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Original Article

Inactivation effects of photodynamic technology with toluidine blue O and laser illumination on *Cronobacter sakazakii* biofilm on a glass surface

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ABSTRACT

Cronobacter sakazakii (*C. sakazakii*) is an opportunistic foodborne pathogen that is commonly found in infants. *C. sakazakii* mainly survives with the aid of biofilms, which are difficult to remove. The work described here focuses on the effect of photodynamic technology (PDT), using a 659.5 nm laser and toluidine blue O (TBO), on *C. sakazakii* biofilms under different incubation time, illumination time and TBO concentration. As the incubation time, TBO concentration and illumination time all increased, the sterilization rate of *C. sakazakii* increased first and then decreased. At an incubation time of 20 min, a TBO concentration of 50 μg/mL, and an illumination time of 30 min, the inactivation rates reached 99.92%, 99.94% and 99.99%, respectively. Response surface methodology (RSM) analysis showed that PDT had the greatest bactericidal effect on *C. sakazakii* at the incubation time of 20.5 min, TBO of 56 μg/mL, and the 659.5 nm laser illumination time of 30.5 min. Confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM) observation showed that PDT disrupted the biofilm structure and altered the morphology and extracellular matrix membrane of *C. sakazakii*, leading to their death. Results obtained from this study indicate that TBO-PDT is a promising inactivation strategy for *C. sakazakii* biofilm.

KEYWORDS: *Cronobacter sakazakii*, biofilms, photodynamic technology, Toluidine Blue O, response surface methodology.

1. INRTODUCTION

In 1961, two cases of meningococcal infection from *Cronobacter sakazakii* were first reported in England [1] while many additional cases have been reported in other countries, including the United State of America [2-4], Canada [5], Korea [6] and Japan [7]. In recent years, *C. sakazakii* has been detected frequently in many parts of China [8, 9]; thus, contamination with *C. sakazakii* is now a global issue. *C. sakazakii* is a gram-negative foodborne pathogen that is a member of the *Enterobacteriaceae* family. It can be isolated from meat, cheese, fruits, milk, infant formula, and other foods. It can also be found in the air, water, soil and in food-manufacturing operations [10-13]. *C. sakazakii* is known as the main cause of life-threatening bacteremia, septicemia, meningitis, and necrotizing enterocolitis in humans, especially for low-weight, premature and immune compromised

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infants [14-17]. Its generation time is shorter than for other *Enterobacteriaceae*. Nazarowec-White and Farber (1997) [18] confirmed that *C. sakazakii* bacteria multiply to 10^7 CFU/mL after the addition of 10^1 CFU/mL colonies to a prepared milk powder in a relatively short time. *C. sakazakii* has been reported to form biofilms on a variety of surfaces such as glass, wood, plastics and metals [19]. These biofilms are encapsulated by extracellular polymeric substances (EPS) whose matrix networks serve as protective barriers [20, 21] which allow *C. sakazakii* to survive in harsh environments and have more resistance to disinfectants and antimicrobial agents from living microorganisms. It has been found that *C. sakazakii* are acid-resistant and desiccation-resistant bacteria [22, 23]. The special structure of biofilms and characteristics of *C. sakazakii* make it very difficult to eradicate by traditional methods. Therefore, it has become imperative to develop new approaches which are an effective, safe, and convenient way to eliminate *C. sakazakii* and its biofilm.

Photodynamic technology (PDT) is a newly developed non-thermal sterilization treatment for cancer and pathogenic microorganism. It has been used to relieve symptoms of malignant tumors in patients with bladder [24] and esophageal cancers [25], since reactive oxygen produced by the tumors can damage DNA and proteins, meaning PDT can also have damaging effects on bacteria and cause cell death [26]. Topaloglu *et al*. (2015) [27] described how PDT can be utilized to destroy cell membranes, based on *Staphylococcus aureus* and the use of fullerene as a photosensitizer. PDT is an effective treatment for periodontal disease because of its ability to destroy oral pathogenic bacteria. Studies have shown that PDT can prevent periodontal disease, such as gum swelling [28, 29]. Donnelly *et al*. (2007) [30] reported that PDT killed 99% of *Monilia albican* with toluidine blue as a photosensitiser (PS). PDT is effective against viruses, bacteria, fungi, and yeast as well as against resistant strains of microorganisms [31]. PDT has been found to be relatively less expensive in comparison to other conventional methods, and it has a low probability of producing toxic chemicals. One of the most important advantages of PDT is that the PS has an affinity for bacteria [32]. From the studies above, it can be deduced that PDT would be a potential therapy for cancer, but also a promising technology for inactivation of food-borne pathogenic bacteria.

It should be noted that a PS has more difficulty penetrating into gram-negative bacteria than into gram-positive bacteria, since gram-negative bacteria have a bilayer membrane structure. It has been shown that a cationic PS is more effective than an anionic PS in killing gram-negative bacteria using PDT [33]. Toluidine blue O (TBO) is a cationic phenothiazine dye that binds primarily to the lipopolysaccharide (LPS) of gram-negative bacteria [34, 35]. In comparison to other PS, TBO has certain advantages, including low price, non-toxicity to cells, and high yield of singlet oxygen, making it useful for inactivation of microorganisms [36-39] especially against *Streptococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* [40].

It has been reported that confocal laser scanning microscopy (CLSM) can excite fluorescent probes or fluorescent dyes that bind to a test substance specifically, with a monochromatic laser as the excitation light source. Confocal technology can eliminate lens aberrations and improve resolution, and it brings with it the advantages of electron microscopes that ordinary optical microscopes cannot match [41]. LIVE/DEAD™ BacLight™ Bacterial Viability Kit is a new dual-color fluorescence assay for bacterial viability that can quickly distinguish between dead and live bacteria. Scanning electron microscopy (SEM) has now become an important approach to characterizing microstructures, and it has been applied in various fields, such as the study of starch nanoparticles and changes in the micro-structure of meat during storage [42, 43]. SEM can be used to observe the surface structure and morphology of samples directly through three-dimensional angles and wide magnification ranges with little sample damage.

The objective of this study is to test the inactivation effects of TBO-PDT with 659.5 nm laser illumination on *C. sakazakii* and its biofilm observed by using CLSM and SEM.

2. MATERIALS AND METHODS

2.1. Bacterial strain and formation of bacterial biofilms on the glass surface

The bacterial strain used in this study was *C. sakazakii* ATCC29544 (containing standard lyophilized strains and strain resuscitation fluid, Guangdong Huankai Microbial Technology Co., Ltd). Bacteria were cultured in 5 mL tryptone soy broth (TSB, Qingdao Hope Bio-Technology Co., Ltd) at 37 ºC and grown for 24 h in an orbital shaker at 100 r/min. After full growth, the cells were centrifuged for 10 min at 3532 g and resuspended in 5 mL sterile phosphate buffered saline (PBS) to remove non-adhered cells. The initial turbidity of the suspensions was adjusted to OD595 nm = 0.5 using a micro plate spectrophotometer (Infinite® M200 PRO, Tecan, Switzerland).

A 200 μL cell suspension was added to each well of a sterilized 6-well microtiter plate (Costar Corning, New York, USA) containing 5 mL TSB and a coverslip (18 mm × 18 mm). The plate was incubated while shaking at 75 r/min for 24 h at 37 ºC [44, 45]. After the incubation period, the glass surface with formed biofilms was washed with sterile PBS in triplicate to remove non-adhered cells.

2.2. PS and light source

TBO (Amresco, USA) was dissolved in distilled water to form 100 μg/mL solution, which was then filtered through a sterile 0.22 μm membrane as a stock solution that was stored in the dark at 4 ºC for less than two weeks.

The light source used in this study was a 659.5 nm laser provided by Optoelectronic Devices and Materials Lab from Jinan University, with power being derived from a 320 mW lamp. The lamp was adjusted to give a uniform spot of 5 cm^2 to ensure illumination of the whole coverslip with a light intensity emission of 64 mW/cm^2, as measured by a thermopile power meter (TPM-300CE; Genetic, Canada). The flow chart is shown in Figure 1.

2.3. PDT test and the evaluation of bacterial viability

Biofilms on the glass surface were incubated with 2 mL TBO solution in each well of sterilized

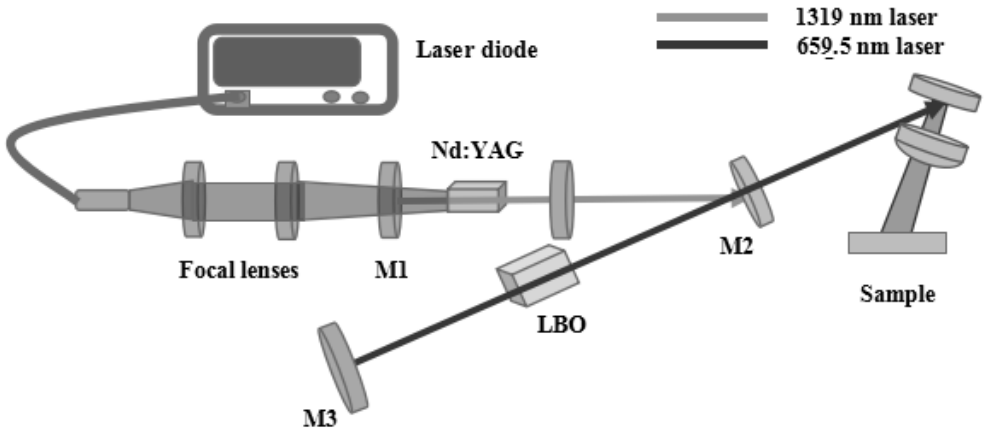

Figure 1. Experimental flow chart of PDT treatment. **M1:** plane mirror, HR@1319 nm HT@808, 1064, 946 nm; **M2:** Radius =+ 100 mm, HR@1319 nm HT@808, 1064, 946, 659.5 nm; **M3:** Radius =+ 100 mm, HR@1319, 659.5 nm HT@808, 1064, 946 nm.

6-well microtiter plates at 37 ºC under dark conditions which were induced using aluminum foil. After incubation, biofilms were treated with a laser beam only. After the treatment, excess TBO solution was washed in triplicate with PBS, and the coverslips were transferred into 50 mL sterile tubes with 10 mL sterile PBS. To detach the biofilm, each tube was sonicated for 14 min at 180 W in an ultrasonic homogenizer (brand). The effect of PDT with TBO as a PS was measured by colony-forming units (CFU/cm^2) [46-48]. Serial suspension dilutions were performed with sterile PBS, and 100 μL of each dilution was plated onto tryptic soy agar (TSA) and incubated for 16-24 h at 37 ºC to calculate the number of CFU.

2.4. Optimization of inactivation conditions using response surface methodology

The different treatment conditions for incubation time, concentration of TBO and illumination time were chosen as independent parameters. Three test groups were chosen:

Group 1: *C. sakazakii* biofilms incubated for different time periods (0, 5, 10, 15, 20, 30 and 40 min) with 10 μg/mL TBO solution, then illuminated for 10 min.

Group 2: *C. sakazakii* biofilms incubated for 10 min with different concentrations of TBO solution (0, 1, 10, 25, 50, 75 and 100 μg/mL), then illuminated for 10 min.

Group 3: *C. sakazakii* biofilms incubated for 10 min with 10 μg/mL TBO solution, then illuminated for different time periods (0, 5, 10, 20, 30, 40 and 50 min).

After that, the level range was determined in one-way test (single factor) experiment. The optimization experiments were designed by response surface methodology (RSM). The Box-Behnken design (BBD) mode was applied to compare interactions among various variable parameters [49]. The response variable (Y) was the log-transformation CFU/cm^2 with BBD, and the optimal conditions for PDT inactivation to reach a maximum were obtained. Verification of the reliability of RSM analysis was conducted by the parallel verification test. The levels of the variables used are shown in Table 1.

2.5. Confocal laser scanning microscope

After being incubated for 10 min with 10 μg/mL TBO solution, *C. sakazakii* biofilms were illuminated for different time periods (0, 10, 30 and 50 min), followed by investigation with confocal laser scanning microscope (CLSM, LSM880, Zeiss, Germany). The TBO solution that was not combined with cells was washed with PBS, and then coverslips were stained with 100 μL LIVE/DEAD^{TM} BacLight^{TM} Bacterial Viability Kit (SYTO9/PI, Thermo Fisher L13152, USA) solution for 15 min at 37 ºC, unbound fluorescent dye with 1 mL of sterile deionized water and a drop of antifade mounting medium was placed on the coverslips. The fluorescent kit contained: one SYTO9 dissolved in 2.5 mL sterile deionized water, one PI dissolved in 2.5 mL sterile deionized water, then 1:1 mixed into a 5 mL EP tube. The stained cells were visualized by CLSM using a 63×/1.4-NA oil immersion objective. Three-dimensional biofilm images were created with Zen blue edition.

2.6. Scanning electron microscopy

Conditions that were optimized by RSM in PDT were chosen to treat *C. sakazakii* biofilms and then visualized by scanning electron microscopy (SEM, LSM700, Germany). Excess TBO solution was

Table 1. The design of response surface methodology.

Independent variable	Levels		
	-1	**0**	**1**
A: Incubation time (min)	15	20	25
B: Concentration of TBO (μg/mL)	40	50	60
C: Illumination time (min)	25	30	35

washed with sterile PBS and then placed in new 6-well microtiter plates. Samples were then fixed with glutaraldehyde for 4 h at 4 ºC and washed with sterile PBS. Fixed samples were dehydrated in several ethanol washes (30%, 50%, 70%, 80% for 15 min and at 100% for 20 min twice) and stored at 70% ethanol overnight. After this period, coverslips were inserted into sample holders for vacuum drying, coverslips were stuck on a sample stage with tape, and samples were then coated with gold-palladium.

2.7. Statistical analysis

Statistical values were reported based on the means of three experiments and their standard deviations. To determine model validity, F value and p value tests for different parameters in BBD were performed using Design-Expert.V8.0.6.1 software. $P < 0.05$ was considered significant.

3. RESULTS AND DISCUSSION

3.1. Optimized incubation time

The effect of different incubation times on PDT is shown in Figure 2A. When incubation time varied from 0 to 5 min, the viable count decreased sharply. An increase in incubation time did not significantly reduce the log transformation CFU/cm^2 of *C. sakazakii*. The inactivation rate reached a maximum of 99.92% in 20 min, but it then

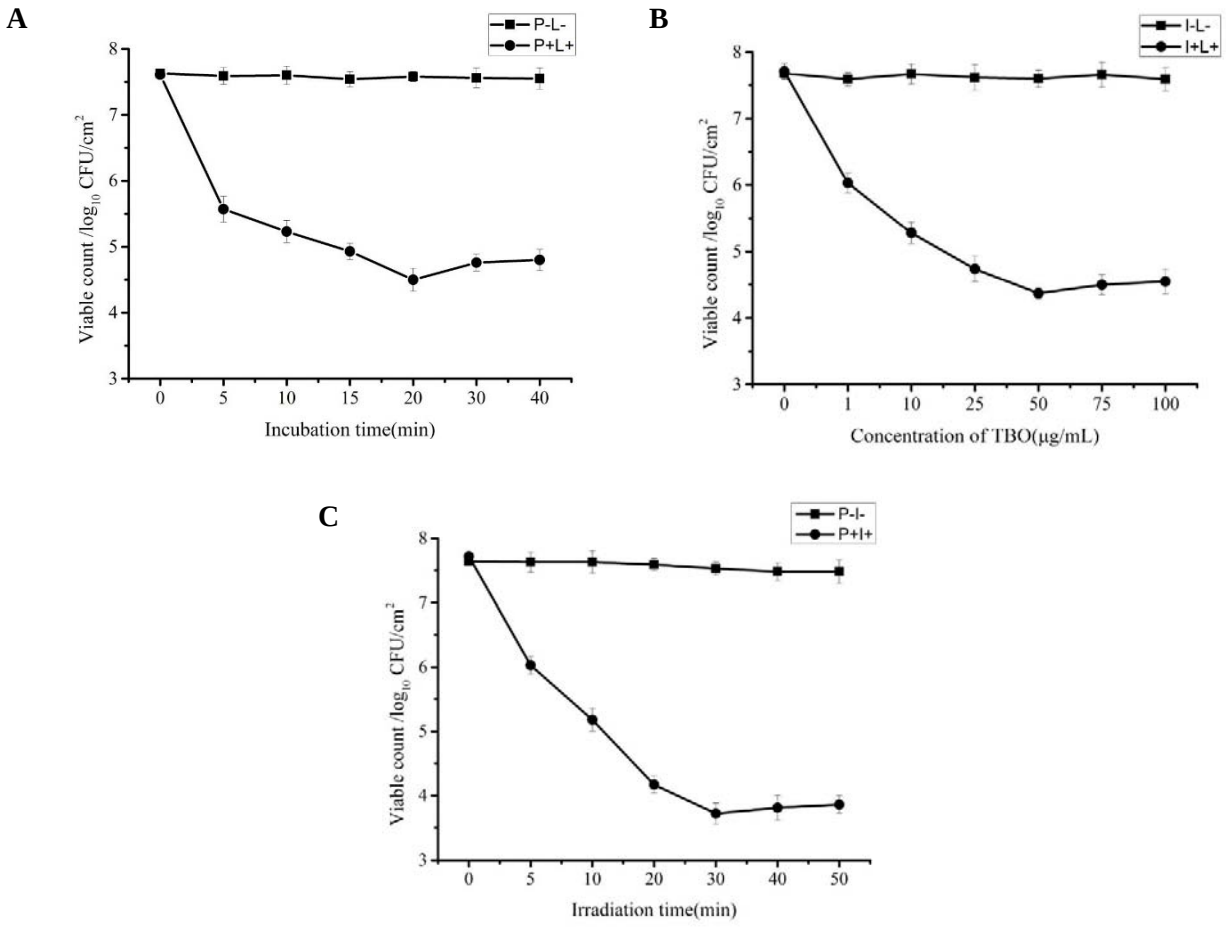

Figure 2. Effects of PDT on viable count of *C. sakazakii*. **A.** Experimental parameters were as follows: TBO concentration = 10 μg/mL, illumination time = 10 min for the PDT. P-L-: incubated without TBO and light; **B.** Experimental parameters were as follows: incubation time = 10 min, illumination time = 10 min for the PDT. I-L-: incubated without incubation and light. **C.** Experiment parameters were as follows: incubation time = 10 min, TBO concentration = 10 μg/mL for PDT. P-I-: incubated without incubation and TBO.

decreased slightly. A possible reason for this is that TBO becomes oversaturated with cells if the incubation time is excessive, which would not be conducive to PDT effects [47]. To optimize incubation time, levels of 15, 20 and 25 min are designed for further RSM analysis.

3.2. Optimized TBO concentration

The effect of TBO concentration variation is illustrated in Figure 2B. The log-transformation for CFU/cm^2 of *C. sakazakii* significantly decreased when the TBO concentration raised from 0 to 50 μg/mL, and 99.94% of *C. sakazakii* were killed when incubated with 50 μg/mL. The effect of PDT in the presence of 100 μg/mL TBO was not higher than that when 50 μg/mL TBO was used. This may be due to the light shielding effect caused by the high concentration of TBO, which prevented the laser from entering the cells [50]. TBO possessed no observable antimicrobial activity against *C. sakazakii* without incubation and illumination. To optimize TBO concentration, levels of 40, 50 and 60 μg/mL are designed for further RSM analysis.

3.3. Optimized illumination time

Illumination time plays a key role in the PDT treatment. Effects of different illumination times were evaluated as shown in Figure 2C. The log-transformed CFU/cm^2 units decreased with increased illumination time from 0 to 30 min. When the illumination time was 30 min, the inactivation rate of *C. sakazakii* reached a maximum of 99.99%. However, when the illumination time was prolonged, viable cells no longer decreased in number. This could be due to illumination not only stimulating the photosensitizer, but also playing a role in heating the bacteria. This experiment was conducted at room temperature, and hence when the illumination exceeded a certain time, the temperature increased, making it more favorable for the growth and reproduction of *C. sakazakii*. To optimize illumination time, levels of 25, 30 and 35 min are designed for further RSM analysis.

3.4. Optimized parameter using response surface methodology

Seventeen experiments, including 12 factorial runs and 5 repetitive runs at the central point, as shown in Table 2, were conducted. Table 2 demonstrates actual and predicted values of the viable counts. Second-order polynomial equations were obtained by multiple quadratic regression analysis as follows:

$$Y = 3.48 - 0.029\ A - 0.075\ B - 0.10\ C + 0.017\ AB + 0.075\ AC + 0.072\ BC + 0.054\ A^2 + 0.051\ B^2 + 0.23\ C^2$$

where Y indicates the predicted viable count, and A, B and C are the coded values for the incubation time, the concentration of TBO, and the illumination time.

The analysis of variance (ANOVA) of BBD was calculated to analyze the effectiveness of the quadratic model (Table 3). The significance of each factor in response to the regression equation is determined by the P-value. The model P-value of < 0.0001 and F-value of > 0.05 implied that the model was highly significant. The P-value for Lack of Fit was 0.1740 (> 0.05), which showed that the fit of the model was acceptable. The coefficient of determination (R-Squared) value was 0.9832 (close to 1), which suggested that 98.32% of variability in the response could be explained by this model. This shows that the regression equation was highly reliable. The adjusted R-Square, with a value of 0.9617, was in reasonable agreement with the R-Square of 0.9832, which showed consistency between the predicted and the actual values. The term A, B, C, AC, BC, A^2, B^2, C^2 were significant ($P < 0.05$) and played important roles in killing *C. sakazakii*. We can also consider that the rank order of the standardized coefficients was C > B > A in this model. Illumination time has the greatest potential influence on the PDT effect, followed by the TBO concentration and the incubation time.

3.5. Response surface plots and verification

The main and interactive effects of variables for viable counts are illustrated intuitively in three-dimensions (3D) (Figure 3). The shape of the two-dimensional contour plots further suggests the mutual interaction of independent variables and responses (Figure 4).

The steep slope of the response surface indicates that this factor has a greater impact on the change in the response. The shape indicates the

Table 2. The experimental design and result of Box-Behnken response surface methodology.

Run	Factors			$Log_{10}(CFU/cm^2)$	
	A	B	C	Actual	Predicted
1	-1	-1	0	3.67	3.70
2	1	-1	0	3.61	3.61
3	-1	1	0	3.52	3.52
4	1	1	0	3.53	3.50
5	-1	0	-1	3.98	3.97
6	1	0	-1	3.74	3.77
7	-1	0	1	3.64	3.62
8	1	0	1	3.70	3.71
9	0	-1	-1	4.04	4.01
10	0	1	-1	3.71	3.72
11	0	-1	1	3.67	3.66
12	0	1	1	3.63	3.66
13	0	0	0	3.48	3.48
14	0	0	0	3.46	3.48
15	0	0	0	3.47	3.48
16	0	0	0	3.52	3.48
17	0	0	0	3.46	3.48

significance of mutual interaction between the variables, and the elliptical contour plot indicates that the interaction effect is significant. In contrast, the circular plot indicates that the interaction effect is not significant. The effect of incubation time and TBO concentration on PDT is shown in Figure 3(1). From the slower slope of the response surface and the sparse contour plots (Figure 4(1)), it was concluded that the interaction between incubation time and TBO concentration had no significant effect on the response.

The interactive effect of incubation time and illumination time on PDT is shown in Figure 3(2). The incubation time and illumination time interaction effect was studied, as it was found to have a significant effect on the response. This was further suggested by the elliptical contours of the two-dimensional contour plots (Figure 4(2)). When the illumination time is certain, the viable counts have changed little with the increase in TBO concentration. However, when the TBO concentration is constant, viable counts tend to decrease rapidly but then rise slowly with the increase in the illumination time.

The plots depicting the interaction between TBO concentration and illumination time are given in Figure 3(3). They show that there is a significant interaction between TBO concentration and illumination time. The optimal natural values of the test variables are: incubation time, 20.43 min; TBO concentration, 56.43 μg/mL and illumination time, 30.54 min. The maximum inactivation of PDT obtained by using the above optimized concentrations of the variables is a log-transformation in CFU/cm^2 of 3.44. Owing to actual operation, an incubation time of 20.5 min, a TBO concentration of 56 μg/mL, and an illumination time of 30.5 min were chosen for the

Table 3. Reliability equation and significance test of the model.

Source	Sum of squares	Df	Mean square	F value	P value	
Model	0.45	9	0.050	45.65	< 0.0001	significant
A	6.612×10^{-3}	1	6.612×10^{-3}	6.05	0.0435	*
B	0.045	1	0.045	41.15	0.0004	**
C	0.086	1	0.086	78.74	< 0.0001	**
AB	1.225×10^{-3}	1	1.225×10^{-3}	1.12	0.3250	
AC	0.022	1	0.022	20.57	0.0027	**
BC	0.021	1	0.021	19.23	0.0032	**
A^2	0.012	1	0.012	11.02	0.0128	*
B^2	0.011	1	0.011	10.01	0.0158	*
C^2	0.23	1	0.23	209.92	< 0.0001	**
Residual	7.655×10^{-3}	7	1.094×10^{-3}			
Lack of Fit	5.175×10^{-3}	3	1.725×10^{-3}	2.78	0.1740	not significant
Pure error	2.480×10^{-3}	4	6.200×10^{-4}			
Corrected total	0.46	16				

$p < 0.01$, significant"**"; $0.01 < p < 0.05$, significant"*"; $p > 0.05$, not significant.

verification experiment. The actual data from the experiment provided a log-transformation value in CFU/cm^2 of 3.46. Thus, the model was reliable and reasonable for predicting the viable count for *C. sakazakii*.

3.6. Visualization of bacterial biofilms on glass surfaces

The inactivation effect of PDT after the treatment of incubation time 10 min, the TBO concentration 10 μg/mL and different illumination time was visualized by CLSM. Our results demonstrated that most of the cells in the biofilms were inactivated by TBO-PDT. The advantage of LIVE/DEAD staining is that it does not destroy the complete structure of the biofilms during exposure to dye. Green fluorescence represents living bacteria, since SYTO9 enters viable bacteria, and PI does not bind to live bacteria. The red fluorescence represents dead bacteria due to the combination of PI and dead bacteria. The orange fluorescence is caused by the overlap of dead bacteria and live bacteria. Green fluorescence is demonstrated in the absence of light (Figure 5a), and after 10 min illumination, the proportion of green bacteria decreased. Most bacteria were live, but a small portion was yellow green, meaning that they were in an apoptotic phase (Figure 5b). In 30 min, obviously, most *C. sakazakii* died, and the PDT effect was considerable (Figure 5c). Following 50 min light (Figure 5d), most of the cells became orange, because the lengthy illumination increased the temperature, which was more suitable for the growth of *C. sakazakii*, and the newly grown cells overlapped with the dead bacteria.

Images without illumination illustrated highly dense and compacted cells with smooth surfaces (Figure 6a). The biological membrane structure became loose, cells ruptured, and grooves appeared, and the cell size grew following PDT (Figure 6b). These results showed that TBO-PDT can destroy the *C. sakazakii* biofilm structure. A series of bacterial cells that have not been treated by PDT appear smooth, clear, and regular (Figure 6c), but a series of bacterial cells with PDT appear completely damaged, and they have different shapes. Furthermore, cellular aggregates surrounded by an extracellular matrix can be observed (Figure 6d).

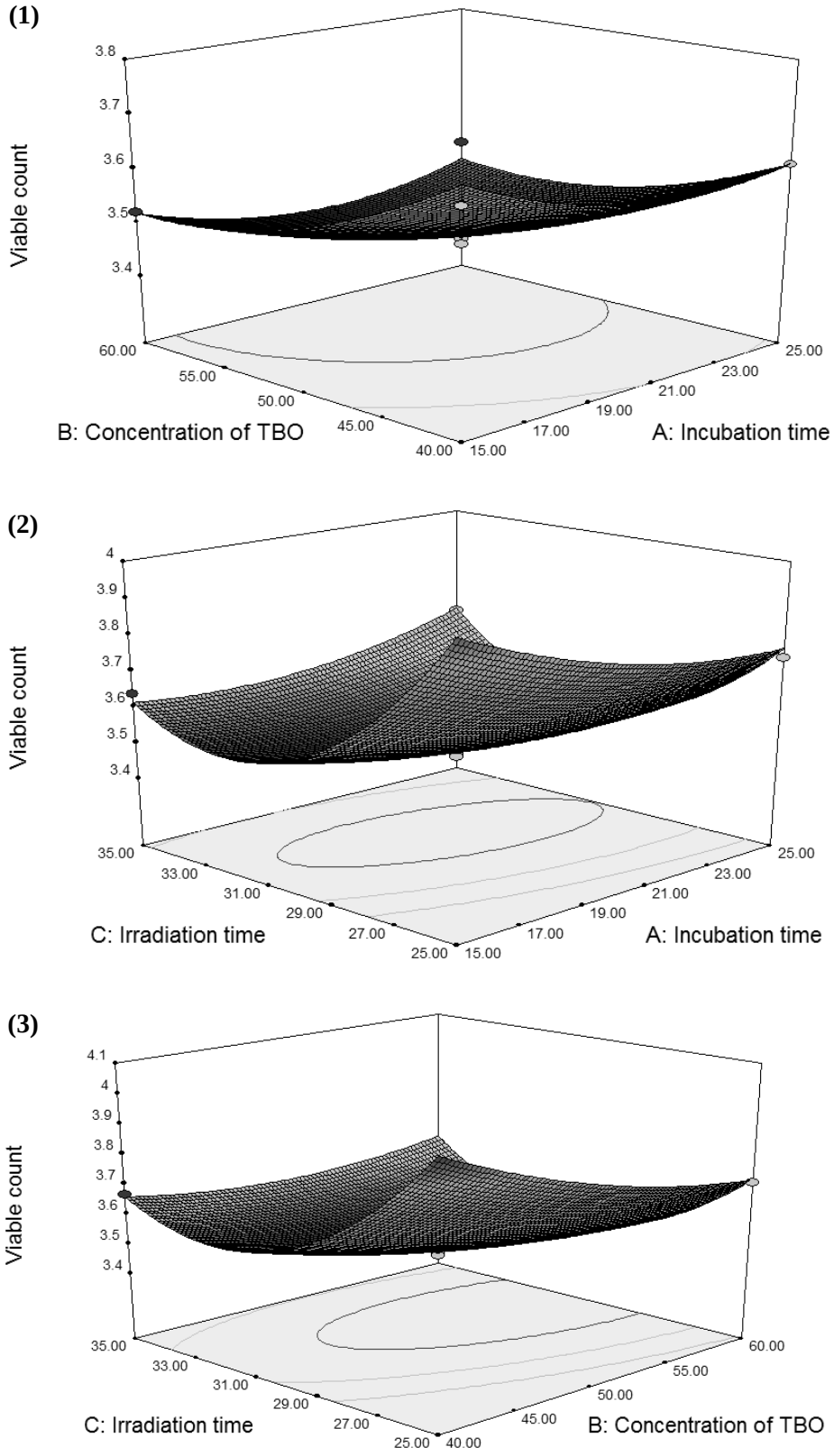

Figure 3. Response surface for the mutual interactions of independent variables on PDT.

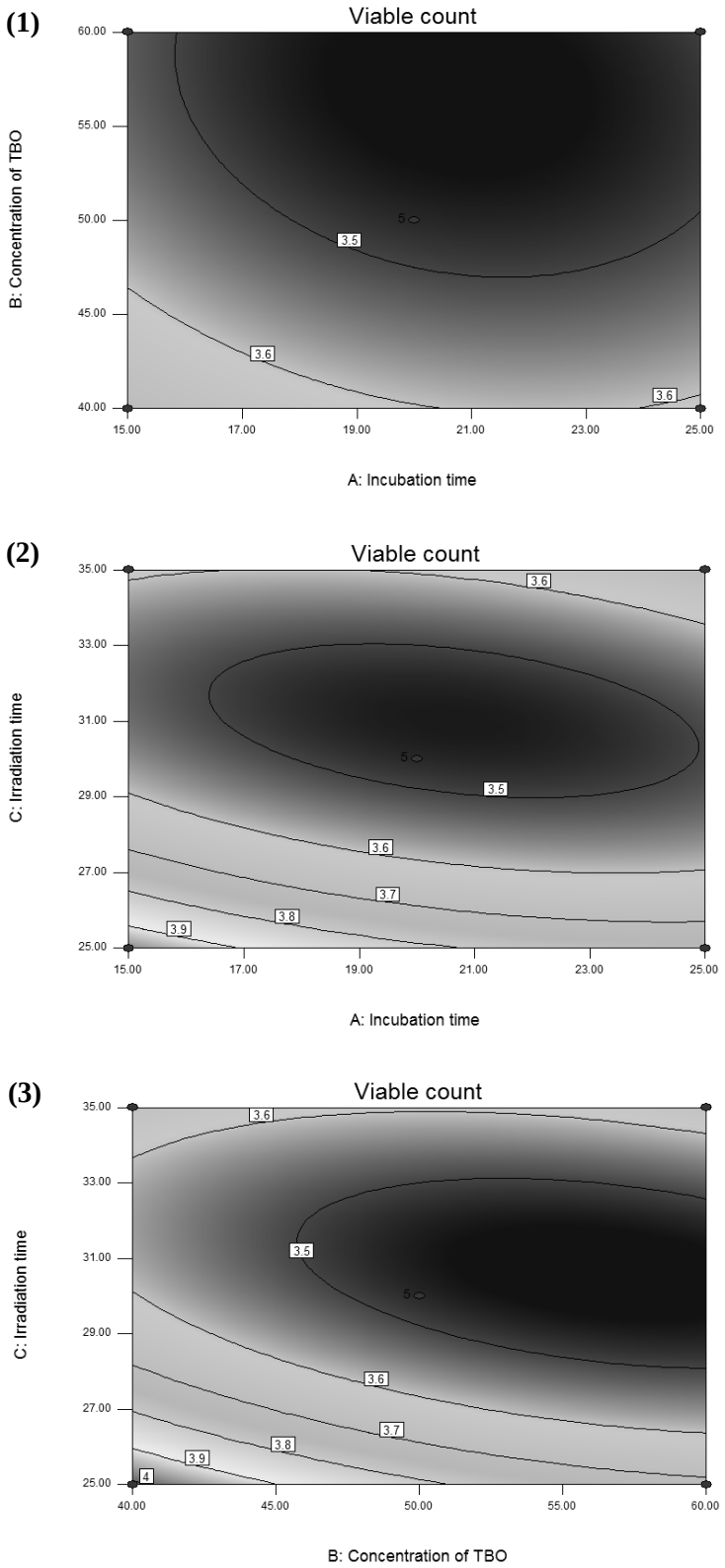

Figure 4. Two-dimensional contour plots for the mutual interactions of independent variables on PDT of A-B **(1)**, A-C **(2)**, B-C **(3)**.

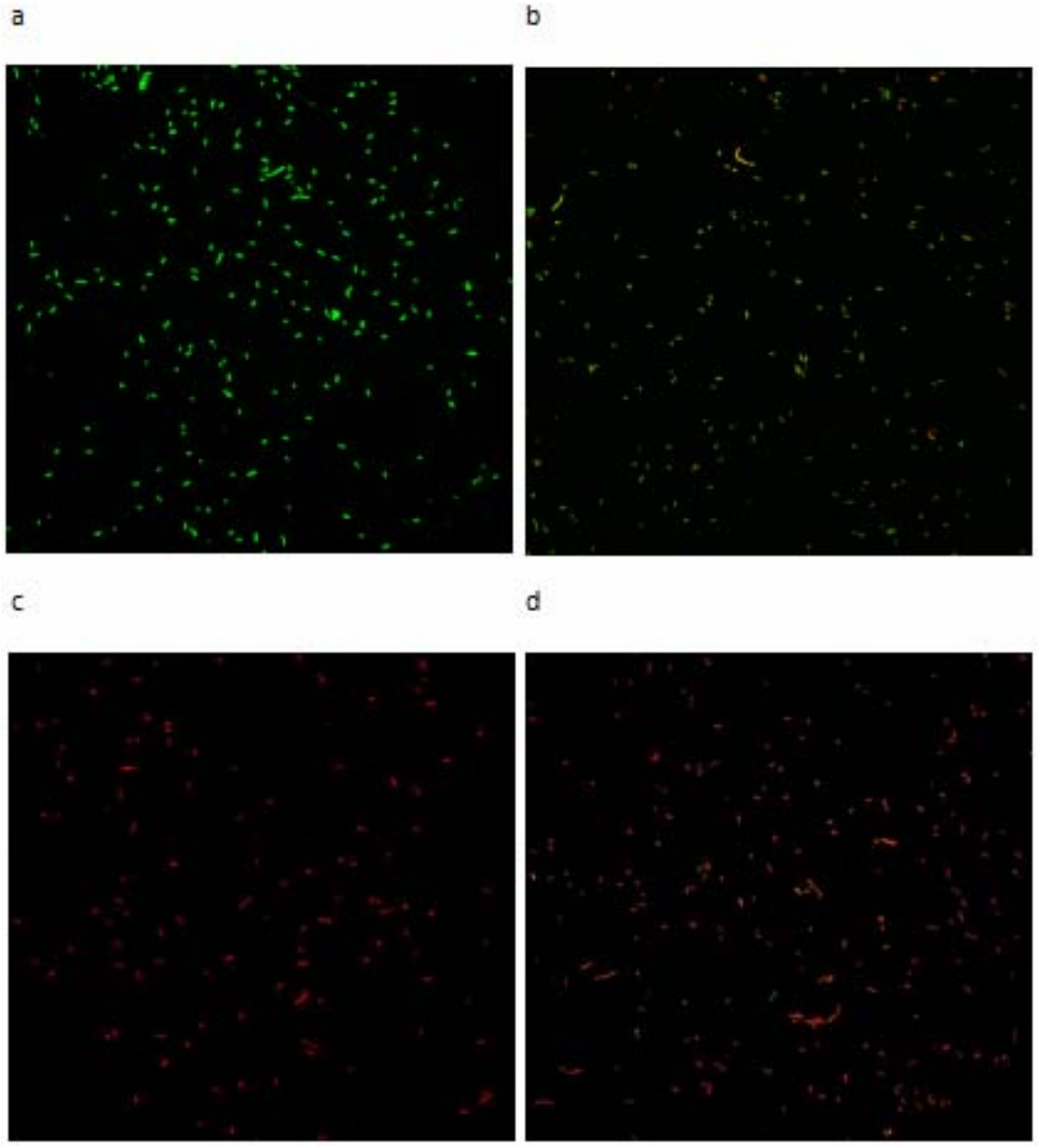

Figure 5. Representative CLSM images of *C. sakazakii* biofilms with LIVE/DEAD staining. **a.** Control: Biofilms were treated with 10 mM ALA for 2 h in dark. **b.** Biofilms were incubated for 10 min with a 10 μg/mL TBO solution and irradiated for 10 min. **c.** Biofilms were incubated for 10 min with a 10 μg/mL TBO solution and illuminated for 30 min. **d.** Biofilms were incubated for 10 min with a 10 μg/mL TBO solution and illuminated for 50 min. A 63×/1.4-NA oil immersion objective was used.

PDT influences the biofilm structure by causing a decrease in membrane thickness. This is a promising technology, and it presents a positive result against the growing reality of resistant microorganisms [51]. Research shows that PS does not produce drug resistance in bacteria [52]. There are two ways to destroy bacteria: electron transfer produces excited triplet oxygen, and energy transfer releases reactive oxygen species (ROS) and singlet oxygen [53, 54].

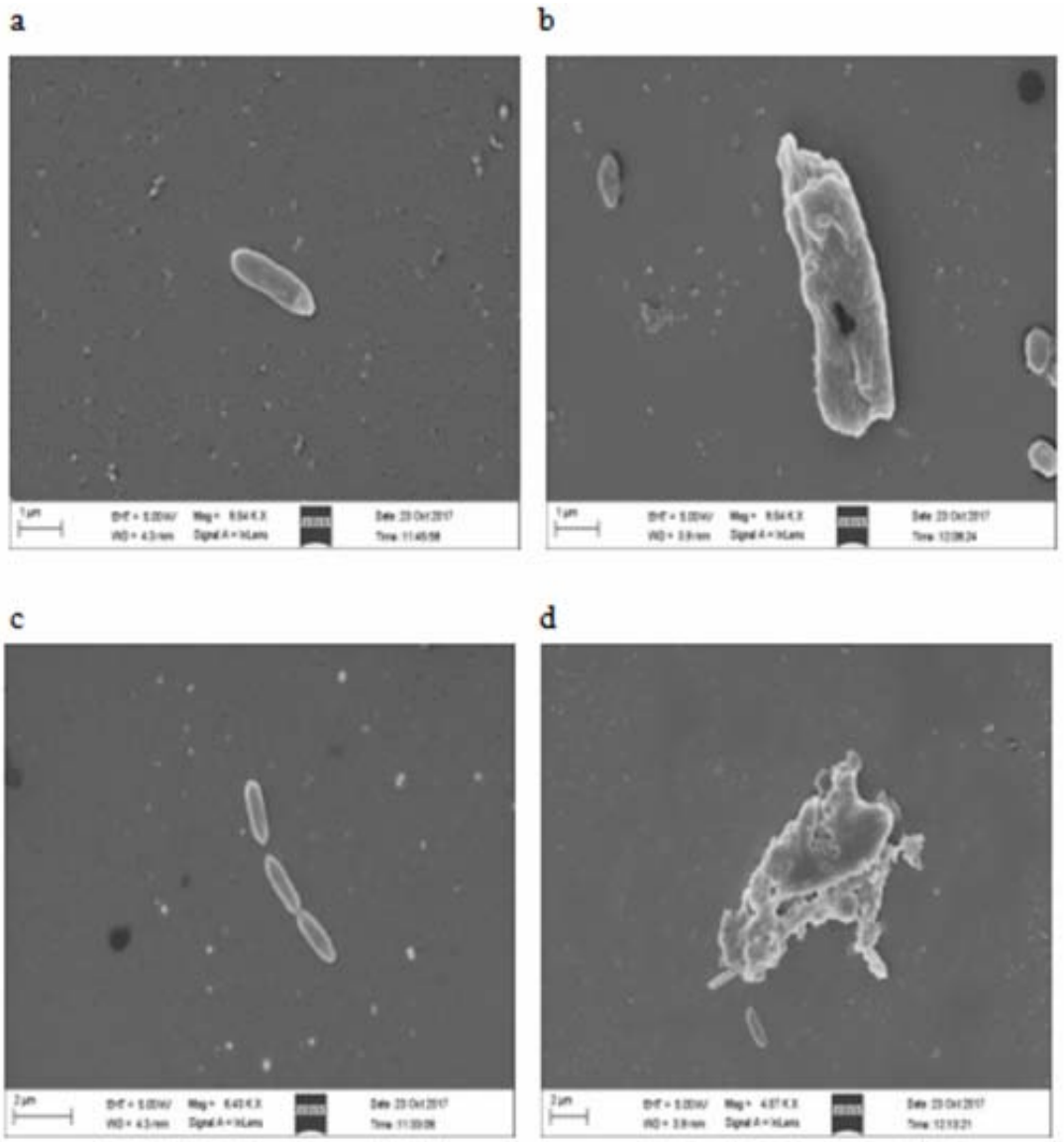

Figure 6. SEM images of PDT-subjected *C. sakzakii* biofilms. Images of *C. sakzakii* treated without PDT (**a** and **c**) and treated with PDT with an incubation time of 20.5 min, TBO concentration of 56 μg/mL, and an illumination time of 30.5 min using a 659.5 nm laser (**b** and **d**). Images a and c show an approximate 10000x amplification, and images b, d show an approximate 5000x magnification.

The PDT effect on biofilms has been studied by other researchers with various PS, such as rose bengal, methylene blue, malachite green, and others [55, 56]. In recent years, many researchers have confirmed the effectiveness of TBO in PDT [57-59]. In this study, TBO proved to be an effective PS with PDT in treating *C. sakazakii* biofilms, and it is compatible at illumination wavelengths from 620-650 nm. Interestingly, it is also effective on *C. sakazakii* biofilms at the 659.5 nm wavelength used in this study, indicating that TBO may also be a promising PS for PDT in the future, thus widening the range of effective illumination wavelengths.

This study was carried out to set appropriate conditions for the use of different incubation times, PS concentrations, and illumination times for *in vitro* study by RSM. The analyses adequately

explained the effects of independent variable responses. This model was found to be highly significant for checking the effect of PDT based on the P-value and F-value, and it was also demonstrated that illumination time was the most important factor influencing PDT, followed by the TBO concentration and then incubation time. Incubation time and illumination time, TBO concentration and illumination time have mutual significant effects on PDT. The results demonstrated that TBO-PDT promoted a statistically significant reduction in the viability of *C. sakazakii* biofilms, with 99.99% of the bacteria in the biofilm being killed after the TBO-PDT treatment.

The effect of TBO-PDT on the *C. sakazakii* biofilms was also visualized by CLSM and SEM in this study. The distribution of live and dead cells can be observed easily and vividly by CLSM through different colours. LIVE/DEAD staining was used in CLSM mainly because it can preserve the intact structure of the biofilms. Our CLSM result showed that most bacteria died after exposure to PDT. On the contrary, the cells were alive without PDT treatment. The SEM analysis with *C. sakazakii* biofilms demonstrated a significant disruption of biofilms after PDT, and in addition, there were changes in bacterial morphology, such as larger size and chain aggregation.

4. CONCLUSION

The inactivation of *C. sakazakii* biofilms was achieved by using TBO-PDT with laser, and conditions were optimized by using RSM. RSM was confirmed to be a valuable tool to exploit the mutual effects and optimization of the various factors and obtain therefore maximum effect of PDT. The present study has shown that PDT can inactivate bacteria due to membrane alterations to its extracellular matrix and disruption of the biofilm structure. It suggests that TBO-PDT is a promising alternative approach to control *C. sakazakii* biofilms.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

REFERENCES

1. Farmer Iii, J. J., Asbury, M. A., Hickman, F. W. and Brenner, D. J. 1980, Int. J. Syst. Evol. Micr., 30, 569-584.
2. Monroe, P. W. and Tift, W. L. 1979, J. Clin. Microbiol., 10, 850-851.
3. Kleiman, M. B., Allen, S. D., Neal, P. and Reynolds, J. 1981, J. Clin. Microbiol., 14, 352-354.
4. Adamson, D. M. and Rogers, J. R. 1981, Clin. Microbiol. Newsl., 3, 19-20.
5. Tekkök, I. H., Higgins, M. J., Ventureyra, E. C. G. and Baeesa, S. S. 1996, Childs Nerv. Syst., 12, 318-322.
6. Kim, J. B., Cho, S. H., Park, Y. B., Lee, J. B., Kim, J. C., Lee, B. K. and Chae, H. S. 2008, Yonsei Med. J., 49, 1017-1022.
7. Teramoto, S., Tanabe, Y., Okano, E., Nagashima, T., Kobayashi, M. and Etoh, Y. 2010, Pediatr. In., 52, 312-313.
8. Bowen, A., Wiesenfeld, H. C., Kloesz, J. L., Pasculle, A. W., Nowalk, A. J., Brink, L. and Tarr, C. L. 2017, MMWR-Morb. Mortal. Wkly. Rep., 66, 761-762.
9. Cui, J. H., Bo, Y. U., Xiang, Y., Zhang, Z., Zhang, T., Zeng, Y. C. and Huo, X. X. 2017, Biomed. Environ. Sci., 30, 601-605.
10. Baumgartner, A., Grand, M., Liniger, M. and Iversen, C. 2009, Int. J. Food Microbiol., 136, 189-192.
11. Coulin, P., Farah, Z., Assanvo, J., Spillmann, H. and Puhan, Z. 2006, Int. J. Food Microbiol., 106, 131-136.
12. Kim, H. and Beuchat, L. R. 2005, J. Food Prot., 68, 2541-2552.
13. Kandhai, M. C. 2010, Detection, occurence, growth and inactivation of *Cronobacter spp*. (*Enterobacter sakazakii*), Wageningen: Wageningen University.
14. Bar-Oz, B., Preminger, A., Peleg, O., Block, C. and Arad, I. 2001, Acta Paediatr., 90, 356-358.

15. Friedemann, M. 2009, Eur. J. Clin. Microbiol., 28, 1297-1304.
16. Lai, K. K. 2001, Medicine, 80, 113-122.
17. Yang, L. H., Deng, Y. M., Zhang, X. T. and Tang, S. Z. 2018, ASIA-PAC J. Food Saf. Secur., 4, 32-44.
18. Nazarowec-White, M. and Farber, J. M. 1997, J. Food Prot., 60, 226-230.
19. Iversen, C., Lane, M. and Forsythe, S. J. 2004, Lett. Appl. Microbiol., 38, 378-382.
20. Mah, T. F. C. and O'Toole, G. A. 2001, Trends Microbiol., 9, 34-39.
21. Stewart, P. S. and Costerton, J. W. 2001, Lancet, 358, 135-138.
22. Chenu, J. W. and Cox, J. M. 2009, Lett. Appl. Microbiol., 49, 153-159.
23. Edelson-Mammel, S., Porteous, M. K. and Buchanan, R. L. 2006, J. Food Sci., 71, M201-M207.
24. Walther, M. M. 2000, Urol. Clin. N. Am., 27, 163-170.
25. Luketich, J. D., Christie, N. A., Buenaventura, P. O., Weigel, T. L., Keenan, R. J. and Nguyen, N. T. 2000, Surg. Endosc., 14, 653-657.
26. Hamblin, M. R. and Hasan, T. 2004, Photochem. Photobiol. Sci., 3, 436-450.
27. Topaloglu, N., Güney, M., Yuksel, S. and Gülsoy, M. 2015, J. Biomed. Opt., 20, 028003.
28. Qin, Y. L., Luan, X. L., Bi, L. J., Sheng, Y. Q., Zhou, C. N. and Zhang, Z. G. 2008, J. Periodont. Res., 43, 162-167.
29. Luan, X. L., Qin, Y. L., Bi, L. J., Hu, C. Y., Zhang, Z. G., Lin, J. and Zhou, C. N. 2009, Lasers Med. Sci., 24, 162-166.
30. Donnelly, R. F., McCarron, P. A., Tunney, M. M. and Woolfson, A. D. 2007, J. Photochem. Photobiol. B-Biol., 86, 59-69.
31. Alves, E., Faustino, M. A., Neves, M. G., Cunha, Â., Nadais, H. and Almeida, A. 2015, J. Photochem. Photobiol. C-Photochem. Rev., 22, 34-57.
32. Pavani, C., Uchoa, A. F., Oliveira, C. S., Iamamoto, Y. and Baptista, M. S. 2009, Photochem. Photobiol. Sci., 8, 233-240.
33. Kharkwal, G. B., Sharma, S. K., Huang, Y. Y., Dai, T. and Hamblin, M. R. 2011, Lasers Surg. Med., 43, 755-767.
34. Usacheva, M. N., Teichert, M. C. and Biel, M. A. 2003, Photochem. Photobiol. B-Biol., 71, 87-98.
35. Usacheva, M. N., Teichert, M. C. and Biel, M. A. 2001, Lasers Surg. Med., 29, 165-173.
36. Wainwright, M., O'Kane, C. and Rawthore, S. 2016, Photochem. Photobiol. B-Biol., 160, 68-71.
37. Graciano, T. B., Coutinho, T. S., Cressoni, C. B., de Paula Freitas, C., Pierre, M. B. R., de Lima Pereira, S. A. and Garcia, M. T. J. 2015, Photodiagnosis Photodyn. Ther., 12, 98-107.
38. Sharma, M., Visai, L., Bragheri, F., Cristiani, I., Gupta, P. K. and Speziale, P. 2008, Antimicrob. Agents Chemother., 52, 299-305.
39. Fekrazad, R., Zare, H. and Vand, S. M. S. 2016, Photodiagnosis Photodyn. Ther., 15, 213-217.
40. Wainwright, M. 2003, Biotech. Histochem., 78, 147-155.
41. Rawlings, D. E. 2005, Microb. Cell. Fact., 4, 1-15.
42. Kim, S. S. and Petrick, J. F. 2005, Tourism Manage., 26, 25-38.
43. Archer, B. and Fletcher, J. 1996, Ann. Touris. Res., 23, 32-47.
44. Lei, X., Liu, B., Huang, Z. and Wu, J. 2015, Arch. Dermatol. Res., 307, 49-55.
45. Bridier, A., Sanchez-Vizuete, M. D. P., Le Coq, D., Aymerich, S., Meylheuc, T., Maillard, J. Y. and Briandet, R. 2012, PLoS One, 7, e44506.
46. Monteiro, J. S., de Oliveira, S. C., Santos, G. M. P., Sampaio, F. J. P., Soares, L. G. P. and Pinheiro, A. L. 2017, Lasers Med. Sci., 32, 29-34.
47. Prochnow, E. P., Martins, M. R., Campagnolo, C. B., Santos, R. C., Villetti, M. A. and Kantorski, K. Z. 2016, Photodiagnosis Photodyn. Ther., 13, 291-296.
48. Menezes, S., Capella, M. A. M. and Caldas, L. R. 1990, J. Photochem. Photobiol. B-Biol., 5, 505-517.
49. Tsai, F. H., Kitamura, Y. and Kokawa, M. 2017, Int. J. Biol. Macromol., 96, 600-610.

50. Vilela, S. F. G., Junqueira, J. C., Barbosa, J. O., Majewski, M., Munin, E. and Jorge, A. O. C. 2012, Arch. Oral Biol., 57, 704-710.
51. Malik, R., Manocha, A. and Suresh, D. K. 2010, Indian J. Dent. Res., 21, 285-291.
52. Maisch, T. 2015, Photochem. Photobiol. Sci., 14, 1518-1526.
53. Pourhajibagher, M., Chiniforush, N., Parker, S., Shahabi, S., Ghorbanzadeh, R., Kharazifard, M. J. and Bahador, A. 2016, Photodiagnosis Photodyn. Ther., 15, 13-18.
54. Chiniforush, N., Pourhajibagher, M., Shahabi, S. and Bahador, A. 2015, J. Lasers Med. Sci., 6, 139-150.
55. Rolim, J. P., De-Melo, M. A., Guedes, S. F., Albuquerque-Filho, F. B., De Souza, J. R., Nogueira, N. A. and Rodrigues, L. K. 2012, J. Photochem. Photobiol. B-Biol., 106, 40-46.
56. Rosa, L. P., da Silva, F. C., Nader, S. A., Meira, G. A. and Viana, M. S. 2015, Photodiagnosis Photodyn. Ther., 12, 276-281.
57. Zanin, I. C. J., Goncalves, R. B., Junior, A. B., Hope, C. K. and Pratten, J. 2005, J. Antimicrob. Chemother., 56, 324-330.
58. Vahabi, S., Fekrazad, R., Ayremlou, S., Taheri, S. and Zangeneh, N. 2011, J. Dent. (Tehran, Iran), 8, 48-54.
59. Zanin, I. C., Lobo, M. M., Rodrigues, L. K., Pimenta, L. A., Höfling, J. F. and Gonçalves, R. B. 2006, Eur. J. Oral Sci., 114, 64-69.