Efficacy of blue LED in microbial inactivation: Effect of photosensitization and process parameters

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ABSTRACT

Efficacy of blue (462 ± 3 nm) Light emitting diode (LED) illumination to inactivate the foodborne pathogens like *Escherichia coli* and *Staphylococcus aureus* in the presence of exogenous photosensitizer (curcumin) was studied *in vitro*. The effect of temperature, concentration of photosensitizer and incubation time with photosensitizer for microbial inactivation was investigated and sublethal injury of cells was determined. Mechanism of inactivation by the combination of photosensitizer and blue light was also examined. A maximum reduction of 5.94 ± 0.22 and 5.91 ± 0.20 log CFU/ml was obtained for *E. coli* and *S. aureus*, respectively, when treated with photosensitizer (20 μM) at 13 J/cm^2 of blue light. There was no significant change in the inactivation of these pathogens both at 9 °C and 27 °C in the presence of photosensitizer. Even, the incubation with the photosensitizer didn't show any significant difference on the inactivation of these food-borne pathogens. Sublethal injury (> 90% injury) was also observed for the cells treated with photosensitizer and blue light simultaneously. Confocal laser scanning microscopy analysis revealed that membrane integrity was disturbed due to photodynamic activity of curcumin in both the bacteria. Further, both cells produced intracellular reactive oxygen species by the action of photosensitizer and blue light. Scanning electron microscopy of *E. coli* and *S. aureus* cells treated with photosensitizer and blue light showed morphological changes in the cell wall compared to untreated group. The study indicated that photodynamic inactivation of foodborne pathogens using LED-based photosensitization can be explored as a potential technique for food safety.

1. Introduction

The consumption of processed foods is on the rise, particularly in urban areas, due to change in lifestyle. Meanwhile, the awareness among customers about food quality and safety is increasing rapidly. The consumers' demand for minimally processed foods and, growing competition in the market has made the processors to adopt newer technologies that preserve food without affecting nutritional and sensory properties. Traditional thermal-based food processing methods ensure prolonged shelf-life and food safety, but also contribute to undesirable changes in food matrix (Devlieghere et al., 2004). Researchers are focusing on developing alternative techniques that not only meet the consumer demand but are also energy-efficient, simple and cost-effective. Many novel non-thermal technologies like High hydrostatic pressure, Pulsed electric field, Ultrasound, Cold Plasma, Dense phase carbon dioxide, Ozone, and Pulsed light processing have been developed to inactivate micro-organisms that hold promises by preserving sensory and nutritional qualities of food (Ortega-Rivas and Salmerón-Ochoa, 2014). The novel and emerging athermal processing by light

such as pulsed light technology is gaining focus in the recent years and are being used for decontamination of foods like fruit juices, meat products, vegetables, and fruits (Anderson et al., 2000; Bhavya and Umesh Hebbar, 2017; Dunn et al., 1995; Ferrario et al., 2015; Gomez-Lopez et al., 2005; Krishnamurthy et al., 2010). Ramos-Villarroel et al. (2012) reported that ultraviolet (UV) was the only agent responsible for the inactivation of pathogens and no antibacterial effect is attributed to infrared (IR), or visible (VIS) light. In contrast, Elmnasser et al. (2007) stated that both the VIS and IR regions also contribute to the destructive effect on microorganisms with high peak power.

Recently, Light Emitting Diode (LED) with visible wavelength is gaining scope in the area of light-based processing of food in spite of its low inactivation efficacy compared to UV light. Processing of foods with UV or Pulsed light technology has limitation due to its harmful effect on human beings, and it also has deleterious effect on quality of foods (Ghate et al., 2017; Kleinpenning et al., 2010; Kim et al., 2017a; Matsumura and Ananthaswamy, 2004; Maclean et al., 2009). Meanwhile, blue region (400–500 nm) of VIS light has higher inactivation effect on microorganisms compared to other regions of VIS

light (Ghate et al., 2013). LED technology being environmental friendly, low energy consuming, safe and high durability, LED based photodynamic inactivation is being explored more and more in food processing (Luksienė and Zukauskas, 2009). Inactivation of microbes by LED illumination is attributed to the production of reactive oxygen species (ROS) by intracellular photosensitizer (PS) excitation, in turn ROS may attack cellular components like DNA, lipids, and proteins ultimately causing bacterial death (Luksienė and Zukauskas, 2009). ROS might preferentially oxidize DNA rather than lipids in cell membrane and cause damage to DNA by targeting guanine bases, forming 8-hydroxy-deoxyguanosine (8-OHdG), an oxidized derivative (Kim et al., 2015; Kim et al., 2017a). Recent studies with blue LED have shown potential application in food safety, especially for decontamination of foods such as fresh-cut papaya (Kim et al., 2017a), mango (Kim et al., 2017b), pineapple (Ghate et al., 2017), milk (Srimagal et al., 2016) and orange juice (Ghate et al., 2016). However, prolonged exposure period was required for inactivation of food-borne pathogens from a range of 7.5 to 48 h (Ghate et al., 2013; Ghate et al., 2017; Kim et al., 2016; Kim et al., 2017a, 2017b).

Hence, the focus of research is to minimize processing time of blue LED illumination by other means such as, use of natural exogenous PS is one such technique. Exogenous PS such as curcumin, chlorophyllin, hypericin, alpha-terthienyl, and 5-Aminolevulinic acid are known to aid the photodynamic inactivation by blue light (BL) by generating ROS (D'Souza et al., 2015). A few studies have been carried out in the field of clinical research on the application of curcumin and blue light (BL) on different pathogenic bacteria that cause dental complications (Araújo et al., 2012; Bulit et al., 2014; Leite et al., 2014; Manoil et al., 2014; Paschoal et al., 2013; Pileggi et al., 2013). Photosensitisation based treatment in food industry can be one of the hurdle technologies for decontamination of food packaging material, vegetables and fruits (Luksiene and Brovko, 2013). Natural PS-curcumin is a food additive that belongs to the group of diphenylheptanoids can be used for photodynamic inactivation of microbes in food stuffs to improve the food safety (Tim, 2015). Recently, photodynamic inactivation on food borne pathogens has also been focused to implement the technique for decontamination of foods. Oliveira et al. (2018) have studied the antimicrobial activity of curcumin in light (400–800 nm) against *Escherichia coli* O157:H7 and *Listeria innocua* in fresh produce wash water. Even, Penha et al. (2017) have performed the studies on the combined effect of curcumin and blue LED illumination (470 nm) on food-borne pathogens such as *Salmonella typhimurium*, *S. aureus*, *E. coli*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. Ribeiro et al. (2013) have studied the effect of curcumin and BL (455 nm) on methicillin resistant *S. aureus* and L929 fibroblasts. Haukvik et al. (2009) have also examined the photokilling effect of curcumin in different aqueous preparations on *Streptococcus intermedius*, *Enterococcus faecalis* and *E. coli* by fluorescent tubes emitting BL.

Practically, there is no comprehensive study on the effect of process parameters in inactivating food-borne pathogens. The major objective of this study is to investigate the effect of exogenous PS, namely, curcumin on the inactivation of food borne pathogens by blue LED illumination (462 nm). The process parameters like temperature of treatment, concentration of PS and incubation time on inactivation efficiency have been studied. Also, attempts were made to understand the mechanism of action of curcumin mediated blue LED illumination against *E. coli* and *S. aureus* through Confocal Laser Scanning microscopy and Scanning Electron microscopy.

2. Materials and methods

2.1. Chemicals

Tryptone soy broth, tryptone soya agar, peptone were procured from HiMedia, India. Phosphate buffered saline was procured from Thermo Fisher Scientific, India. Curcumin was procured from Sigma-Aldrich, India. Propidium iodide was obtained from Cayman chemical company, UK. All the chemicals used in the study were of analytical grade.

2.2. Bacterial strains and culture conditions

E. coli (ATCC 11775) and *S. aureus* (ATCC 12600) were obtained from Food Safety and Analytical Quality Control Laboratory at CSIR-CFTRI, Mysuru and stored in 50% glycerol at −80 °C till further use. Vials containing frozen stock cultures were thawed at room temperature, and were reactivated in 10 ml of sterile tryptone soy broth (TSB) at 37 ± 2 °C for 24 h under static conditions. Further, to obtain working cultures, at least two consecutive transfers were made in TSB at 24 h interval and a daily subculture (24 h) was also performed to maintain cell viability prior to use. Five ml of TSB containing culture was centrifuged at 4000 ×g for 5 min at 4 °C and washed twice with sterilized phosphate buffered saline, pH 7.4 (PBS). The resultant pellet was resuspended in PBS at an initial concentration of approximately 10^9 CFU/ml and the bacterial suspension was used for further experiments.

2.3. Bacterial inactivation by curcumin and blue light illumination

A stock solution of PS (10 mM) was prepared by dissolving curcumin in ethanol. Overnight (18 h) incubated *E. coli* and *S. aureus* culture were diluted in PBS and later diluted in 10 ml of maximum recovery diluent (MRD) solution (1 g peptone and 8.5 g saline in 1 l of water) to achieve final concentration of ~7 log (CFU/ml). A batch type LED illumination system developed at CSIR-CFTRI was used for the experiments. The system consists of height adjustable product holding tray, LED bulb assembly, UV lamps for sterilization of chamber, jacketed chamber for circulation of chilled water and temperature probe (Pt100). During treatments, bacterial suspension in MRD solution with PS (10 μM and 20 μM) was placed in a sterile Petri dish (75 mm diameter) below the light source and exposed to BL for 1 h in the LED illumination chamber. The distance of 8.5 cm was adjusted between the sample and the LED bulb. High intensity 462 ± 3 nm LED (6 × 6 mm shape) (Melux Control Gears P. Ltd., India) was used in this study. The irradiance (W/cm^2) of 462 ± 3 nm LED was 3.60 ± 0.02 mW/cm^2 at the surface of bacterial suspension. The dosage provided for each bacterial suspension was calculated by the Eq. (1) (Maclean et al., 2009).

$$E = P \times t \tag{1}$$

where, E = dose (energy density) in J/cm^2, P = irradiance (power density) in W/cm^2, and t = time in s.

To study the role of temperature on microbial inactivation, inoculated MRD solution was illuminated by 462 ± 3 nm LED until 13 J/cm^2 (1 h) at the set temperature of either 9 ± 1 °C or room temperature (27 ± 2 °C). Further, to evaluate the effect of incubation time on simultaneous application of PS and BL, the inoculated MRD solution prior to LED illumination was incubated at either 9 or 27 °C for 1 h. Controls consisted of bacterial suspensions with PS kept in the dark, and bacterial suspensions without PS exposed to BL under experimental temperatures. At the end of the experiment an aliquot of 1 ml was withdrawn and pour plated onto tryptone soy agar (TSA) after serially diluting with 0.85% saline solution. Plates were incubated at 37 ± 2 °C for 24 h, followed by manual counting of the colonies and bacterial count were expressed as log CFU/ml.

2.4. Sublethal injury test

For the sublethal injury determination, the aliquot withdrawn from the Petri plate was plated onto TSA supplemented with sodium chloride (NaCl), after serial dilution (Ghate et al., 2013). The concentration of NaCl was 3% and 2.5% for *E. coli* and *S. aureus* respectively. The plates were incubated for 24 to 48 h at 37 ± 2 °C, the colonies were enumerated and sublethal injury was calculated using the Eq. (2):

$$\text{Sublethal injury}(\%) = \left[1 - \frac{\text{CFU on TSA+NaCl}}{\text{CFU on TSA}} *100\right] \qquad (2)$$

All the experiments conducted for sublethal injury determination was treated at 20 μM concentration of PS.

2.5. Analysis of bacterial membrane integrity

To obtain concrete evidence on cellular membrane damage, membrane integrity of *E. coli* and *S. aureus* during the treatment was analysed by using fluorescent probe, propidium iodide (PI). Red fluorescing PI (485/635 nm) can only enter the damaged cytoplasmic membrane of the cells (Ueckert et al., 1995), due to its high molecular weight (668 Da). Test solution containing ~7 log (CFU/ml) of *E. coli* or *S. aureus* cells + PS was exposed to BL. Bacteria in MRD solution alone, cells treated with PS and BL alone served as controls. After the treatment, cells were then harvested by centrifuging at 8000 rpm at 4 °C for 5 min. The pellet was then re-suspended in PBS containing 5 μM PI solution and incubated in dark at room temperature for 15 min. The incubated solution was then washed twice with PBS to remove excess stain and centrifuged for 3 min at 6000 rpm at 4 °C. Finally, pellet was resuspended in PBS and cells were imaged using confocal laser scanning microscope (CLSM) from Zeiss (LSM700, Carl Zeiss, Oberkochen, Germany) using 63× oil immersion objective lens, with excitation wavelength of 485 nm and emission wavelength of 635 nm.

2.6. Analysis of intracellular oxidative stress

Intracellular oxidative stress of *E. coli* and *S. aureus* during the treatment was analysed using fluorescent staining probe, 2′,7′–dichlorofluorescin diacetate (DCFDA). Cells were treated and centrifuged as mentioned in Section 2.5. The pellet was then re-suspended in PBS containing 10 μM DCFDA solution and incubated in dark at 37 °C for 30 min. The incubated solution was then washed twice with PBS to remove excess dye and centrifuged for 3 min at 6000 rpm at 4 °C. Finally, pellet was resuspended in PBS and cells were imaged using CLSM using 63× oil immersion objective lens, with excitation wavelength of 485 nm and emission wavelength of 520 nm.

2.7. Scanning electron microscopy analysis

Scanning electron microscopy (SEM) was conducted to observe the morphological changes occurred in the bacterial cells after the simultaneous treatment with PS and BL. A treatment sample containing ~7 log (CFU/ml) of *E. coli* or *S. aureus* + PS treated with BL was used. Controls consisted of bacterial suspension in MRD, bacterial suspensions with PS kept in the dark, and cells exposed to BL only in room temperature. Following treatment, bacterial cells were centrifuged at 6000 rpm for 5 min and after subsequent washing with PBS, the cells were fixed with 2.5% glutaraldehyde for 4 h. After incubation, the cells were washed twice with PBS and were dehydrated sequentially in 25, 50, 75, and 95% (v/v) aqueous solutions of ethanol and finally with absolute ethanol. Before the observation on SEM, cells were mounted on a SEM stub and sputter coated with gold to capture the images by LEO 435 VP (LEO Electron microscopy Ltd., Cambridge, UK) at 15 kV.

2.8. Statistical analysis

Values are expressed as mean of three independent experiments done in triplicates. Statistical analysis was performed using Graphpad Instat software and significance between the groups was determined by one-way ANOVA with Tukey's post *t*-test. Value of $p < 0.05$ was considered as significant difference between the groups.

3. Results and discussion

3.1. Antimicrobial efficacy of curcumin mediated blue LED illumination

In the present work, the experiments were designed to study the effect of PS concentration, temperature and incubation followed by BL treatment on inactivation against food borne pathogens, like *E. coli* and *S. aureus*. As per the US FDA regulation, a process should achieve at least 5 log pathogen reduction in order to increase the safety of fruit and vegetable juices (US FDA, 2004). The range of log reduction achieved by combination of PS and BL for *E. coli* was 2.75 to 5.94 log reduction and for *S. aureus*, it was 3.78 to 5.91 log reduction.

In order to investigate the effect of PS concentration on the photoinactivation of food borne pathogens, two concentrations 10 μM and 20 μM of PS were tested. Bacteria were also treated with ethanol (solvent used to dissolve the PS) in order to check its toxicity to the cells. The *E. coli* cells treated with ethanol alone showed 0.25 ± 0.02 log CFU/ml increase after treatment, whereas *S. aureus* resulted in 0.01 ± 0.01 log CFU/ml reduction. Thus it can be confirmed that no cytotoxicity was detected with ethanol treatment alone. Bacterial cells treated with PS alone at 20 μM for 60 min showed < 0.5 log reduction, whereas, Tyagi et al. (2015) reported that about 30% and 70% of killing of *E. coli* and *S. aureus* when cells (10^4 CFU/ml) were treated with curcumin I (dissolved in DMSO) at 25 μM for 60 min. Authors also reported that inactivation of bacteria by PS is directly related to the density/inoculum size of the bacterial cells used. The greater reduction observed in the above study as compared to our present study could be attributed to higher concentration of curcumin and lower density of bacterial cells used, and higher solubility of curcumin in DMSO than ethanol. In the present study, for *E. coli* inactivation, concentration of PS had only a significant effect on inactivation ($p < 0.001$) at 27 °C (Fig. 1b). However, there was a significant difference of 1.26 and 1.37 log reduction ($p < 0.05$) in inactivation of *S. aureus* between 10 μM and 20 μM PS and BL application at both 9 and 27 °C respectively (Fig. 1c and d). Furthermore, for *S. aureus*, the concentration of PS did not affect significantly in PS (1 h) + BL treatment. Ribeiro et al. (2013) also observed that methicillin susceptible *S. aureus* viability decreased in dose-dependent manner when treated with curcumin (dissolved in DMSO) and LED light (455 nm). Penha et al. (2017) observed that *E. coli* and *S. aureus* inactivation was found to be 1.29 and 3.27 log CFU/ml, respectively, by photodynamic inactivation (470 nm) mediated by curcumin (diluted in PBS) at a very high concentration of 75 μM and, high fluence of 139 J/cm^2. Oliveira et al. (2018) has reported that the inactivation of about 4 to 5 log CFU/ml of *E. coli* O157:H7 was achieved, when combination of curcumin at 5 mg/l (diluted in ethanol) and 6 J/cm^2 of visible light (400–800 nm) was applied. Comparison between the independent investigations could be difficult due to difference in experimental environments such as wavelength of light used, source of light, concentration of PS and properties of PS preparation, post-irradiation time, bacterial species, fluence of light and bacterial species (Haukvik et al., 2009).

In order to examine the effect of temperature on inactivation of food borne pathogens, the bacterial cells were treated with PS and exposed to BL at 9 and 27 °C. Sample temperature during LED illumination was found to increase by 3 and 4 °C, when the test samples were treated at 9 and 27 °C respectively. As can be seen from Fig. 1, the photo-inactivation of *E. coli* and *S. aureus* by the application of exogenous PS was effective both at 9 and 27 °C. Control cells that were either exposed to BL alone or treated with PS alone, didn't have any significant antimicrobial effect, regardless of treatment temperatures. At both the temperatures, combination of PS and BL resulted in > 5 log reduction for both food borne pathogens at highest concentration of PS tested. Our present study showed that, influence of PS concentration is greater than temperature in microbial load reduction. Similarly, Oliveira et al. (2018) have also observed that refrigeration condition (4 °C) didn't
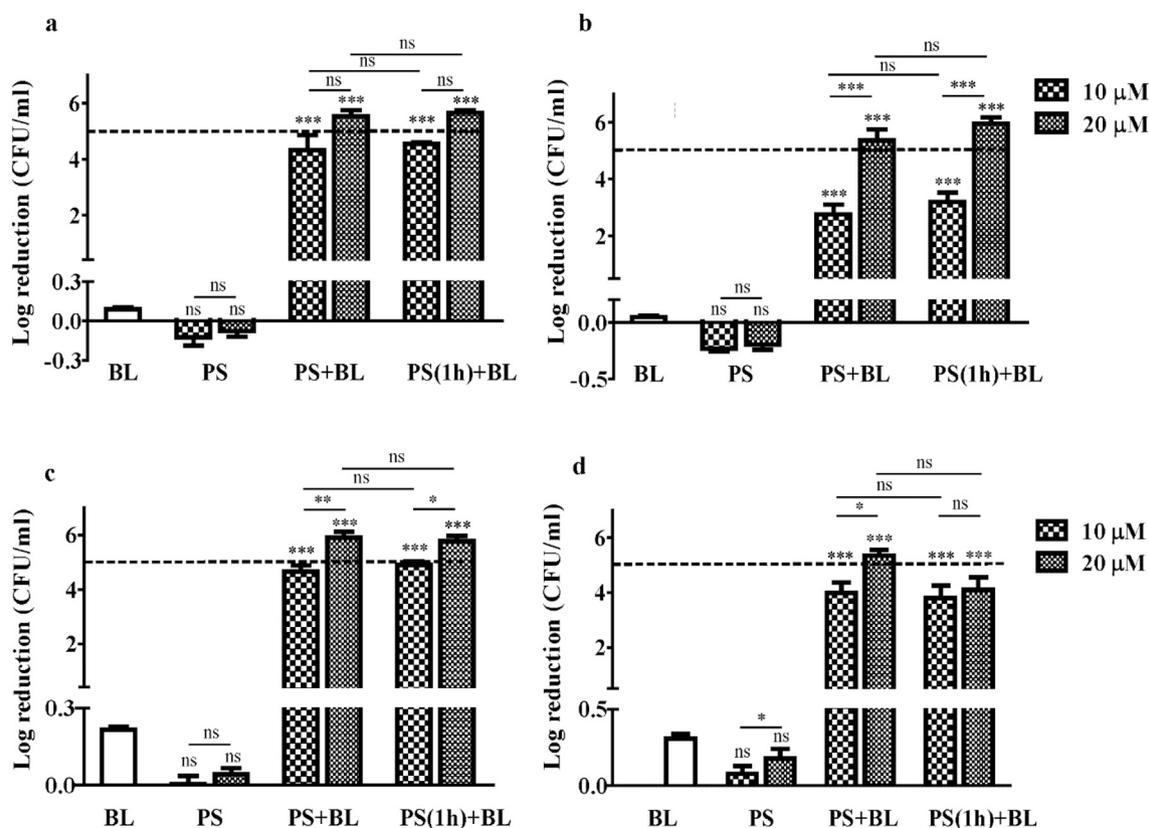
Fig. 1. Graphic representation of inactivation counts (in CFU/ml) observed after experiments with *E. coli* (a, b) and *S. aureus* (c, d) at 9 °C (a, c) and at 27 °C (b, d). Effect of curcumin concentration and incubation time on antimicrobial activity by blue LED illumination at 13 J/cm². Values are represented as mean ± SEM of 3 independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, and ns (non-significant) compared to BL treatment (BL - blue light, PS - photosensitizer).

Table 1
Percentage sublethal injury of *E. coli* and *S. aureus* treated with curcumin (20 μM) followed by illumination with blue LED at 13 J/cm².

| Treatment	*E. coli* (3% salt concentration)		*S. aureus* (2.5% salt concentration)	
	9 °C	27 °C	9 °C	27 °C
BL	10.57 ± 3.9[a]	9.42 ± 2.29[a]	35.44 ± 19.00[a]	21.89 ± 2.89[b]
PS	4.53 ± 2.19[a]	9.47 ± 1.61[a]	7.73 ± 4.94[a]	5.02 ± 5.53[a]
PS + BL	96.67 ± 5.77[b]	73.95 ± 3.56[b]	100 ± 0.00[b]	91.99 ± 0.49[c]
PS (1 h) + BL	100 ± 0.00[b]	97.06 ± 4.16[c]	100 ± 0.00[b]	100 ± 0.00[c]

Values are represented as mean ± standard deviation of 3 independent experiments. Values in the same column with different superscripts (a, b, c) are significantly different ($p < 0.05$). BL - blue light; PS - photosensitizer.

significantly influence the antimicrobial activity of curcumin mediated UV-A light exposure against *E. coli* O157:H7 and *L. innocua* when compared to that of treatment at 25 °C. However, Ghate et al. (2013) observed a reduction of 5.1 and 4.7 log CFU/ml against *E. coli* and *S. aureus*, respectively, when treated with 461 nm LED illumination, for 7.5 h at 10 °C, whereas, only bacteriostatic effect was observed at 20 °C. This might be due to the increased membrane fluidity at lower temperatures, which results in damage of cell membrane by ROS during LED illumination.

To evaluate the effect of incubation time with PS prior to LED illumination, cells were treated with PS at particular concentration and were incubated for 1 h at experiment temperature. Pre-irradiation incubation with 20 μM of PS and then LED illuminated *S. aureus* was found to be reduced by 5.78 ± 0.20 and 4.10 ± 0.45 log CFU/ml at 9 and 27 °C, respectively (Fig. 1c and d). Whereas, *E. coli* reduced by 5.65 ± 0.10 and 5.94 ± 0.22 log CFU/ml at 9 and 27 °C respectively (Fig. 1a and b). There was no significant difference in inactivation of bacteria even after incubating with PS of 10 μM and 20 μM

concentrations for 1 h at both the test temperatures. Haukvik et al. (2009) has also observed ≥3 log reduction of *E. coli* at 25 μM of curcumin in DMSO with pre-irradiation incubation of 30 min at 37 °C and exposure to 30 J/cm² of BL.

Inactivation of *E. coli* by PS (20 μM) and LED illumination (13 J/cm²) almost reached limit of detection (1 log CFU/ml) at 27 °C with incubation (Fig. 1b). Whereas, in case of *S. aureus*, inactivation almost reached limit of detection at 9 °C without incubation (Fig. 1c). Studies on curcumin mediated BL treatments at 20 μM concentration showed that, except for *S. aureus* at 27 °C with incubation, microbial load was reduced to the desired level (as per US FDA regulation), as shown in Fig. 1. Further, treatment with BL and PS alone offered practically no antibacterial effect in both the bacterial cells studied. These results confirm that the combination of PS and blue LED illumination has a stronger synergistic effect on *E. coli* and *S. aureus in vitro*. Log reductions of *S. aureus* were found to be 3.98 ± 0.38 CFU/ml and for *E. coli*, it was 2.75 ± 0.34 CFU/ml at 10 μM PS and 27 °C. The study showed that curcumin mediated BL treatment was more effective to *S. aureus* (Gram-
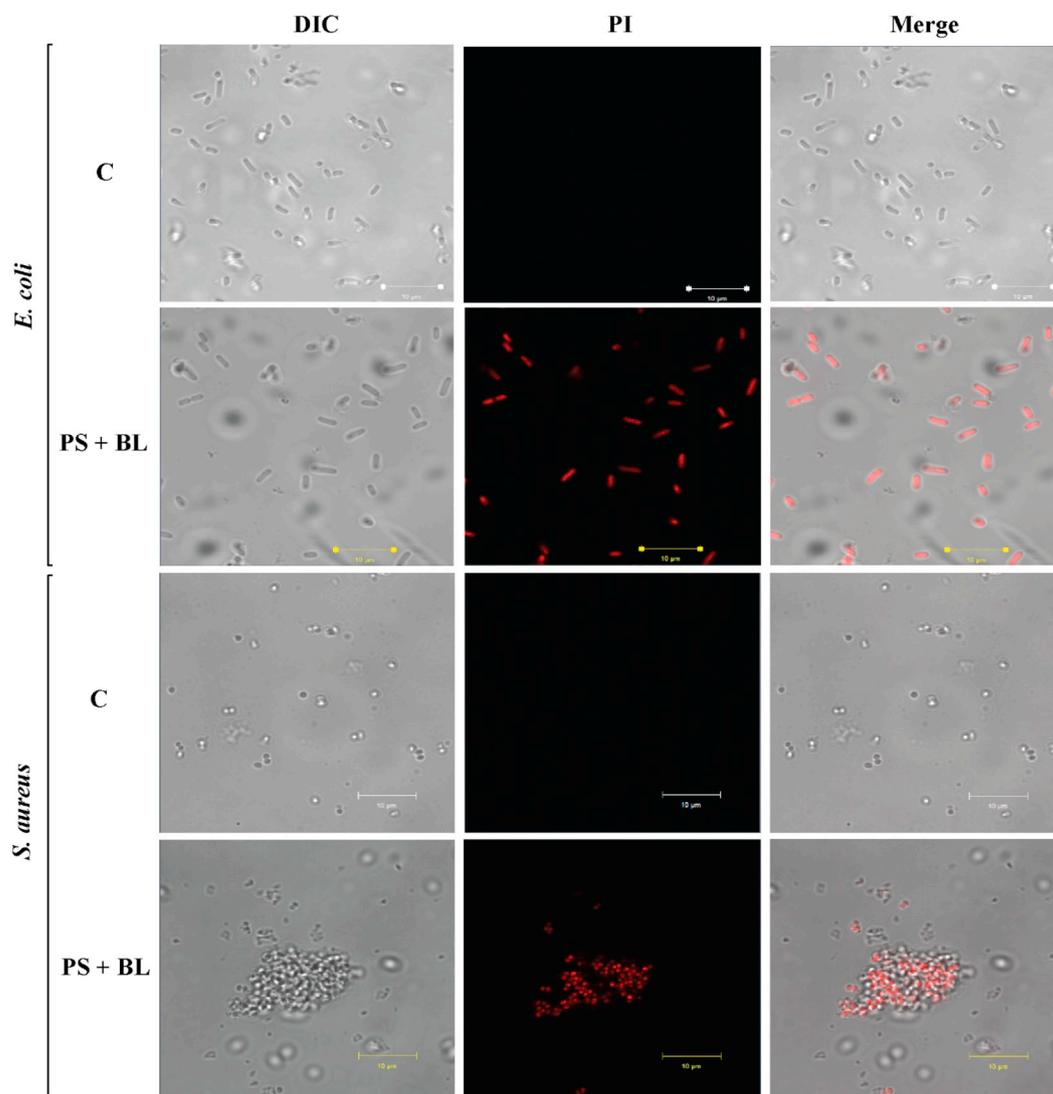
Fig. 2. CLSM images of *E. coli* and *S. aureus* incubated with PI. Photodynamic inactivation of *E. coli* and *S. aureus* was evaluated at 27 °C in the presence of curcumin (20 μM) and simultaneously illuminated by blue LED at 13 J/cm^2 (BL - blue light, PS - photosensitizer, PI - propidium iodide, DIC - differential interference contrast). Pictures were taken using ×63 oil immersion lens with zoom factor of 2.

positive bacteria) as compared to *E. coli* (Gram-negative bacteria). The variation in degree of inactivation may be due to the structural differences between *E. coli* having typical bilayered lipid external structures (diderm bacteria) and *S. aureus* having single lipid bilayer (monoderm bacteria). Pereira et al. (2014) has reported that the photodynamic inactivation efficiency is mainly associated with the chemical composition of bacterial external surface and lipids seem to be playing an important role. Also, the outer cell membrane of Gram-positive bacteria has relatively high degree of porosity and permeability for bioactives like photosensitizers, particularly when the molecular weight is below 1800 Da (Jori and Brown, 2004; Jori et al., 2006; Pereira et al., 2014).

### 3.2. Investigation of antimicrobial mechanism of curcumin mediated blue LED illumination

3.2.1. Sublethal injury of photodynamically inactivated E. coli and S. aureus

Based on the above parameters tested, further experiments (except sublethal injury studies) were conducted at 20 μM of PS without incubation at 27 °C with *E. coli* and *S. aureus* for analysing the mechanism of action. The sublethal injury of the bacteria were determined in order to check whether the curcumin mediated LED illumination caused damage to the bacterial cytoplasmic membrane. The loss of osmotic functionality of cytoplasmic membrane or damage may not necessarily lead to cell death and surviving cells may subsequently recover in medium containing sublethal concentration of NaCl (Somolinos et al., 2008). Sublethal injury of *E. coli* was found to be > 90% irrespective of the temperatures, except for the cells treated at 27 °C without pre-illumination incubation (Table 1). Even, *S. aureus* showed > 90% injury at both the experimental conditions. However, there was no appreciable percentage of sublethal injury in *E. coli* and *S. aureus* treated with BL and PS alone at both temperatures. Ghate et al. (2013) observed 31 and 17% sublethal injury in *E. coli* and *S. aureus*, respectively, when treated at 461 nm illumination for 7.5 h at 20 °C. Whereas, the corresponding sublethal injury was 98.9 and 92.8% when treated at 461 nm illumination for 4.5 h at 10 °C. Our results illustrate the possibility of damage in cellular membrane by PS + BL treatment, since bacterial cells became more sensitive to NaCl compared to control cells.

3.2.2. Analysis of cell membrane integrity by PI staining

The CLSM images of *E. coli* and *S. aureus* clearly demonstrate the loss of membrane integrity due to photo-inactivation (PS + BL)

DIC DCFDA Merge
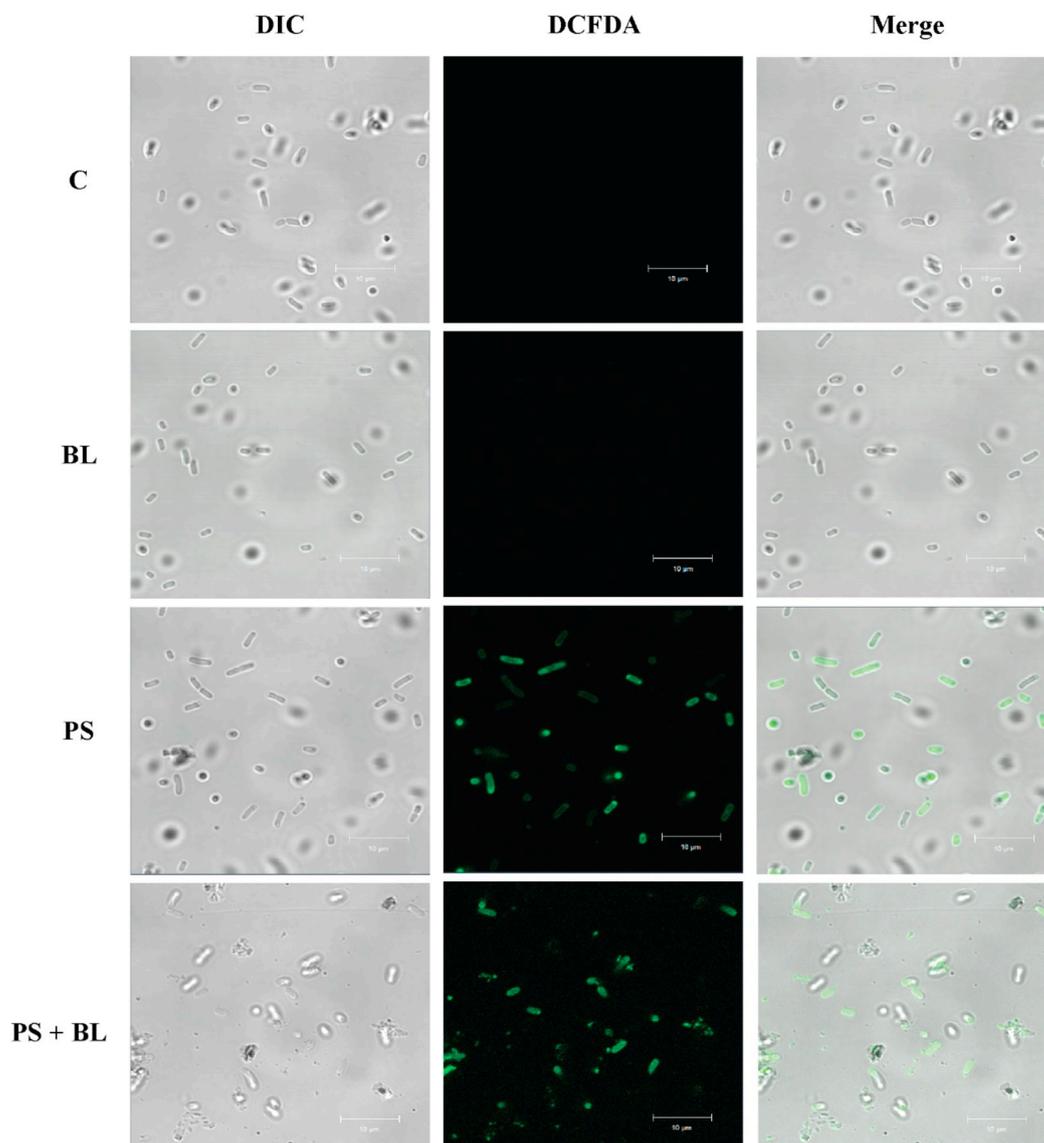
Fig. 3. CLSM images of *E. coli* incubated with DCFDA. Photodynamic inactivation of *E. coli* was evaluated at 27 °C in presence of curcumin (20 μM) and simultaneously illuminated by blue LED at 13 J/cm² (BL - blue light, PS - photosensitizer, DCFDA - dichlorofluorescin diacetate, DIC - differential interference contrast). Pictures were taken using ×63 oil immersion lens with zoom factor of 2.

compared to that of control cells (Fig. 2). Cells treated with BL and PS alone didn't show any significant loss of membrane integrity (data not shown). In contrary to our results, Tyagi et al. (2015) reported that curcumin I at 100 μM concentration led to leaky membrane of bacteria, indicating damage to bacterial membrane as the major cause of inactivation. The observed higher degree of membrane damage could be due to the difference in concentration of curcumin used and also its solubility in solvent. Jiang et al. (2014) also studied the inactivation of *S. aureus* pneumonia by blue light-activated curcumin and it showed noticeable damage to membrane permeability, resulting in cell death caused by intracellular ROS increase that could cause photodynamic killing of *S. aureus*. Oxidative stress will target the lipids, particularly unsaturated fatty acids present in the cell membrane and initiate lipid oxidation. The lipid oxidation in the membrane decreases its fluidity and thus disrupts the membrane-bound proteins significantly (Cabiscol Català et al., 2000).

3.2.3. Intracellular ROS analysis by DCFDA staining

Further, intracellular oxidative stress was analysed by using DCFDA staining, where in presence of ROS, DCFDA is converted into 2′, 7′–dichlorofluorescin (highly fluorescent). Reactive oxygen species are known to excite the intracellular PSs which absorb photon of visible light. While returning to ground state, these molecules transfer energy to oxygen molecule, resulting in the production of ROS. Reactive oxygen species like superoxide anion, hydrogen peroxide, hydroxyl radicals and singlet oxygen, may attack cellular components like DNA, lipids, and proteins, which ultimately causes bacterial death (Luksienė and Zukauskas, 2009). The bacteria, *E. coli* and *S. aureus* treated with PS + BL produced intracellular ROS (Figs. 3 and 4). Although, cells treated with PS alone also showed production of intracellular ROS, there was no significant reduction in microbial load. Zhao and Drlica (2014) mentioned that bacteria have defence mechanism against detoxification of ROS such as antioxidant enzymes and protective proteins. Antioxidant enzymes like superoxide dismutases (SODs), catalase, alkyl hydroperoxide reductase, and glutathione peroxidase have the ability to nullify the killing effect of ROS (Kashmiri and Mankar, 2014). Apart from enzymes, protective proteins like thioredoxin, glutaredoxin and other non-enzymatic antioxidants such as NADH and NADPH pools, also have defence systems against oxidative stress (Cabiscol Català et al., 2000). Similar to our results, Wang et al. (2017) also observed intracellular ROS production in *E. coli* treated with gallic acid alone and combination of gallic acid + UV-A light. However, SOD activity was
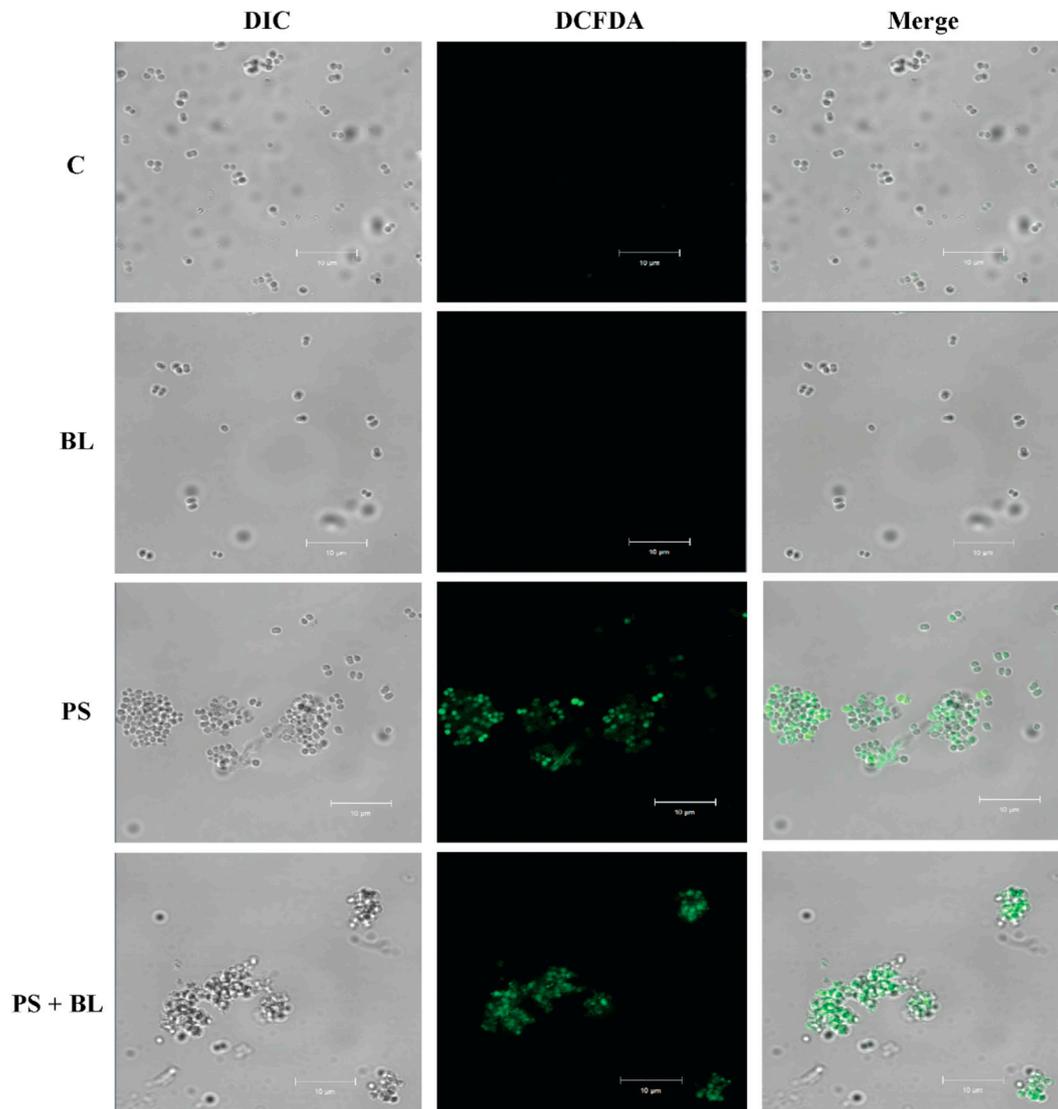
Fig. 4. CLSM images of *S. aureus* incubated with DCFDA. Photodynamic inactivation of *S. aureus* was evaluated at 27 °C in presence of curcumin (20 μM) and simultaneously illuminated by blue LED at 13 J/cm² (BL - blue light, PS - photosensitizer, DCFDA - dichlorofluorescin diacetate, DIC - differential interference contrast). Pictures were taken using ×63 oil immersion lens with zoom factor of 2.
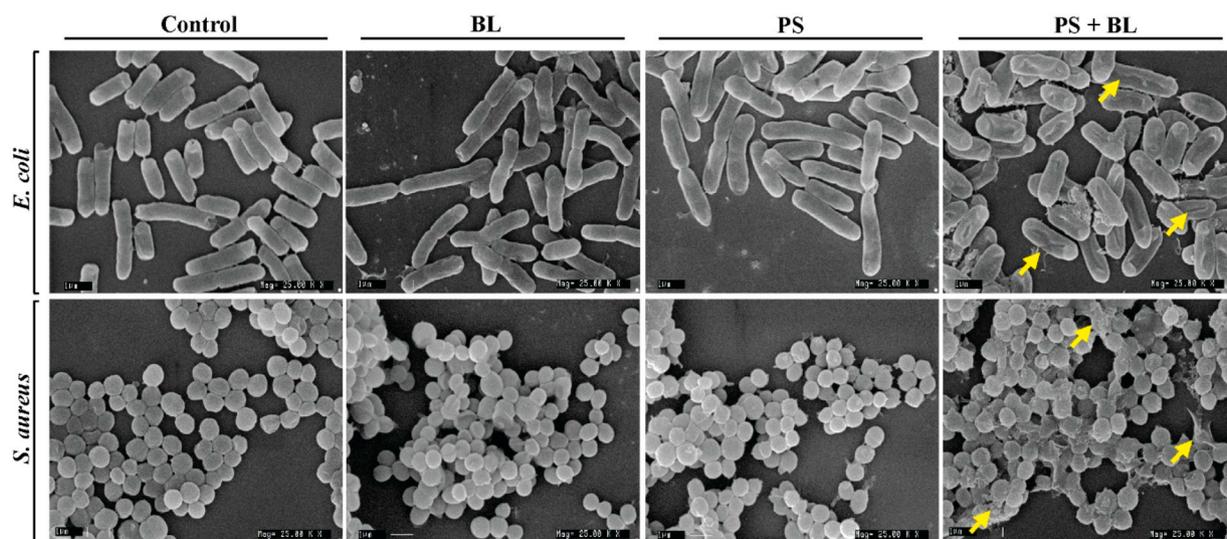

Fig. 5. SEM images showing morphology of *E. coli* and *S. aureus* treated with curcumin (20 μM) and irradiated by blue LED (13 J/cm²) at 27 °C (BL - blue light, PS - photosensitizer).

increasing when cells were treated with gallic acid alone, whereas gallic acid + UV-A light combination showed lower SOD activity. This may be the probable reason for the cells treated with PS alone, survived due to their defence mechanism system.

3.2.4. Determination of morphological changes in bacterial cells by SEM

To confirm the findings on membrane damage induced by combination of PS and BL, SEM was used to directly visualize the morphological changes in *E. coli* and *S. aureus* (Fig. 5). *E. coli* treated with PS (20 μM) and subsequent illumination with BL showed evident proof of shrinkage and flattening with groove formation on the uneven cell surface, whereas control cells with other treatments had smooth cell surface. In case of *S. aureus*, the cells were found to be lysed and partial leakage of cytoplasmic contents. Similarly, transmission electron microscopy images of *S. aureus* pneumonia treated blue light-activated curcumin showed vacuole formation and subsequently resulting in cell death (Jiang et al., 2014). Thus, cell membrane damage can be the probable mechanism of action for inactivation of *E. coli* and *S. aureus* by PS and BL in combination.

In summary, combination of PS and BL showed eloquent effect of > 5 log reduction on the inactivation of *E. coli* and *S. aureus* compared to PS or BL alone. The concentration of PS had a notable effect (1.19 to 2.61 log reduction) on photo-inactivation of *E. coli* and *S. aureus*. Processing temperature and incubation time didn't significantly affect the bacterial reduction. Mode of action could be attributed to membrane damage and loss of integrity by the subsequent production of intracellular ROS. Further studies are needed to decipher the molecular mechanism associated with photodynamic inactivation mediated by curcumin. Also, decontamination studies using real systems like liquid/solid foods will provide greater insight into the suitability of this technique for industrial application to achieve food safety levels.

Conflict of interest

The authors declare that there is no conflict of interest.

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