (NBT) was purchased from Bio Basic Inc. (Markham, Ontario, Canada). The riboflavin and phosphate buffered solutions (pH 7.8) were prepared before the experiment. Ultra-pure water purified by a Milli-Q system was used as a solvent throughout this study.

2.2. Effects of Light Quality on Riboflavin Photolysis

The photolysis reactions were implemented in a plastic box (104 × 74 × 55 in cm) and its exterior was covered with black cloth as described previously [25]. Three LED lamps (58 cm in length) in blue, green and red (VITALUX T8HO LED tube lights, Vita LED Technologies Co., Tainan, Taiwan) were used as light sources. The irradiance was checked by a solar power meter (TM-207, Tenmars Electronics Co., Taipei, Taiwan). As applied in a previous study [25], the wavelengths of the emitted maxima of the blue, green and red lights were 463, 529, and 632 nm, with the spectral widths at half heights ($W_{1/2}$) of 23, 31, and 14 nm, respectively.

NBT reduction was carried out based on Beauchamp and Fridovich's method [28], with minor modifications, to determine the production of $O_2^{\cdot -}$ in this study [23,25]. Each chemical was freshly prepared prior to the experiment. The total volume of the reaction aliquot was 3 mL and the concentrations of riboflavin, methionine and NBT were 0.0024, 10.0 and 0.16 mM in 100 mM phosphate buffer at pH 7.8, respectively. The reaction solution was irradiated by blue, green and red lights at 2.0 mW/cm^2 for 10, 20, and 30 min, respectively. The $O_2^{\cdot -}$ was generated from riboflavin photolysis by reducing NBT to form formazan, which was detected at 560 nm via a UV/vis spectrometer (Lambda35, Perkin-Elmer).

2.3. Effects of Riboflavin Photolysis on CV Degradation

The effects of riboflavin photolysis on CV degradation were examined in an opaque plastic cup (8 cm in height and 7 cm in diameter). Blue LED lamps (DC 12 V 5050, vitaLED Technologies Co., Tainan, Taiwan) were pasted onto the plastic cylinder as described previously [20]. The reaction solution was held within a glass tube, which was set at the top-end of the cylinder. The cylinder was placed in a cold room where the temperature was monitored by an infrared thermometer (MT 4, Raytek Co., Santa Cruz, CA) at 9 ± 1°C. The irradiance was validated by a solar power meter (TM-207, Tenmars Electronics Co., Taipei, Taiwan). The DC power supply (YP30-3-2, Chinatech Co., New Taipei City, Taiwan) was employed to gauge the light irradiance. The intensity attenuation of blue light caused by the glass tubes used in this study was < 4%.

The effects of riboflavin photolysis on CV degradation were examined in a cold room at the temperature of 9 ± 1°C. All solutions were made in 100 mM of phosphate buffer (pH 7.8). In brief, solutions of CV (10 mg/L) in the presence of 240 μM riboflavin were exposed to

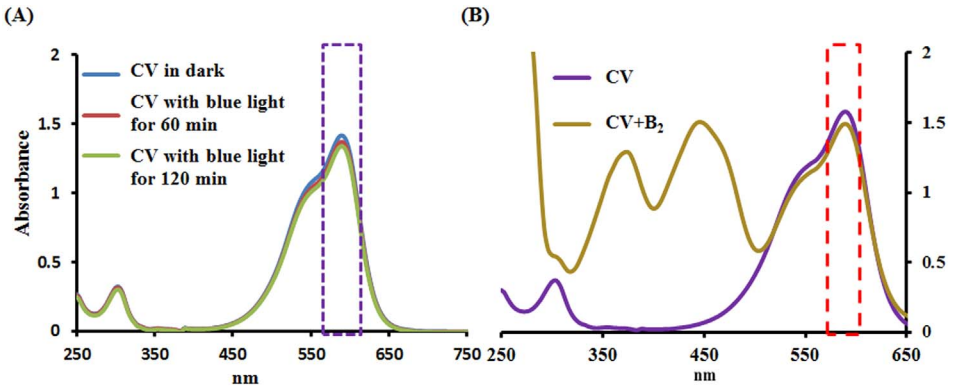

Fig. 1. Spectra of (A) 10 μg/g CV illuminated by blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min, respectively, and (B) 10 μg/g CV alone and CV in 240 μM riboflavin (B_2) solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

blue light irradiation at 2.0 mW/cm^2 for 60, 120, 240, 360, 480, and 600 min, with CV being prepared under the same conditions but left in the dark as control. The absorbance of the mixed solution was scanned at 250–650 nm spectrophotometrically.

2.4. HPLC Analysis of CV after Riboflavin Photolysis

The CV solutions were analyzed as part of the HPLC analysis. The irradiation settings were prepared as described in Section 2.3 and examined in a cold room at the temperature of 9 ± 1°C. Briefly, in (A), the solution of 25 mg/L CV in 240 μM riboflavin was exposed to blue light irradiation at 0.5 mW/cm^2 for 2.5, 5, 10 and 15 min, while, in (B), the solution of 10 mg/L CV in 240 μM riboflavin was exposed to blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min. The solution of CV in 240 μM riboflavin solution was prepared in the dark as control.

HPLC was performed on a Hitachi L system equipped with a quaternary pump (Hitachi-L2130) connected to a vacuum degasser, a thermostatted column compartment, a manual injector, a photodiode array detector (DAD, Hitachi-L2450), and an EZChrom Elite workstation. The Mightysil RP-18 GP Aqua column (5 μm, 4.6 mm id × 250 mm, Kanto Chemical Co., Tokyo, Japan) was eluted at a rate of 1.0 mL/min. The mobile phase was 0.2% H_3PO_4 in 80% methanol. The CV solution and the sample solution were filtered through a 0.45 μm filter (Millipore) prior to injection. DAD detector was set at 588 nm for acquiring chromatograms with UV–vis spectra being recorded between 220 and 650 nm during analysis.

2.5. Effects of CV and Degraded CV on Viability of S. aureus

The effects of CV and degraded CV solutions on the viability of *S. aureus* were investigated. The irradiation settings were prepared as described in Section 2.3. The degraded CV solutions were prepared by dissolving CV in a 240 μM riboflavin solution (pH 7.8) and the final CV concentration was 10 mg/L. Then, the CV solutions were exposed to blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min. As for the control treatment, a thick aluminum foil was used to wrap tubes holding the CV solution that was left in the dark.

S. aureus (BCRC Taxonomy ID: 10,451) was grown overnight in LB broth at 37°C. After overnight growth, 500 μL of *S. aureus* was loaded into a 1.5 mL centrifuge tube and diluted five times with double sterilized water. Cultures were grown to an optical density of 600 nm (OD_{600}) at 0.5 (~6 × 10^7 CFU/mL). After centrifugation at 10,000 rpm for 5 min, the supernatant was removed. One mL of CV or degraded CV in riboflavin solution was added to the pellet, followed by resuspension, while riboflavin solution without CV was used as the control. The bacterial solution diluted with CV or degraded CV solution was incubated in a cold room for 60 min where the temperature was monitored by an infrared thermometer at 9 ± 1°C. Then, the 200 μL bacterial solution was transferred to LA plates for overnight growth at 37°C. The survival of *S. aureus* following treatment was examined by counting the number of viable colony forming units (CFUs) after treatment. The inactivation rate of *S. aureus* was calculated by the decreasing percentage (= [1 – *C*/*N*] × 100%, where *C* and *N* are the numbers of CFUs after the placing CV in the dark, with degraded CV treatment [*C*] and without CV treatment [*N*], respectively). Thus, the reduction percentage was defined as the negative value of the inactivation rate.

2.6. Statistics

Data are represented by mean ± standard deviation (SD) of three separate experiments. A homoscedastic two-tailed Student's *t*-test was employed to determine whether the two sets of measurements were different. The level set for statistical significance was $p < 0.05$.

3. Results

3.1. Spectra of CV in Riboflavin Solution

The effects of blue light and riboflavin on the CV degradation were first investigated in this study. Fig. 1(A) shows the spectra of CV after blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min. The maximal absorbance of CV appears at 588 nm and the integrity of CV molecules was not changed under blue light irradiation. Fig. 1(B) shows the spectra (250–650 nm) of CV and CV in riboflavin solution. Three absorption peaks of CV in riboflavin solution, at 373, 445 and 588vnm, are observed.

3.2. Effect of Light Quality on Riboflavin Photolysis

As shown in Fig. 2(A), the spectra of riboflavin after blue light irradiation at 2.0 mW/cm^2 for 60 and 240 min. Two absorption peaks of riboflavin were observed, at 373 and 445 nm, while the absorbance of riboflavin at 445 nm was dramatically decreased when treated with blue light irradiation for 240 min. Our previous study reported that the spectra of riboflavin were measured during the course of color illuminations in photo-decomposition reactions [29]. Irradiation with blue light showed the highest photo-decomposition efficiency of riboflavin in visible light, while the absorbance of riboflavin at 445 nm decreased dramatically upon irradiation. The green and red lights showed fewer effects because the spectral changes were not significant as described previously [29].

The effects of the light quality on the NBT reduction during the riboflavin photolysis are shown in Fig. 2(B). $O_2\cdot^-$ generated from light-excited riboflavin can be determined by the NBT reduction method [23]. As shown in Fig. 2(B), the levels of riboflavin photolysis were increased along with the reaction time. The highest efficiency of riboflavin photolysis was observed under blue light irradiation. The photochemical effects of green and red lights were 11.1% and 2.44% of that of blue light, respectively. The green and red lights showed slight

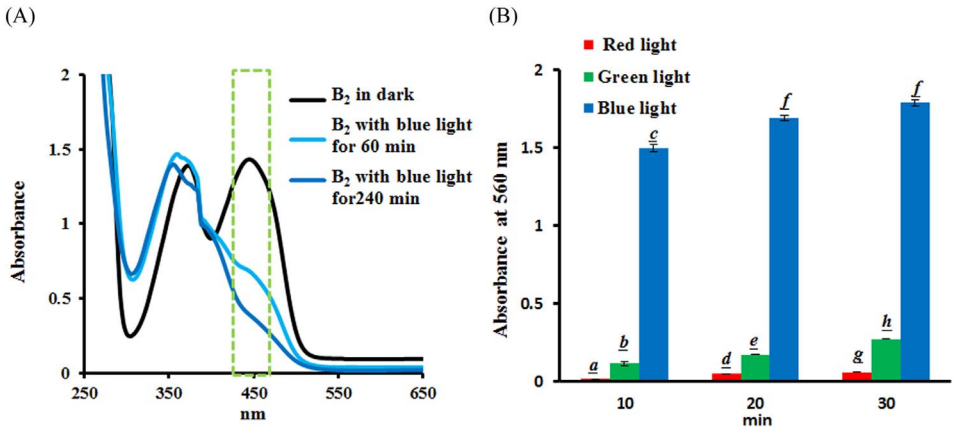

Fig. 2. (A) 240 μM riboflavin illuminated by blue light irradiation at 2.0 mW/cm^2 for 60 and 240 min. (B) Effects of light quality on NBT reduction by riboflavin (B_2) photolysis at 2.0 mW/cm^2 with irradiation for 10, 20, and 30 min. Data were represented by mean ± SD, where $n = 3$. Significant differences ($p < 0.05$) between groups are indicated by the different letters above each bar.

effects, a minor observation noticed in this study. The irradiation with blue light exhibited the highest photo-decomposition efficiency of riboflavin in visible light, whereas green and red lights showed little effects as described previously. Blue light is therefore much more efficient and critical in terms of $O_2^{\cdot -}$ generation from riboflavin photolysis in visible light.

In this study, $O_2^{\cdot -}$ was analysed by the riboflavin/NBT method. The spectra of the riboflavin/NBT method upon irradiation by a blue LED at 2.0 mW/cm^2 for 30 min are shown in Fig. 3(A). The photochemically reduced riboflavin generated $O_2^{\cdot -}$. The $O_2^{\cdot -}$ then reduced NBT to form formazan, which can be detected at 560 nm with the mechanism of formazan formation being detailed in Fig. 3(B) as described previously [30].

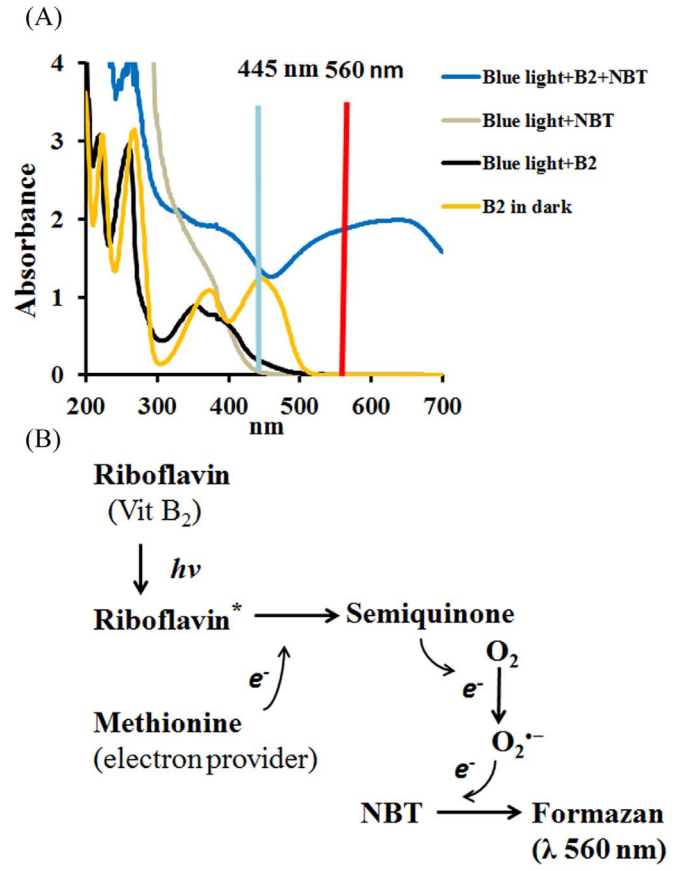

Fig. 3. (A) Spectra of B_2/NBT method upon irradiation by a blue LED at 2.0 mW/cm^2 for 30 min. (B) The generated $O_2^{\cdot -}$ from riboflavin (B_2) photolysis and assayed by measuring the photoreduction of nitro blue tetrazolium (NBT). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. HPLC-DAD Analysis of CV after Riboflavin Photolysis

The chromatograms of the 25 mg/L of CV in riboflavin solution with blue light irradiation were recorded by a photodiode-array detector operating at 588 nm (Fig. 4A). For the aqueous solution of CV, the major chromatographic signal appears at 3.61 min (line a). After riboflavin photolysis, the other chromatographic peaks are observed at 3.39 and 3.13 min in line (b–e). As shown in Fig. 4(B), the degradation percentage of CV in riboflavin solution was increased along with irradiation time. A ninety-percent degradation rate on CV (25 mg/L) was achieved in riboflavin solution with blue light irradiation at 0.5 mW/cm^2 for 15 min. Then, the effects of degraded CV in riboflavin solution on microbial viability were also investigated as described in Section 2.5. A 99% inactivation rate on *S. aureus* was achieved by CV (25 mg/L) in riboflavin solution with blue light irradiation at 0.5 mW/cm^2 for 15 min, as shown in Fig. 4(C).

3.4. Effects of Riboflavin Photolysis on CV Degradation and Decolorization

The effects of riboflavin photolysis on CV degradation and decolorization were also investigated. Prior to riboflavin photolysis, 10 mg/L of CV in 0.1 M of phosphate buffer solution (pH 7.8) appeared to be deep blue. Upon riboflavin addition and photolysis, CV in riboflavin solutions showed different color changes during blue light irradiation. As seen in Fig. 5, CV in 240 μM riboflavin solution was treated with blue light irradiation at 2.0 mW/cm^2 for 240 min, the solution appeared to be transparent. CV may have reacted with riboflavin during the photolytic process, producing deviations of CV through structural rearrangements

Fig. 6(A) shows the spectra (250–650 nm) of CV in riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 60, 120, 240, 360, 480, and 600 min. CV is a polycyclic aromatic compound; its main absorbance is at 588 nm in the visible light region. The absorbance of CV in riboflavin solution at 588 nm was dramatically decreased by blue light irradiation. By quantitating the absorbance at 588 nm, the extent of CV decreased upon riboflavin photolysis, with reduction percentages of 77.0, 77.8, 84.4, 85.0, 88.9 and 90.1% for 60, 120, 240, 360, 480 and 600 min of blue light irradiation, respectively, suggesting that CV is fragmented after riboflavin photolysis.

The absorbance of CV in riboflavin solution was decreased, whereas riboflavin photolysis was increased in a time-dependent manner as shown in Fig. 6(A). However, when the light irradiation intensity was enhanced to 5.0 mW/cm^2 as shown in Fig. 6(B), the CV decreased upon riboflavin photolysis with reduction percentages of 69.1, 70.4, and 72.1% for 10, 20, and 30 min under blue light irradiation at 5.0 mW/

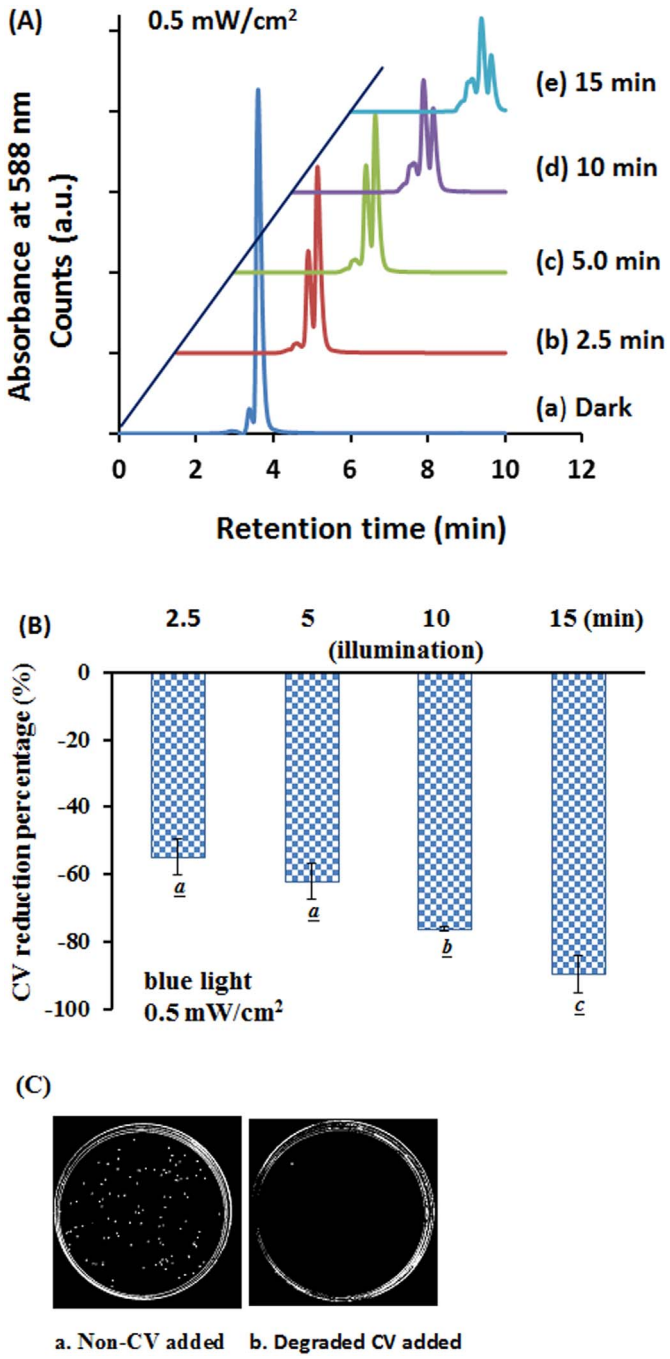

Fig. 4. (A) Chromatograms of 25 mg/L CV in riboflavin (B_2) solution treated with blue light irradiation. The chromatogram of CV without blue light irradiation is shown in line (a) as a control. Other chromatograms represent those treated with blue light irradiation for (b) 2.5, (c) 5.0, (d) 10, and (d) 15 min. (B) Effects of riboflavin photolysis on CV degradation. The reduction percentage was the degradation of CV in a riboflavin solution after treatment with blue light irradiation at 0.5 mW/cm^2 for 2.5, 5, 10, and 15 min, as detected by an HPLC system at 588 nm. (C) Effects of degraded CV in riboflavin solution on viability of *S. aureus*. 25 mg/L of CV in riboflavin solution was treated with blue light irradiation at 0.5 mW/cm^2 for 15 min. Data were represented by mean ± SD, where $n = 3$. Statistical differences ($p < 0.05$) between groups are indicated by the different letters below each bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

cm^2, respectively.

The self-photolysis of CV under blue light irradiation does not decompose effectively. Because the activated species was produced after the riboflavin was excited by blue light, a good amount of photo-decomposed CV in riboflavin solution was expected. According to the pseudo-stationary hypothesis, the activated species, $O_2{\cdot}^-$ and ·OH, can be considered as a constant. Rate expression (Eq. 1) is simplified into a pseudo-first order kinetic model (Eq. 2). The Eq. (3) could be obtained by the Eq. (2) integrated between $t = 0$ and t = t. Hence, the photo-decomposition rate of the CV photolysis in riboflavin solution was simulated by pseudo-first order kinetic model.

$$-\frac{dC_{cv}}{dt} = k_2 \times C_{cv} \times C_{activ.} \quad (1)$$

$$-\frac{dC_{cv}}{dt} = k_{appa.} \times C_{cv} \quad (2)$$

$$-\ln\left(\frac{C}{C_0}\right) = k_{appa.} \times t \quad (3)$$

where, C_0 and C are the initial concentration of CV and the concentration of CV at reaction time t, respectively (mg/L). The k_{appa} is the apparent decomposition rate constant. The results showed a linear regression from the CV photolysis in riboflavin solution experimental data, suggesting that the decomposition rate of CV can be described by the pseudo-first order kinetics with respect to CV concentration. The values of k_{appa}, apparent degradation rate constant, is 0.2352 (h^{-1}), estimated through linear regression analysis of the data, and the better quality photocatalytic ability of riboflavin might be ascribed to its efficient utilization of blue light. The results are shown in Fig. 7.

3.5. Effects of CV Treated with Riboflavin Photolysis on Microbial Viability

The chromatograms of the 10 mg/L CV in riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min have been recorded by a DAD operating at 588 nm. A 98.7% degradation rate on CV was achieved with riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 120 min, as shown in Fig. 8(A).

CV is used as a mutagenic and bacteriostatic agent in medical solutions, and it has been reported that CV raises toxic effects in the environment [3]. The effects of degraded CV on microbial viability were investigated in this study. Fig. 8(B) shows that the higher the irradiation time of CV treated with riboflavin, the lower the level of reduction percentage of *S. aureus* after 1 h of incubation. A 94.3% inactivation rate on *S. aureus* was achieved with 10 mg/L CV treatment, as seen in Fig. 8(B). When the degraded CV was added, as shown in Fig. 8(B), with 10 mg/L of CV in riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min, 23.7 and 14.8% inactivation rates were observed in *S. aureus*, respectively.

4. Discussion

The micronutrient riboflavin is non-toxic and is an essential vitamin required for the functions of the human body. The absorbance of riboflavin at 445 nm was decreased by 52.1% for 60 min and 72.3% reduction was observed for 240 min blue light irradiation at 2.0 mW/cm^2 as shown in Fig. 2(A). Ahmad et al. (2008) [31] used a mercury vapor fluorescent lamp (emission at 405 and 435 nm) for riboflavin photolysis experiments. A gradual decrease in absorbance of aqueous phase at 445 nm indicated the loss of riboflavin, while an increase in absorbance of chloroform extract at 356 and 445 nm suggest the timely formation of lumichrome and lumiflavin, respectively [20]. The absorption spectra of riboflavin at 445 nm exhibited the major absorbance in visible light. Under blue light irradiation, however, the lower absorbance of riboflavin at 445 nm implies a higher quantum yield of the riboflavin photolysis.

It has been reported that buffer pH, light illuminance and NBT concentrations are the major factors correlated with the absorbance of the NBT reduction method [23]. As shown in Fig. 2(B), the energy doses of blue light irradiation for 20 and 30 min at 2.0 mW/cm^2, were equivalent to 2.4 and 3.6 J/cm^2, respectively. The NBT reduction under

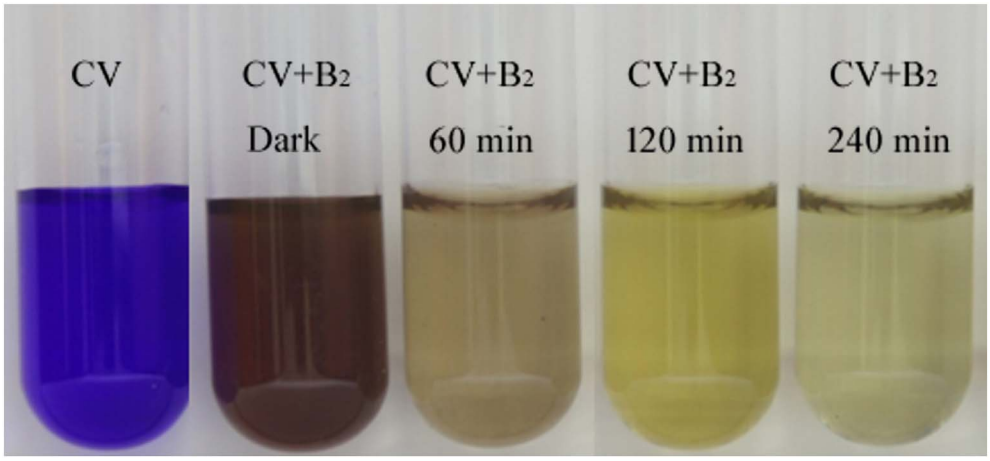

Fig. 5. Color changes of CV in 240 μM riboflavin (B_2) solution treated with blue light irradiation at 2.0 mW/cm^2 for 60, 120, and 240 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

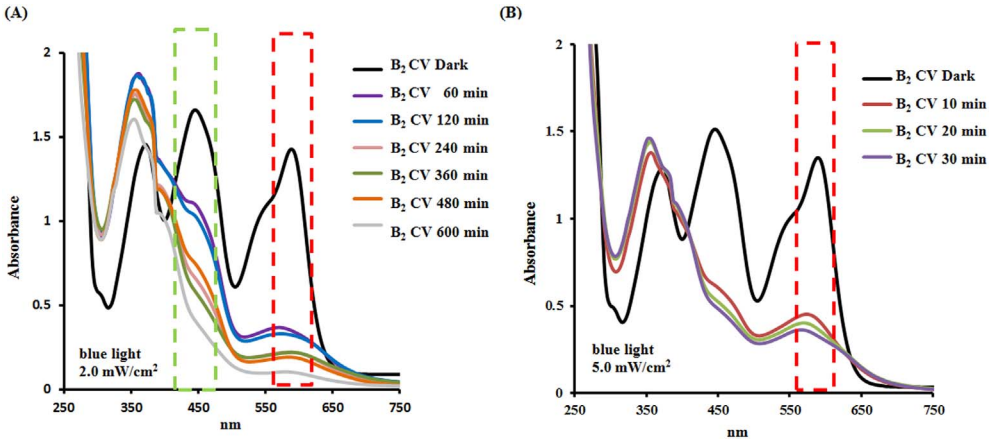

Fig. 6. (A) The absorbance spectra of CV in 240 μM riboflavin (B_2) solution treated with blue light irradiation at 2.0 mW/cm^2 for 60, 120, 240, 360, 480 and 600 min. (B) The absorbance spectra of CV in 240 μM riboflavin solution treated with blue light irradiation at 5.0 mW/cm^2 for 10, 20 and 30 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

blue light treated with 20 min and 30 min irradiation were both insignificant in these time frames. This implies that riboflavin can be used to induce free radical species under blue light irradiation at 2.4 J/cm^2.

The effects of light quality on the riboflavin photolysis were investigated in Fig. 2(B). The generation of $O_2{\cdot}^-$ from the intermediates during the decomposition of riboflavin in aqueous solution was detected via NBT reduction [20]. The highest efficiency under blue light irradiation was used to inspect the riboflavin photolysis, so to carry out photo reduction of the isoalloxazine ring by electrons donated by the ribityl side chain [15]. Riboflavin (RF) is endogenously present in living organisms, and it is a well-known sensitizer for the light-induced oxidation of different substrates. Aerobic photo-oxidative processes for sensitized events in solution containing the superoxide radical anion ($O_2{\cdot}^-$) participating in these reactions, generated by electron transfer with quantum yield $\Phi O_2{\cdot}^-$ being 0.009 and singlet molecular oxygen [$O_2(^1\Delta_g{}^-)$] generated by energy transfer with quantum yield ΦΔ being 0.47, have been addressed [32,33]. The processes of photochemical reactions of riboflavin are represented in Eqs.4–6, where ^{1}RF· and ^{3}RF· are the electronically excited singlet and triplet states of RF, respectively. RF$^{\cdot+}$ is the molecular ion of RF with one electron dislodged from the molecule, while O_2 ($^3\Sigma g-$) is the dissolved ground-state molecular oxygen [34].

$$RF + h\nu \rightarrow {}^1RF\cdot \rightarrow {}^3RF\cdot \quad (4)$$

$$^3RF\cdot + O_2(^3\Sigma g^-) \rightarrow RF^{\cdot+} + O_2{\cdot}^- \quad (5)$$

$$^3RF\cdot + O_2(^3\Sigma g^-) \rightarrow RF + O_2(^1\Delta g^-) \quad (6)$$

Sel et al. confirmed the generation of ·OH after UVA irradiation of riboflavin by the characteristic 1:2:2:1 ESR spectrum of the ·DMPO-OH adduct [35]. Riboflavin can serve as an effective electron mediator with molecular oxygen being reduced stepwise via the $O_2{\cdot}^-$, which can be converted to diffusible hydrogen peroxide (H_2O_2) and in turn generate oxygen-dependent hydroxyl radicals (·OH).

Based on the report demonstrated above, several possible major processes were proposed for the riboflavin-sensitized photo-degradation under blue light irradiation, as shown in Eqs.7–12.

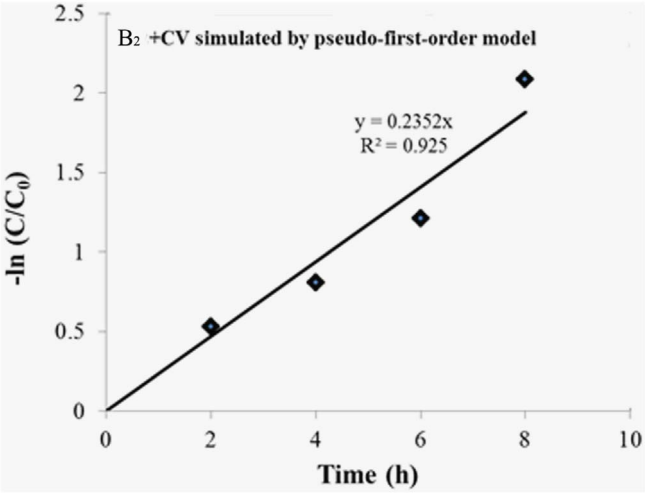

Fig. 7. Pseudo first-order apparent degradation rate constants of CV at initial riboflavin (B_2) concentration of 240 μM, treated with blue light irradiation at 2.0 W/cm^2.

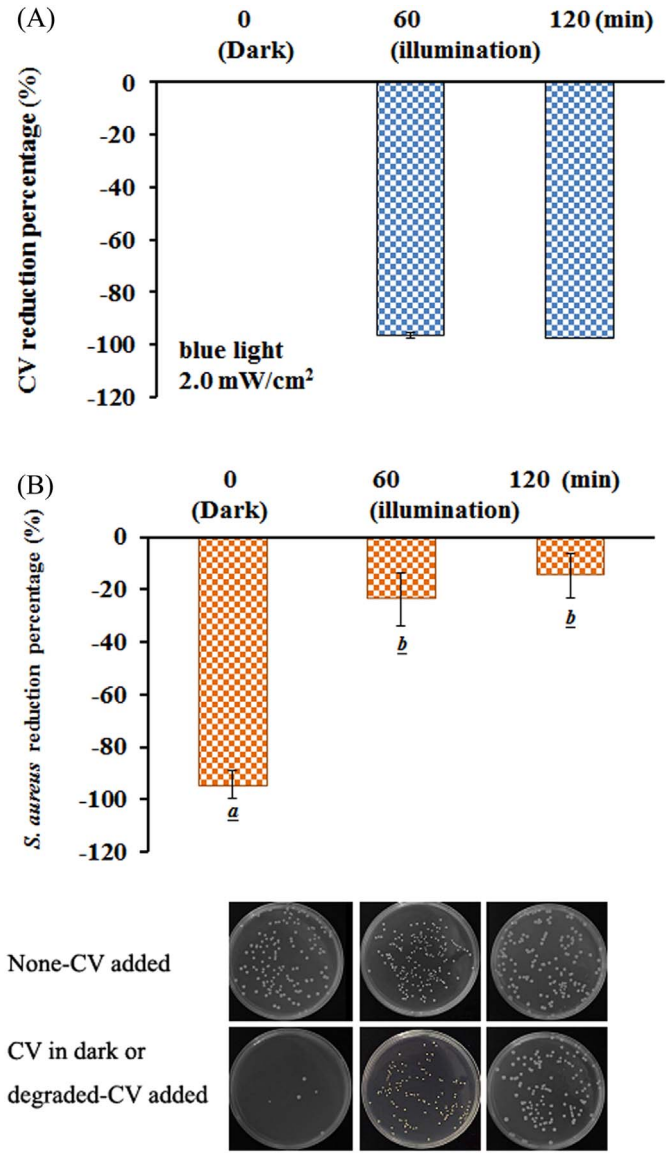

Fig. 8. (A) Effects of riboflavin photolysis on CV degradation. The reduction percentage was the degradation of CV in a riboflavin solution after treatment with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min, as detected by an HPLC system at 588 nm. (B) Effects of CV or degraded CV in riboflavin solution on viability of *S. aureus*. CV of 10 mg/L in the dark without blue light irradiation was used as a control. Other treatments were 10 mg/L CV in riboflavin solution treated with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min. Data are represented by mean ± SD, where $n = 5$. Statistical differences ($p < 0.05$) between groups are indicated by the different letters below each bar. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$$O_2\cdot^- + H^+ + e^- \rightarrow HOO\cdot \quad (7)$$

$$HOO\cdot + H_2O \rightarrow \cdot OH + H_2O \quad (8)$$

$$O_2\cdot^- + 2H^+ \rightarrow H_2O_2 \quad (9)$$

$$H_2O_2 + RF\cdot^- \rightarrow \cdot OH + OH^- \quad (10)$$

$$RF\cdot^+ + OH^- \rightarrow \cdot OH \quad (11)$$

$$RF\cdot^+ + H_2O \rightarrow \cdot OH + H^+ \quad (12)$$

As observed in Fig. 6(A), the wavelength of the maximum absorption of the spectral bands of CV under blue light irradiation at 2.0 mW/cm^2 was shifted from 588 nm to 572 nm and from 372 nm to 360 or 356 nm. Also observed in Fig. 2 (B), the wavelength of the maximum absorption of the spectral bands of riboflavin under blue light irradiation at 2.0 mW/cm^2 was shifted from 373 nm to 356 nm. As described above, Ahmad et al. (2008) reported that riboflavin photolysis can decrease the absorbance of aqueous phase at 445 nm and increase the absorbance of chloroform phase at 356 nm (lumichrome) and 445 nm (lumiflavin) [31]. It was also reported that the spectral bands of the maximum absorption of CV dye were shifted from 585.5 nm to 543.8 nm, from 377.1 nm to 342.6 nm, and from 288.3 nm to 274.8 nm, presumably due to formation of a series of *N*-demethylated intermediates via the BiO_xCl_y/BiO_mI_n-mediated photocatalytic processes [36]. But the possibility that the series of intermediates of CV photodegradation have negative effects on the ecosystems cannot be excluded at this stage.

As shown in Fig. 4, CV degraded by 90% was shown to cause a 99% inactivation rate in *S. aureus* by riboflavin photolysis via blue light irradiation at 0.5 mW/cm^2 for 15 min. It has been reported that CV is very effective, with a low critical concentration, against *Staphylococcus* species [4]. It has also been suggested that CV treated with riboflavin photolysis under blue light irradiation at 0.5 mW/cm^2 for 15 min still retains the efficacy of its antimicrobial ability. The effect of CV degradation on microbial viability is therefore an important indicator of the efficiency of the wastewater treatment.

As seen in Fig. 5, for CV treated with riboflavin under blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min, the mixed CV solutions exhibited color changes during blue light irradiation. Under the same circumstances, the CV decreased upon riboflavin photolysis with reduction percentages of 97.8 and 98.7% for 60 and 120 min via blue light irradiation at 2.0 mW/cm^2, respectively, as shown in Fig. 8(A). The 76.3 and 85.2% viabilities of *S. aureus* were still retained in the CV dissolved riboflavin solutions when treated with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min, respectively, as shown in Fig. 8(B). These results suggest that the degradation of CV in the presence of riboflavin treated with blue light irradiation at 2.0 mW/cm^2 for 60 and 120 min also decreases its toxicity toward microorganisms. The application of blue light irradiation in riboflavin photochemical treatment reported in this study can be applied as an environmentally friendly wastewater treatment for the degradation of CV after the process of dyeing.

The ROS generated from riboflavin photolysis could attack CV, leading to the destruction of CV and in turn reduction of its antimicrobial ability. These cycles continuously occurred when the system was exposed to the blue light irradiation. Ultimately, after several cycles of photo-oxidation, the degradation of CV by the formed oxidant species could be expressed by Eqs.13–15:

$$CV + RF\cdot^+ \rightarrow CV\cdot^+ \rightarrow \text{degraded compounds} \quad (13)$$

$$CV + \cdot OH/O_2\cdot^- \rightarrow \text{degraded compounds} \quad (14)$$

$$CV\cdot^+ + \cdot OH/O_2\cdot^- \rightarrow \text{degraded compounds} \quad (15)$$

The process and mechanism of the degradation of CV dye by metal-based photocatalyst have been analyzed [9,37], In the present study, the CV degradation process is similar to the results obtained previously.

The photodegradation pathway of CV is achieved through photosensitized riboflavin. CV absorbing a photon was promoted to an excited electronic state CV·, from which an electron could be transferred into riboflavin:

$$CV + h\nu \rightarrow CV\cdot \quad (16)$$

$$CV\cdot + RF \rightarrow CV\cdot^+ + RF(e^-) \rightarrow CV\cdot^+ + RF\cdot^- \quad (17)$$

$$O_2 + RF\cdot^- \rightarrow O_2\cdot^- + RF \quad (18)$$

where, $RF\cdot^-$ is the radical anion reduced form.

$RF\cdot^-$ induces the generation of ROS (Eq. 18). The major compounds and activated intermediates were proposed in the six reaction steps (Eqs.7–12) of RF photolytic ractions, resulting in the decomposition of

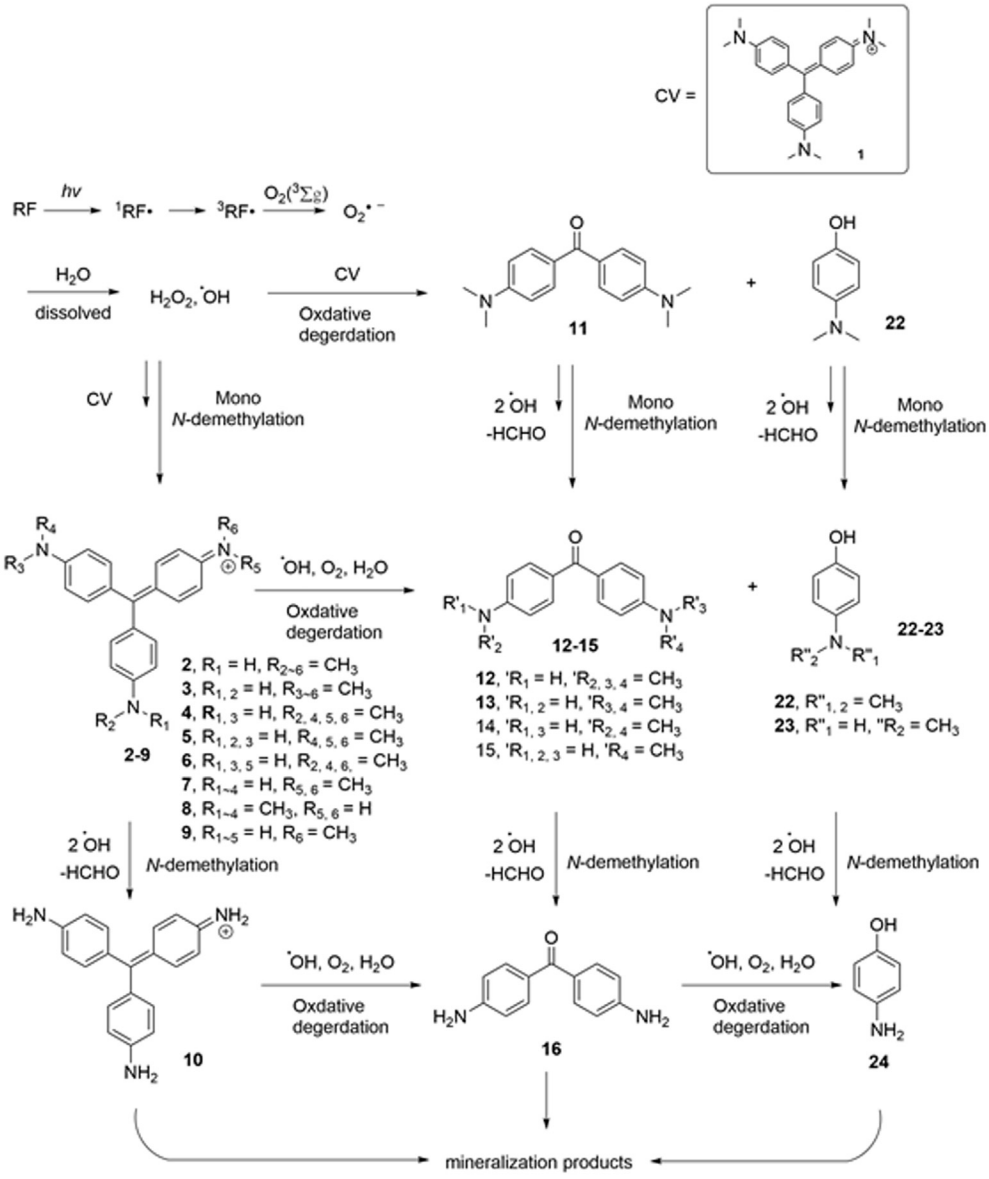

Fig. 9. Scheme for the photodegradation mechanisms of RF/CV.

CV. Apparently, the photodecomposition of CV occurred via the ROS generated from these photosensitized processes.

The probability for the formation of ·OH is much lower than that of $O_2{}^{\cdot -}$.·OH is an extremely reactive and non-selective oxidant, leading to the partial or complete mineralization of several organic chemicals. All the above active radicals drove the photodegradation or mineralization of the dye molecule were studied previously [1,2,6,38–40]. The intermediates identified in this study showed the same results as those of previous studies. The initial period of CV photodegradation by riboflavin, reactions between *N*-demethylation and oxidative degradation took place based on the intermediates identified with the first pathway involving hydroxyl radical attack on the *N,N*-methylamino group of CV, the results indicated that the *N*-demethylation degradation of CV dye took place in a stepwise manner to yield mono-, di-, tri-, tetra-, penta-, hexa- *N*-demethylated CV species during the process. The second pathway involves in a hydroxyl radical attack on the central carbon atom of CV, yielding a reactive cationic radical, with a bond between the central carbon atom and the *N,N*-dimethylamino phenyl ring, as shown in Fig. 9.

CV was not degraded under blue light irradiation as shown in Fig. 1, whereas the spectra of CV in riboflavin solutions at 445 nm and 588 nm were dramatically decreased by blue light irradiation, as shown in Fig. 6. CV degradation caused by riboflavin is most likely ascribed to efficient utilization of riboflavin photolysis via blue light and the high reactively of the ROS generated. As observed in Fig. 6(A), the absorbance of CV in riboflavin solution decreased, while the level of riboflavin photolysis was found to increase with reaction time. As shown in Fig. 6, the reduction percentages of CV at 588 nm under blue light irradiation at 5.0 mW/cm^2 for 30 min (9.0 J/cm^2) and 2.0 mW/cm^2 for 120 min (14.4 J/cm^2) were numerically similar. This suggests that light intensity is more effective than irradiation time when it comes to CV degradation efficiency under the photochemical reaction of riboflavin.

The maximum water solubility of riboflavin was found to be approximately 240 μM in this study. Riboflavin-5′-phosphate (flavin mononucleotide, FMN) is produced from phosphorylation at the 5′-position of the ribityl side-chain of riboflavin. The water solubility of FMN is 200-times higher than that of riboflavin [15]. Our previous study showed that the ROS formed was higher in FMN than in riboflavin under the same level of blue light irradiation with NBT reduction [20]. Therefore, FMN could be more effective than riboflavin in terms of CV degradation efficiency under the photochemical reaction of blue light.

Light irradiation of riboflavin under UV [14–18] and blue light [19,20] were examined. When the weather is sunshine, the solar

radiation could be the major irradiation source for CV degradation during riboflavin photolysis treatment. Blue light irradiation at 2.0 mW/cm^2 for 240 min and solar radiation with sunshine for 30 min showed almost the same efficiency for CV degradation during riboflavin photolysis (data not shown).

Our previous study reported that the spectrum of white LED was composed of multiple color lights with the major portion of the emitted maxima wavelength being at 454 nm [20]. The wavelength of the emitted maxima of blue light was 463 nm in this study. White LED may replace the blue LED lamps for CV degradation during riboflavin photolysis treatment.

5. Conclusions

Riboflavin photolysis by blue light (λ = 463 nm) irradiation was shown to degrade CV. CV was degraded by the ROS generated, such as $O_2^{\cdot -}$, H_2O_2, HOO· and ·OH. The riboflavin photolysis-treated CV solution appeared to be transparent and the conformational transformation was caused by pathways involving reactive free radicals. Degradation resulting from riboflavin photochemical procedures can greatly reduce the antimicrobial ability of CV and decrease its impact on microbial ecosystems. Riboflavin photochemical treatment via blue light irradiation is a simple and safe way to degrade CV and perhaps other types of dye molecules.

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