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Biofilm Formation and Methylene Blue-mediated Photodynamic Inactivation of *Vibrio Parahaemolyticus* in the Sea Food Industry

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Abstract

This study was conducted to better understand the mechanism of *Vibrio parahaemolyticus* biofilm formation and to assess the inactivation effects of methylene blue-mediated photodynamic inactivation (PDI) technology as a preventative measure. Optical microscopy, following crystal violet staining, was used to observe the kinetics of *V. parahaemolyticus* biofilm formation. The crystal violet-based assay was performed in microtiter plates, and it was employed to determine which factors were most influential in the formation of the biofilms. Colony counting and confocal laser scanning microscopy (CLSM) were used to test the inactivation effect of methylene blue-mediated photodynamic technology on the biofilms. *V. parahaemolyticus* has the ability to form biofilms, as evidenced by their immediate adherence to glass surfaces and rapid maturity, within 24 h. High (7%) or low (0.5%) salinity was not conducive to the formation of biofilms, and rotational speed greater than 130 rpm also inhibited the process. A 4.05 log reduction in the concentration of viable biofilm cells was obtained with 100 μg/mL methylene blue and 20 min irradiation (24.996 J/cm^2), but planktonic cells were more susceptible to the methylene blue-mediated photodynamic reaction (5.46 log reduction). The results presented here show that the methylene blue-mediated PDI technology is an effective means to inactivate *V. parahaemolyticus* by disrupting its membrane integrity and to inhibit the pathogen's formation of protective biofilms. This technology is a valid tool that can be used to enhance food safety in the sea food industry.

Keywords: *Vibrio parahaemolyticus*; Biofilm formation; Photodynamic inactivation technology; Methylene blue

Introduction

Vibrio parahaemolyticus, an aquatic food-borne pathogen, is responsible for most incidences of seafood-associated gastroenteritis in several countries, and especially those in Asia [1]. This pathogen has elicited considerable attention in the scientific and medical communities due to its high level of pathogenicity. *V. parahaemolyticus* commonly colonizes certain types of seafood, and it forms biofilms in aqueous environments, on aquaculture and line equipment, and on packaging materials by forming organized structures of extracellular polymeric substances (EPS) [2-3]. Biofilms are more resistant to sanitizers and other adverse conditions than are planktonic free cells, and they cause persistent food hygiene problems by cell dispersion during the maturation process [4]. Therefore, the prevention or inhibition of biofilm formation and the disruption of mature biofilms are indispensable in effectively eliminating *V. parahaemolyticus*.

The biofilm formation process occurs in a stepwise manner, being divided into five continuous stages: i. initial reversible attachment, ii. irreversible attachment by quorum sensing (QS) and EPS secretion, iii. micro-colony formation, iv. biofilm maturation encased in EPS, and v. detachment of cells from the mature biofilm [5-6]. Mature biofilms are formed by Salmonella typhimurium at 48 h on glass surfaces, and they can be observed directly by crystal violet staining [7], while *Candida albicans* biofilms form through three distinct developmental phases: early (0 to 11 h), intermediate (12 to 30 h), and mature (38 to 72 h) [8]. However, the growth kinetics and the formation characteristic on glass surfaces of *V. parahaemolyticus* biofilms are yet to be characterized.

Biofilm formation is a dynamic process that can be affected by many environmental factors, such as pH, temperature, growth medium composition, and surface attachment characteristics [9,10]. These factors can be used to control the production of biofilms in the seafood and other related industries. Han et al. [11] reported that *V. parahaemolyticus* biofilm formation was significantly stronger at higher (15-37 °C) rather than at lower

(4-10 °C) temperatures. *V. parahaemolyticus* can readily attach to glass and stainless steel surfaces, and the attachment process can be reduced by various stress treatments, such as heat shock at 42 °C, two-phase acid adaptation at pH 5.8 and pH 5.0, or the presence of sugars in the culture medium [12]. However, little is known about the influence of other factors on biofilm formation, such as the growth phase of the bacteria, the media salt concentration, or the shear stress that is imposed.

When a mature *V. parahaemolyticus* biofilm has already formed, eradication is far more difficult. Approximately 76% of mature biofilms remain attached to a surface, with this residual amount being susceptible only to physical treatments, such as heat shock and ultrasound [13]. Several novel strategies to control biofilm formation have been reported, including exposure to chitosan, bacteriophages, and probiotics [4]. The most commonly used strategy to control formation is the application of chemical reagents, such as sodium hypochlorite or quaternary ammonium compounds [14]. Due to the resistance of *V. parahaemolyticus* biofilms to substrate starvation, desiccation, and exposure to disinfectants and antibiotics [15-16], removal of biofilms from seafood and food-processing equipment by routine cleaning and sanitation methods is challenging [17]. Therefore, it's imperative that an effective alternative inactivation technology be developed to eradicate biofilms produced by multidrug resistant bacteria and, hence, minimize seafood contamination within processing lines.

Photodynamic inactivation (PDI) technology is a novel and promising alternative approach that can be used to eliminate biofilms, as its input requirements are low-cost, and it is environmentally friendly [18]. PDI technology involves the destruction of microorganisms by the combined action of light, a photosensitizer, and molecular oxygen [19]. Molecular oxygen forms highly cytotoxic, reactive oxygen species that cause oxidative damage to cytoplasmic membranes and DNA [20]. It has been shown that PDI is effective against the planktonic form of *V. Parahaemolyticus* [21], however, decontamination of the more resistant biofilm form of *V. parahaemolyticus* has rarely been identified.

Photosensitization, which is the key factor in using PDI technology for cancer treatment, has been extensively reviewed by Sobotta et al. [22-23]. Ren et al. [24] used a hematoporphyrin monomethyl ether PDI technology to induce a significant bactericidal effect in *Staphylococcus aureus*, while methylene blue combined with tungsten-halogen lamp light irradiation was employed to inactivate *Listeria monocytogenes* [25]. Methylene blue is a common cationic and water-soluble photosensitizer that belongs to the phenothiazinium family [26]. Moreover, methylene blue has been widely used in clinical treatments due to its low toxicity against human cells [27], and it has been shown to inactivate several types of microorganisms, due to its high quantum yields of singlet oxygen and its ability to bind to cell walls while not penetrating membranes [28]. Nevertheless, little information is available on methylene blue's application to biofilms. Therefore, the objective of this study was to investigate the effects of methylene blue mediated PDI technology on *V. parahaemolyticus* biofilm formation and the combined inactivation effects of this technology on *V. parahaemolyticus* biofilms in the seafood industry.

Materials and Methods

Bacterial strain and culture conditions

V. parahaemolyticus ATCC 17802 was obtained from the Guangzhou Center for Disease Control and Prevention and preserved at -80 °C using 20% (v/v) glycerol as a cryoprotectant. Stored bacterial cells were grown for 24 h in tryptone soy agar (TSA) containing 3% (w/v) sodium chloride (NaCl, Damao Chemical Reagent, Tianjin, China) at 37 °C. A single colony was transferred to a 15 mL tryptone soy broth (TSB with 3% (w/v) NaCl) and cultivated at 37 °C for 16 h in a vibrating incubator (Stab S2, RADOBIO, Shanghai, China) at 120 rpm under aerobic conditions. Cells were harvested by centrifugation (2,200 × g for 10 min at 4 °C) (ST16R, Thermo Sorvall, USA), washed twice and re-suspended in a phosphate buffered saline solution (PBS; pH 7.2). The initial concentration of the bacterial culture was approximately 10^8 CFU/mL. This was the bacterial concentration that was then used in all subsequent experiments. All media and PBS used were supplied by Hope Bio-Technology Co., Ltd. (Qingdao, China).

Observation of biofilm formation

A 200 μL suspension of bacteria was added to each well in sterile 6-well polystyrene microtiter plates. Individual wells contained 20×20 mm cover slips. Five mL of TSB with 3% (w/v) NaCl were included to enhance biofilm production before incubating and agitating bacteria at 37 °C and 50 rpm, respectively. Biofilm formation was investigated by varying the cultivation time as follows: 0, 6, 12, 18, 24 or 36 h. Progress was determined by crystal violet staining (Yeasen Biotech Co., Ltd, Shanghai, China) following removal of the culture medium and vigorous rinsing of the slides with PBS. The biofilms were observed using an optical microscope (XSP-2CA, Shanghai, China) at 10×100 magnification.

Biomass quantification for biofilm formation in microtiter plates

Biomass was quantified with a modified crystal violet-based assay, as described by Kwiecinska-Piróg et al. [29]. The biofilm formation procedures were performed by adding 10 μL of the bacterial suspensions to sterile 96-well polystyrene microtiter plates along with 200 μL TSB + 3% (w/v) NaCl and incubating the suspensions at 37 °C at a rotational speed of 50 rpm for varying lengths of time (0-120 h). The culture medium was changed the following day, and 10 μL sterile PBS was used as the blank control. The effect of salinity and shear stress on biofilm formation was determined by using one of eight NaCl concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 or 7.0 percent) combined with five rotational

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speeds (0, 50, 70, 100 or 130 rpm) to analyze the effect of salinity and shear stress on biofilm formation, and bacterial suspensions were incubated at 37 °C for 24 h. Orbital shaking, by varying the rotational speed, was used to differentiate the shear stress.

Bacterial suspensions were removed, and all microtiter plates were rinsed three times with 200 μL PBS to flush non-adherent bacteria. Plates were air-dried for 30 min at room temperature. Then, 200 μL 0.1% (w/v) crystal violet were added for 5 min, the stain was removed, and wells were rinsed with PBS to eliminate excess crystal violet. Plates were dried for 15 min at room temperature, and the bound dye in each well was dissolved using 200 μL of 33% (v/v) acetic acid. After 5 min, the biomass was determined by measuring absorbance at 595 nm with a multi-mode microplate reader (Infiniti M200pro, Switzerland). The OD_{595} values for the experimental group minus the blank control provided the final reading. All experimental assays were performed in triplicate.

Photodynamic inactivation assays

Photosensitizer and light source: Methylene blue, the photosensitizer used in this study, was purchased from Sigma-Aldrich (USA). The stock solution was dissolved in PBS (pH=7.4) to obtain a concentration of 10 mg/mL, which was then sterilized by filtration through a 0.22 μm Millipore membrane (Sartorius Stedim Biotech, Germany) and stored in the dark at 4 °C until used. Irradiation procedures were performed using a photocatalysis-xenon lamp (250–1800 nm; LSH-X150; Zolix Instruments Co., Ltd, Beijing, China) located on the inside-top of a plastic chamber, with a power density of 20.83 mW/cm^2. The light source was focused on the sample distal to a convex mirror, and the diameter of the spot was 4 cm. Samples were positioned at the center of the chamber, 20 cm from the lamp. The dosage provided was calculated as follows [30]:

$$E = Pt$$

where E = Dose (energy density) in J/cm^2, P=Irradiance (power density) in W/cm^2, and t = time in sec.

Experimental procedures for PDI inactivation: The *V. parahaemolyticus* biofilms were formed on the cover slips (20×20 mm) in the sterile 6-well polystyrene microtiter plates as previously described, followed by 24 h cultivation. The media was removed, and the cover slips were rinsed with PBS. To investigate the effect of light dose on *V. parahaemolyticus* biofilm inactivation, 2 mL of 100 μg/mL methylene blue solution were added to each well, and the biofilms were incubated in the dark for 30 min. After incubation, irradiation times of 1 min (1.2498 J/cm^2), 5 min (6.249 J/cm^2), 10 min (12.498 J/cm^2), and 20 min (24.996 J/cm^2) were employed. The controls consisted of biofilms without photosensitizer and exposure to different doses of light. To study the role of methylene blue concentration combined with light dose on microbial inactivation, the mature *V. parahaemolyticus* biofilms were incubated in the dark with different concentrations of methylene blue (0.1, 1, 10, 100 or 1000 μg/mL) at 37 °C for 30 min, and they were subsequently exposed to light for 1, 5, 10 or 20 min. The control samples included were the following: no treatment (L- S-), irradiation only (L+ S-), and methylene blue only (L- S+). The groups that did not receive methylene blue did receive the same volume of PBS (pH=7.4). The methylene blue only groups and the no treatment group were protected from light for 20 min.

Following irradiation, the biofilm samples on the cover slips were transferred to 50 mL centrifuge tubes, and biofilms were then removed from the cover slip surfaces using glass bead sonication (KQ-500DE, Kunshan Ultrasonic Instruments Co., Ltd, China) at 50 kHz for 5 min (paused for 30 s after each 30 s treatment). Each group of samples was serially diluted 10-fold in PBS, and the 100 μL dilutions were each spotted onto a TSA plate. Plates were incubated at 37 °C for 24 h, and the results were expressed as log CFU/mL. Each experimental condition was performed in triplicate.

A comparison of photodynamic inactivation of *V. parahaemolyticus* biofilms and planktonic cells was undertaken by respectively transferring 200 μL of the cell suspensions and a biofilm-coated cover slip in each well in 6-well plates with 2 mL methylene blue at different concentrations (0, 0.1, 1.0, 10, 100 or 1000 μg/mL) and then irradiating for 20 min. Cell survival was determined by a plating method, and each experimental condition was tested in triplicate.

Observation of PDI with confocal laser scanning microscopy: A confocal laser scanning microscopy (CLSM) experiment was performed to visualize the effect of PDI on the integrity of bacterial biofilms and to assess cell viability. The covers slips were placed in sterile 6-well polystyrene microtiter plates with inoculated TSB containing 3% (w/v) NaCl, and they were incubated and agitated at 37 °C and 50 rpm for 24 h. The preformed biofilms were treated with different concentrations of methylene blue (0, 1.0, 10 or 100 μg/mL) and irradiated for 20 min. Another set of samples were treated with 100 μg/mL methylene blue and irradiated for varying lengths of time (0, 1.0, 10 or 20 min). Bacterial membrane integrity and cell viability were assessed by CLSM (LSM710, ZEISS, Germany) using a living-dead cell staining Kit (Biovison, USA). After irradiation, the samples were rinsed with PBS three consecutive times. The samples were stained with SYTO®9 (for intact live bacteria; Ex/Em=488/518 nm) and propidium iodide (for membrane damaged or non-viable bacteria; Ex/Em=488/615 nm) according to the manufacturer's instructions, incubated in the dark at 37 °C for 30 min without shaking, and subsequently analyzed with a CLSM equipped with an Argon laser (488 nm), and a He–Ne laser (555 nm). Images were extracted with Zen 2010 software. For each condition, three independent biofilm samples were obtained.

Statistical analysis

All experiments were carried out independently in triplicate. Values were expressed as the mean ± standard deviation (SD). The

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data reported were statistically validated using one-way analysis of variance (ANOVA) with Tukey's post *t*-test, by the IBM SPSS statistics 21 (USA). A *p*-value of < 0.05 was considered significant.

Results and Discussion

Kinetics of *V. parahaemolyticus* biofilm formation

V. parahaemolyticus biofilms formed on glass surfaces were viewed under optical microscopy after staining the adhered bacteria with crystal violet. Figure 1 shows that *V. parahaemolyticus* biofilm formation on glass occurred over five distinct developmental phases:

i) initial attachment (0 to 6 h),

ii) irreversible attachment (6 to 12 h),

iii) micro-colony formation (12 to 18 h),

iv) maturation (18 to 24 h), and

v) detachment (24 to 36 h).

In Figure 1a, the control group without incubation can be seen. Initially, *V. parahaemolyticus* exists in the planktonic mobile cell form, and only a few red fine particles are evident. From 0 to 6 h, the red fine particles increased significantly, and fine, filamentous substances appeared on the glass (Figure 1b). In this stage, the number of adherent bacteria increase markedly, and the pili or flagella are responsible for the motility and adhesion of bacteria. Only a small quantity of extracellular polymeric substances (EPS) are evident, and many moves independently [31]. Bacteria are loosely attached and can return to the planktonic state following intervention. At 6 to 12 h, a large number of agglomerated particles appeared, and the red color was intensified (Figure 1c). The biofilm biomass can be quantified by color gradients [32], and irreversible attachment is complete within 12 h due to the action of quorum sensing molecules and EPS secretion. EPS are responsible for binding cells together (cohesion) and facilitating microcolony formation [33]. Distinct micro-colonies appeared on the surface, and the biofilm structure tended to be closed and orderly over a period of 18 h (Figure 1d). Micro-colony formation is the basic building block of biofilms, resulting from the concurrent growth of microorganisms and the accumulation of EPS [5]. By 24 h, the biofilm achieved its darkest color, and the annular connecting structure appeared (Figure 1e). Maturation is the most stable stage in biofilm formation, and the thickness of the extracellular matrix increased continuously until *V. parahaemolyticus* communities were completely encased within this material. Details of the complex architecture of biofilms, such as interstitial channels, pores within bacterial colonies, and water channels between colonies could be visualized. Cell detachment at 36 h is the final stage in the biofilm cycle, when the color of the biofilms becomes lighter due to numerous cells being released from the mature biofilm (Figure. 1f). Pathogens released from the biofilm invariably cause food hygiene problems. In addition, due to limited nutrient availability or the accumulation of inhibiting metabolites, starvation leads to accelerated detachment, and it forces bacteria inside the biofilm to find new nutrient environments [34].

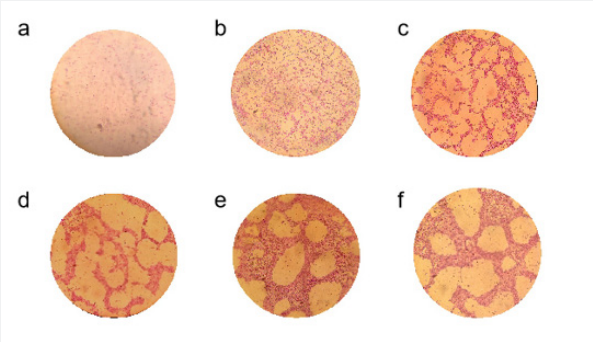

Figure 1: Images of the *V. parahaemolyticus* biofilm formation process visualized after crystal violet staining. Representative images (magnification, 10×100) of *V. parahaemolyticus* biofilms grown on glass for 0 h (a), 6 h (b), 12 h (c), 18 h (d), 24 h (e) and 36 h (f).

The ability to form biofilms can vary among species. The results reported here for *V. parahaemolyticus* were similar to those reported for Listeria monocytogenes and *Salmonella typhimurium* biofilm formation on glass surfaces [7,35], but *V. parahaemolyticus* may have a greater ability to form biofilms than do *L. monocytogenes* or *S. typhimurium*, as it more quickly adheres to glass surfaces and matures within 24 hours.

Influence of time, salinity, and shear stress on *V. parahaemolyticus* biofilm formation

The transition of microorganisms from the planktonic to the biofilm state is influenced by a number of extrinsic factors. The effect of culture time, salinity and shear stress on biofilm growth were explored in this study. Biofilm production as a function of growth time from 0 to 120 h is described in Figure 2. Biomass increased gradually with prolonged incubation up to 24 h, reaching a maximum at 24 h. The differences in biomass produced at 4, 8 and 12 h were significantly less than at 24 h ($p<0.05$), while there were no significant differences in biomass production from 24 to 120 h ($p>0.05$). The fastest growth period was from 8 to 24 h due to EPS secretion and micro-colony formation, which was in agreement with Lizcano's report [36]. Results from Korenová

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et al. [37] highlighted the fact that OD-values based on a crystal violet-based assay are relevant to producing EPS secretion and the formation of micro-colonies. During the development of *V. parahaemolyticus* biofilms, the growth of biomass increases and then reaches a critical point, and a dynamic equilibrium is obtained when a supply of sufficient nutrients is available. These results further support the exceptional ability of *V. parahaemolyticus* strains to form biofilms.

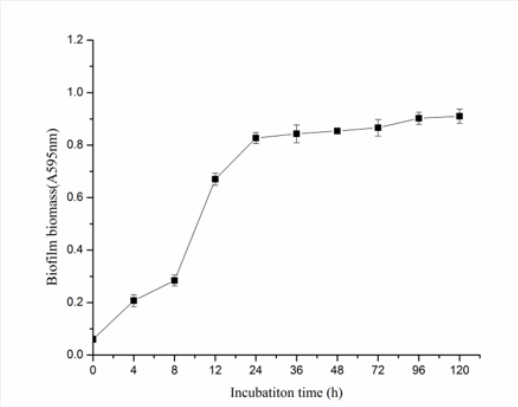

Figure 2: Biomasses of *V. parahaemolyticus* biofilms during 120 h incubations using OD_{595} measurements.

Data on *V. parahaemolyticus* biofilm formation at varying salinity concentrations and degrees of rotational speed are shown in Figure 3. The test results demonstrated that these two variables had a significant influence on biofilm formation ($p<0.05$). The formation rate was enhanced by increasing salinity from 0.5 to 3% and at relatively low rotational speed (at 0-70 rpm). Biofilm formation was significantly stronger at 3% NaCl + 70 rpm rotational speed than in other groups ($p<0.05$). Furthermore, the OD_{595} data revealed that the formation of *V. parahaemolyticus* biofilm was inhibited at low salinity (0.5%) and at high salinity (7%) as well at high rotational speed (130 rpm). The *V. parahaemolyticus* bacterium is a halophile, and it requires a minimum of 0.5% NaCl for growth, with optimal salinity being 2-4%, and it is able to survive at NaCl concentrations up to 10.5% [38-39]. In another report, Mizan et al. [16] found that the largest *V. Parahaemolyticus* biofilm was formed on shrimp surfaces in the presence of 0.01-0.015% glucose + 2% NaCl. In addition, Yang et al. [40] determined that biofilm formation can be inhibited by either low or high salinity due to unbalanced intracellular osmotic pressure, which can obstruct gene expression. Low rotational speed increases opportunities for contact among cells during initial rotations, which would help the cells to detach or disperse from the matrix at their maturation stage. Roosjen et al. [41] found that the initial attachment of bacteria can be active or passive which, depending on motility, diffusion or shear stress of the surrounding fluid phase, increases fluid flow to the attachment surface and leads to faster adhesion of plankton. *Candida krusei*, maintained in a high velocity flow system, must overcome higher shear stress to attach to surfaces and maintain stable matrix structures [9]. Therefore, high shear stress or adjustment to salinity can be used to prevent cells from firmly attaching to contact surfaces, thus preventing *V. parahaemolyticus* biofilm formation.

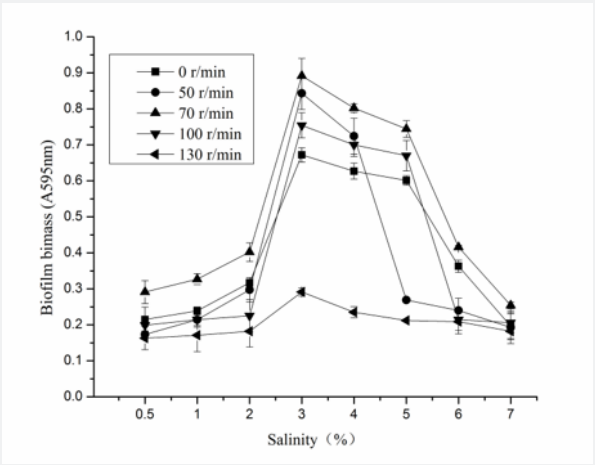

Figure 3: Influence of salinity and rotational speed on the OD_{595} of *V. parahaemolyticus* biofilms.

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Anti-biofilm efficacy of methylene blue-mediated PDI against *V. parahaemolyticus*

Herein, experiments were designed to study the effect of light dose and methylene blue concentration on inactivation against *V. parahaemolyticus* biofilms. If an approximate 3 log CFU/mL reduction in a microbial population is reached, this is considered to be an effective antimicrobial treatment, based on the general guidelines of the American Society for Microbiology [42].

The effects of irradiation time (light dose) on *V. parahaemolyticus* inactivation are shown in Figure 4A. Biofilms exposed to methylene blue solutions without light exposure presented no significant reduction in bacterial count ($p>0.05$). The combination of white light and methylene blue presented strong antimicrobial activity against the biofilms. After 1 min PDI treatment, the combination of light and 100 μg/mL methylene blue caused a significant decrease in bacterial concentration ($p<0.05$) with a 1.61 log reduction in cell count in the biofilm matrix. With the light dose increasing to 24.996 J/cm^2, the PDI treatment showed enhanced light dose dependent bactericidal activity. There was a significant difference of 2.69 and 4.05 log reduction in inactivation of *V. parahaemolyticus* biofilms between 10 min (12.498 J/cm^2) and 20 min (24.996 J/cm^2), respectively ($p<0.05$). Therefore, a higher light dose resulted in greater PDI inactivation, which may have been due to the light dose influencing the amount of methylene blue being activated, and thus the production of reactive oxygen species.

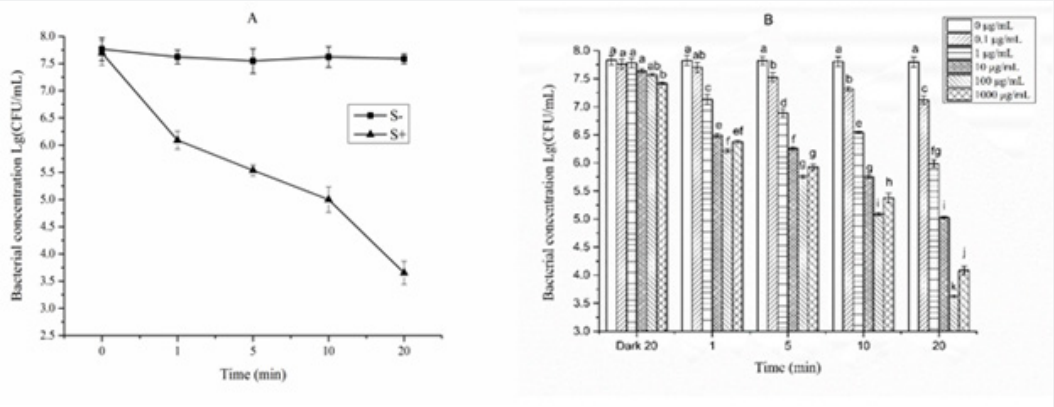

Figure 4: Photodynamic inactivation of *V. parahaemolyticus* biofilms using methylene blue. (A) Effects of irradiation time on V. *parahaemolyticus* biofilms cfu under PDI treatment; (B) Effects of methylene blue concentration on V. *parahaemolyticus* biofilms cfu under PDI treatment. Different letters indicate significant differences between treatment groups ($p<0.05$).

The effects of five methylene blue concentrations and different light doses on the inactivation of *V. parahaemolyticus* biofilms are represented in Figure 4B. The control experiments for light alone (L+S-) further verified that this treatment had a negligible effect on the inactivation of *V. parahaemolyticus* biofilms ($p>0.05$) while methylene blue (100 and 1000 μg/mL) by itself (L-S+) exerted but a slight inhibitory effect (<1 log unit). Huang et al. [43] showed that methylene blue (200 μg/mL) by itself reduced biofilm viability by 32% in *Streptococcus mutans*, while Nie et al. [44] detected no dark toxicity with up to 64 μg/mL of methylene blue. Therefore, it is concluded that relatively low concentrations of methylene blue impart no dark toxicity (<100 μg/mL). Considering all control samples (L-S-; L-S+; L+S-), there were significant differences among all control and PDT treated groups ($p<0.05$), except at the lowest methylene blue concentration (0.1 μg/mL) with 1 min exposure. This indicates that photodynamic inactivation treatment can successfully kill matrix-enclosed *V. parahaemolyticus*. After 20 min irradiation with 10 μg/mL methylene blue, the viability of cells within *V. parahaemolyticus* biofilms was reduced 2.61 log units, which was not significantly different from the 10 min irradiation + 100 μg/mL methylene blue group ($p>0.05$). Therefore, developing a more powerful light source to increase the light dose per unit time could further shorten the illumination time and the methylene blue concentration. The results shown in Figure 4B indicate that the optimal methylene blue concentration range for killing biofilm cells varies from 10 μg/mL to 100 μg/mL for V. parahaemolyticus. Similar results were reported for the combination of 18 J/cm^2 red light + 78 µM (24.95 μg/mL) methylene blue, where greater than a 3 log reduction in the concentration of biofilm cells was achieved in *Escherichia coli* [45]. However, biofilm killing efficiency at higher concentrations of methylene blue (1000 μg/mL) was significantly lower than at 100 μg/mL after 10 min and 20 min illumination ($p>0.05$). Gao and Matthews [46] reported that the antimicrobial efficacy of PDI was lower at higher concentrations of curcumin (2000 ppm) than at 200 ppm of curcumin. This might have been due to a self-shielding effect of the light in a highly concentrated solution, as a higher solute concentration may have blocked light and interfered with the light activation process [47]. In conclusion, the combination of 100 μg/mL methylene blue and 20 min (24.996 J/cm^2) irradiation was the superior PDI treatment condition to use against *V. parahaemolyticus* biofilm cells.

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The inactivation of planktonic cells with PDI was also tested for comparison with the biofilm form. Different concentrations (0, 0.1, 1.0, 10, 100 or 1000 μg/mL) of methylene blue and 20 min irradiation were tested (Figure 5). As expected, planktonic bacteria were more susceptible to PDI than were biofilms, and the difference was particularly obvious in the experiments with methylene blue. Using 10 μg/mL of methylene blue and 24.996 J/cm^2 of white light, inactivation of cells achieved a 3.98 log reduction in the planktonic form. When exposed to 100 μg/mL methylene blue with 20 min irradiation, cell viability within the *V. parahaemolyticus* biofilms and the planktonic form was reduced by 3.95 log units and 5.46 log units, respectively. This demonstrated that *V. parahaemolyticus* biofilms have a 10-100 fold tolerance to different PDI treatment compared to the planktonic state, probably due to the lower amount of methylene blue that was bound to biofilm cells, and owing to the mechanical barrier to methylene blue diffusion posed by the extracellular matrix and to alterations in gene expression [45].

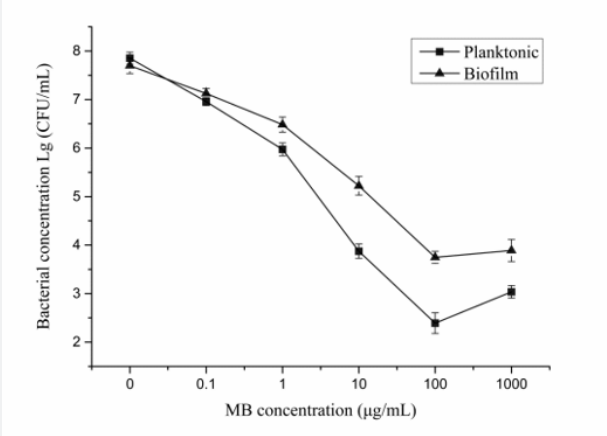

Figure 5: Comparison of PDI effects between *V. parahaemolyticus* biofilms and planktonic forms.

Visualizing effects of PDI under CLSM

The CLSM images of *V. parahaemolyticus* biofilms clearly demonstrate the loss of membrane integrity and biofilm inactivation due to PDI (Figure 6). Images of biofilms treated with different methylene blue concentrations and for varying irradiation times are presented in Figure 6A and Figure 6B. In the negative controls (L+S-; L-S+), homogeneous and intensively distributed structures that cover entire available surfaces can be seen, yet there is no loss of membrane integrity or green bacteria evident (Figure 6A-a and Figure 6B-a). The green color indicates intact live bacterium while red color represents membrane damaged or non-viable bacteria. The yellow color indicates cells that are in the last stages of apoptosis or live and dead cells that have overlapped in the same position. After PDI treatment with increasing concentration and light dose, the images obtained in the two experimental groups showed similar trends, with green bacteria being significantly decreased, indicative of an increase in the number of dead cells (Figure 6A-bcd and Figure 6B-bcd). When the biofilms were treated with 100 μg/mL methylene blue + exposure to irradiation for 20 min, the cells within the biofilm matrix were almost completely eliminated (Figure 6A-d and Figure 6B-d). Li et al. [48-49] demonstrated similar effects of PDI against *L. monocytogenes* biofilms using CLSM visualization. In conclusion, CLSM can rapidly and visually supply qualitative images for a large number of biofilms. The visual results obtained by CLSM were in agreement with the plate counting method, demonstrating that methylene blue mediated PDI can rapidly and effectively inhibit and eradicate *V. parahaemolyticus* biofilms.

Conclusion

V. parahaemolyticus readily forms a thick matrix-enclosed biofilm community on glass surfaces, resulting in increased resistance to hygienic and chemical treatments. The formation of *V. parahaemolyticus* biofilm is influenced by time, salinity and shear stress. A better understanding of these factors can help to avoid *V. parahaemolyticus* biofilm formation in food industry applications. PDI technology is useful in destroying *V. parahaemolyticus* biofilms when cationic methylene blue is employed as the photosensitizer, and the technology is effective at a relatively low light dose and low methylene blue concentration. Therefore, the use of a more powerful light-source or natural sunlight could further shorten illumination time, which would enhance the application of this technology for the seafood industry. Additional studies are needed to determine the precise sterilization mechanism involved in PDI on seafood sensory characteristics under real processing conditions.

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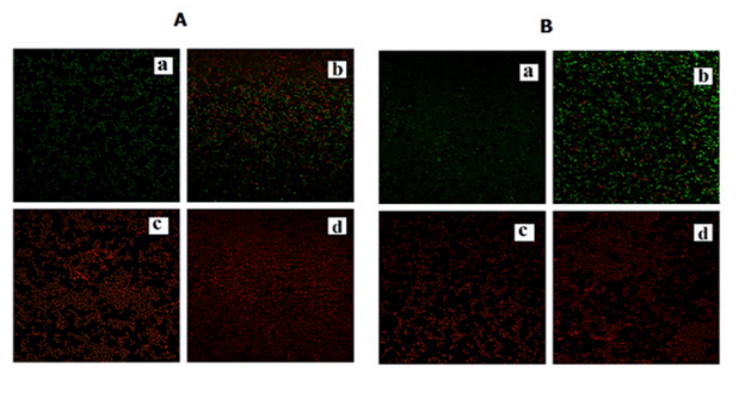

Figure 6: Changes in living and dead cells of *V. parahaemolyticus* biofilms before and after methylene blue-medicated PDI treatment: (A) Treatment with different concentrations of methylene blue a=L+S-; b=L+S+ (1.0 μg/mL); c=L+S+ (10 μg/mL); d = L+S+ (100 μg/mL); L+ means irradiation for 20 min (B) Irradiation at different times. a=L-S+; b=L+ (1 min), S+; c=L+ (10 min), S+; d=L+ (20 min); S+ means 100 μg/mL of methylene blue.

References

1. Newton A, Kendall M, Vugia DJ, Henao OL, Mahon BE (2012) Increasing rates of vibriosis in the United States, 1996-2010: review of surveillance data from 2 systems. Clin Infect Dis 54: 391–395.
2. Aagesen AM, Phuvasate S, Su YC, Hase CC (2018) Characterizing the adherence profiles of virulent *Vibrio parahaemolyticus* isolates. Micro Ecol 75: 152-162.
3. Kavita K, Mishra A, Jha B (2013) Extracellular polymeric substance from two biofilm forming Vibrio species: Characterization and applications. Carbohydr Polymer 94(2): 882-888.
4. Xie T, Liao ZL, Lei H, Fang X, Wang J, et al. (2017) Antibacterial activity of food-grade chitosan against *Vibrio parahaemolyticus* biofilms. Microb. Pathog 110: 291-297.
5. Jahid IK, Ha SD (2014) The paradox of mixed-species biofilms in the context of food safety. Comp Rev Food Sci Food Safety 13(5): 990- 1011.
6. Kostakioti M, Hadjifrangiskou M, Hultgren SJ (2013) Bacterial biofilms: Development, dispersal, and therapeutic strategies in the dawn of the postantibiotic era. Cold Spring Harbor. Perspect. Med. 3(4). doi: 10.1101/cshperspect.a010306
7. Deng YM, Yang LH, Zhang XT, Dong DL, Tang SZ (2019) Effects of ethanol adaptation on the resistance to lethal stress of *Salmonella typhimurium* and its biofilm. J Chin Inst Food Sci Tech 19(09): 53-58.
8. Chandra JD, Kuhn DM, Mukherjee PK, Hoyer LL, Ghannoum MA (2001) Biofilm formation by the fungal pathogen Candida albicans: Development, architecture, and drug resistance. J Bacter 183(18): 5385-5394.
9. Brugnoni LI, Cubitto MA, Lozano JE (2011) Role of shear stress on biofilm formation of *Candida krusei* in a rotating disk system. J. Food Eng. 102(3): 266-271.
10. Whitehead KA, Verran J (2015) Formation, architecture and functionality of microbial biofilms in the food industry. Curr. Opin. Food Sci 2: 84-91.
11. Han N, Mizan MFR, Jahid IK, Ha SD (2016) Biofilm formation by *Vibrio parahaemolyticus* on food and food contact surfaces increases with rise in temperature. Food Control 70: 161-166.
12. Wong HC, Chuang YC, Yu JA (2002) Attachment and inactivation of *Vibrio parahaemolyticus* on stainless steel and glass surface. Food Microbiol 19: 341-350.
13. Zhao XH, Zhao FH, Wang J, Zhong NJ (2017) Biofilm formation and control strategies of foodborne pathogens: food safety perspectives. RSC Adv. 7(58): 36670-36683.
14. da Rosa JV, da Conceicao NV, da Conceicao RDD, Timm CD (2018) Biofilm formation by *Vibrio parahaemolyticus* on different surfaces and its resistance to sodium hypochlorite. Ciência Rural 48(12).
15. Elexson N, Afsah-hejri L, Rukayadi Y, Soopna P, Son R (2014) Effect of detergents as antibacterial agents on biofilm of antibiotics-resistant *Vibrio parahaemolyticus* isolates. Food Control. 35(1): 378-385.
16. Mizan MFR, Jahid IK, Kim M, Lee KH, Kim TJ, et al. (2016) Variability in biofilm formation correlates with hydrophobicity and quorum sensing among *Vibrio parahaemolyticus* isolates from food contact surfaces and the distribution of the genes involved in biofilm formation. Biofouling 32(4): 497–509.
17. Myszka K, Czaczyk K (2011) Bacterial biofilms on food contact surfaces - a review Pol J Food Nut Sci 61(3): 173-180.
18. Castro KADF, Moura NMM, Fernandes A, Faustino MAF, Simões MMQ, et al. (2017) Control of *Listeria innocua* biofilms by biocompatible photodynamic antifouling chitosan based materials. Dyes Pigments 137: 265-276.
19. Alves E, Fostino MAF, Tome JPC, Neves MGPMS, Tome AC, et al. (2013) nNucleic acid changes during photodynamic inactivation of bacteria by cationic porphyrins. Bioorg Med Chem 21(14): 4311-4318.
20. Alves E, Faustino MAF, Neves MGPMS, Cunha A, Tome J, et al. (2014) An insight on bacterial cellular targets of photodynamic inactivation. Fut Med Chem 6(2): 141-164.
21. Arrojado C, Pereira C, Tome JPC, Fa MAF (2011) Applicability of photodynamic antimicrobial chemotherapy as an alternative to inactivate fish pathogenic bacteria in aquaculture systems. Photochem Photobiol Sci 10(10): 1691-1700.
22. Sobotta L, Skupin-Mrugalska P, Piskorz J, Mielcarek J (2019) Porphyrinoid photosensitizers mediated photodynamic inactivation against bacteria. Eur J Med Chem 175: 72-106.
23. Sobotta L, Skupin-Mrugalska P, Piskorz J, Mielcarek J (2019) Non-porphyrinoid photosensitizers mediated photodynamic inactivation against bacteria. Dyes Pigments 163: 337-355.

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24. Ren YQ, Tang SZ, Jin H, Wu XY, Bi SL, et al. (2008) Photodynamic inactivation of *Staphylococcus aureus* and its AFM Images. Food Fermentation Ind 34(8): 56-59.

25. Lin SL, Hu JM, Tang SS, Wu XY, Chen ZQ, et al. (2012) Photodynamic inactivation of methylene blue and tungsten-halogen lamp light against food pathogen *Listeria monocytogene*. Photochem Photobiol 88(4): 985-991.

26. Leelanarathiwat K, Katsuta Y, Katsuragi H, Watanabe F (2020) Antibacterial activity of blue high-power light-emitting diode-activated flavin mononucleotide against *Staphylococcus aureus* biofilm on a sandblasted and etched surface. Photodiagnosis Photodyn Ther 31.

27. Thomas M, Craik JD, Tovmasyan A, Batinic-Haberle I, Benov LT (2015) Amphiphilic cationic Zn-porphyrins with high photodynamic antimicrobial activity. Fut. Microbiol 10(5): 709-724.

28. Anane YA, Apalata T, Vasaikar S, Okuthe GE, Songca SP (2020) *In vitro* antimicrobial photodynamic inactivation of multidrug-resistant *Acinetobacter baumannii* biofilm using protoporphyrin IX and methylene blue. Photodiagnosis Photodyn. Ther. 30. doi: 10.1016/j.pdpdt.2020.101752.

29. Kwiecinska-Piróg J, Bogiel T, Skowron K, Wieckowska E, Gospodarek E (2014) Proteus mirabilis biofilm-Qualitative and quantitative colorimetric methods-based evaluation. Braz JMicrobiol 45(4): 1423-1431.

30. Maclean M, MacGregor SJ, Anderson JG, Woolsey G (2009) Inactivation of bacterial pathogens following exposure to light from a 405-nanometer light-emitting diode array. Appl Environ Microbiol 75(7): 1932-1937.

31. O'Toole GA, Kolter R (1998) Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol Microbiol 30(2): 295-304.

32. de Mele MFL, Cortizo MC (2000) Biodeterioration of dental materials: influence of bacterial adherence. Biofouling 14: 305-316.

33. Gerke C, Kraft A, Süssmuth R, Schweitzer O, Götz F (1998) Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. J Biol Chem 273(29): 18586-18593.

34. O'Toole GA, Kaplan HB, Kolter R (2000) Biofilm formation as microbial development. Ann Rev Microbiol 54: 49-79.

35. Li HA, Tang SS, Liu YQ, Deng X, Liu S, et al. (2014) Antimicrobial photodynamic activity of methylene blue against *Listeria monocytogenes* biofilms. Food Sci 35(03): 144-147.

36. Lizcano A, Chin T, Sauer K, Tuomanen EI, Orihuela CJ (2010) Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of *Streptococcus pneumoniae*. Microb Pathog 48(3-4): 124-130.

37. Korenová J, Lopasovská J, Kuchta T (2008) Comparison of three microtiter plate-based methods for quantification of biofilm formation ability of bacteria contaminating food technologies. J Food Nutr Res 47(2): 100-104.

38. Gao L, Ouyang M, Zhang H, Rao SQ, Yin YQ, et al. (2018) Biological characteristics of *Vibrio parahaemolyticus* during growth in adverse environment. Food Sci 39(6): 177-182.

39. Whitaker WB, Parent MA, Naughton LM, Richards GP, Blumerman SL, et al. (2010) Modulation of responses of *Vibrio parahaemolyticus* O3:K6 to pH and temperature stresses by growth at different salt concentrations. Appl. Environ Microbiol 76(14): 4720-4729.

40. Yang L, Zhan LJ, Han HH, Gao H, Guo ZBet al. (2010) The low-salt stimulon in *Vibrio parahaemolyticus*. Int J Food Microbiol 137(1): 49-54.

41. Roosjen A, Boks NP, van der Mei HC, Busscher HJ, Norde W (2005) Influence of shear on microbial adhesion to PEO-brushes and glass by convective-diffusion and sedimentation in a parallel plate flow chamber. Colloids Surface B 46(1): 1-6.

42. ASM. Antimicrobial Agents and Chemotherapy (2016) Instructions to Authors.

43. Huang TC, Chen CJ, Chen CC, Ding SJ (2019) Enhancing osteoblast functions on biofilm contaminated titanium alloy by concentration-dependent use of methylene blue-mediated antimicrobial photodynamic therapy. Photodiagnosis Photodyn Ther 27: 7-18.

44. Nie M, Deng DM, Wu Y, de Oliveira KT, Bagnato VS, et al. (2020) Photodynamic inactivation mediated by methylene blue or chlorin e6 against *Streptococcus mutans* biofilm. Photodiagnosis Photodyn. Ther. 31. doi: 10.1016/j.pdpdt.2020.101817.

45. Gulias O, Mckenzie G, Bayo M, Agut M, Nonell S (2020) Effective photodynamic inactivation of 26 *Escherichia coli* strains with different antibiotic susceptibility profiles: A planktonic and biofilm study. Antibiotics 9(3): 98-109.

46. Gao JW, Matthews KR (2020) Effects of the photosensitizer curcumin in inactivating foodborne pathogens on chicken skin. Food Control 109.

47. Barr H, MacRobert AJ, Tralau CJ, Boulos PB, Bown SG (1990) The significance of the nature of the photosensitizer for photodynamic therapy: Quantitative and biological studies in the colon. Br J Can 62(5): 730-735.

48. Li HA, Hu HD, Deng X, Liu S, Tang SZ, et al. (2014) Using confocal laser scanning microscope visualizing the effect of anti-microbial photodynamic technology against *Listeria monocytogenes* biofilms. Sci. Tech Food Ind 35(04): 157-160.

49. Deng X, Tang SZ, Wu Q, Tian J, Riley WW, et al. (2016) Inactivation of *Vibrio parahaemolyticus* by antimicrobial photodynamic technology using methylene blue. J Sci Food Agri 96(5): 1601-1608.

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