In vitro and in silico approaches to investigate antimicrobial and biofilm removal efficacies of combined ultrasonic and mild thermal treatment against *Pseudomonas fluorescens*

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**ABSTRACT**

A combined ultrasonic and thermal (US-TM) treatment was developed in this study to achieve a high efficacy of *P. fluorescens* biofilm control. The present study demonstrated that combined a moderate ultrasound treatment (power \(\geq 80\) W) and a mild heat (up to 50 °C) largely destroyed biofilm structure in 15 min and removed \(\geq 65.63\)% of biofilm from a glass slide where cultivated the *P. fluorescens* biofilm. Meanwhile, the viable cell count was decreased from 10.72 to 6.48 log_{10} CFU/mL. Differences in biofilm removal and lethal modes of US-TM treatment were confirmed through microscopies analysis in vitro. The ultrasound first contributed to releasing the bacteria in the biofilm to the environment and simultaneously exposing inner bacteria at the deep layer of biofilm depending on shear force, shock waves, acoustic streaming, etc. When the biofilm structure was destroyed, US-TM treatment would synergistically inactivate *P. fluorescens* cells. In silico studies adopted COMSOL to simulate acoustic pressure and temperature distribution in the bioreactor; both of them were significantly influenced by various factors, such as input power, sonotrode position, materials and volume of container, etc. Facing the biofilm issue existing on the surface of container, boundary conditions were exported and thereby pointing out potential “dead ends” where the ultrasound may not be effectively transduced. Both in vitro and in silico results may inspire the food industry to adopt US-TM treatment to achieve biofilm control.

1. Introduction

*Pseudomonas fluorescens* is widely distributed in plants, soil, and the marine environment [1]. *P. fluorescens* has a strong biofilm-forming ability and secretion of extracellular enzymes that leads to the spoilage in meat, vegetables, and dairy products [2]. Biofilm refers to a structured community of microorganisms colonizing on a biotic or abiotic surface and self-producing a matrix composed of extracellular polymeric substances (EPS) [3,4]. When the biofilm is formed on food contact surfaces, the foods are persistently contaminated by inner microorganisms in the biofilm which becomes a big threat to food safety. In addition, the biofilm is also a shelter for inner microorganisms to promote resistances of chemical disinfectants, thermal treatment, and mechanical cleaning, etc. [5]. In the food industry, the residue of *P. fluorescens* biofilm is of concerned due to the prevalence on food contact surfaces and the strong biofilm-forming ability. The matured *P. fluorescens* biofilm would co-culture with pathogenic bacteria to enhance their resistance and repeatedly pollute the food products [6].

Considering practical conditions, conventional thermal treatment may not be sufficiently enough to inactivate inner microorganisms in the biofilm formed on food contact surfaces in the food industry, such as equipment, convey belts, and food containers [7]. O’Toole, Ricker and Nuxoll [8] evaluated thermal mitigation of *Pseudomonas aeruginosa* biofilms which were subjected to mild heating at 50 °C. The bacterial density only decreased by 1.54 log_{10} CFU/cm\(^2\) in 30 min, which indicated that the heat resistance of bacteria in the biofilm is relatively strong. Furthermore, such a mild heat treatment could only inactivate via a sublethal injury process [9]. Ultrasound is an innovative
Bacillus stearothermophilus that an ultrasonic treatment at 40 kHz for 10 s would effectively detach biofilm on stainless steel, which was only decreased 1.4 Log$_{10}$ CFU/mL. Although the biofilm removal efficacy of ultrasound is relatively high, it is noticeable that the mechanism responsible for the biofilm removal and microbial inactivation are physical forces (i.e. agitation, turbulence, shear stress, and high pressure) and free radicals with high bactericidal reactivity generated by the cavitation bubbles in liquid media [11,12]. Although the biofilm removal efficacy of ultrasound is relatively high, it is noticeable that the antibacterial effect of ultrasound was unsatisfactory when uses alone. Lee, Kim and Ha [13] determined viable Listeria monocytogenes inoculated on stainless steel, which was only decreased 1.4 Log$_{10}$ CFU/mL after a 100-min ultrasonic treatment (frequency: 37 kHz, equipment power: 1200 W). In other cases, the viability of bacteria could be even promoted when the ultrasonic intensity was applied below the bacterial tolerance threshold [14]. This phenomenon may be attributed to mechanotransduction that activated the bacterial stress response system and bacterial mechanosensitive channels [15,16]. Therefore, combined ultrasonic and thermal (US-TM) treatment is possible to effectively remove biofilm on the food contact surfaces and sterilize inner microorganisms for achieving a reasonably high bacterial effect. Sublethal injury of cells would be also decreased when treated the biofilm using the combined treatment.

There are two more issues that should be considered when adopting ultrasound to achieve biofilm control. The first is the attenuation of ultrasound in liquid media. An ultrasonic attenuation coefficient depends on various factors, such as type of liquid, particle size, etc. [17]. The second is the unevenly distribution of ultrasound on the surface biofilm. Although nearly all previous studies have indicated power/intensity/frequency of ultrasonic treatment, the effectiveness of dislodging biofilms could be affected by many other factors, e.g. distance from ultrasonic transducer, biofilm position, and shape of container. For example, an acoustic pressure at high level occurs mainly in the solution around the probe. The rest areas respond slower. The surface of container where grows the biofilm should be fully considered. Therefore, it is necessary to develop a new methodology to propose a standardized parameter making data comparable and predictable. Considering many factors would influence ultrasonic treatment, the acoustic pressure distribution on the biofilm is a suitable candidate, which can be obtained by a simulation in silico. During the COMSOL simulation, the above-mentioned factors can be considered together and eventually output the acoustic pressure distribution on the biofilm [18].

In this study, ultrasound and moderate heat were to be applied together as a hurdle approach to achieve Pseudomonas fluorescens biofilm. COMSOL was first to be simulated acoustic pressure and temperature distribution on the surface of biofilm in silico followed by determining antimicrobial and biofilm removal efficacy in vitro using US-TM treatment at various conditions. Simulation results may inspire researchers to consider distribution of acoustic pressure on the boundary of container when treating the biofilm in practical use.

2. Materials and methods

2.1. Bacterial strain and cultivation of P. fluorescens biofilm

P. fluorescens (ATCC 13525) was purchased from Beina Science & Technology Co., Ltd. (Beijing, China). It was first cultivated in a nutrient broth (Sinopharm Chemical, Shanghai, China) for 48 h at 28 °C with stirring at 180 rpm. In this study, the biofilm was formed on a glass side (Φ = 20 mm). The glass slide was pre-washed to remove organic compounds and then sterilized following the method described in [7]. The glass slide was transferred to a well of microtitre plate and then added 2 mL of nutrient broth with suspended bacteria (10$^8$ CFU/mL) into the well. The microtitre plate was then incubated at 28 °C for 48 h. At the end of incubation, the P. fluorescens biofilm reached the maturity stage.

2.2. US-TM treatment

The glass slide with matured biofilm was placed in the center of a culture dish (Φ 35 mm). The 5 mL of sterilized saline water was transferred into the culture dish and then sealed by a parafilm. The culture dish was then placed at the bottom of a bioreactor (SDA-SCSB500, Shendi Glassware, China) followed by adding 500 mL distilled water. An ultrasound system was utilized to generate high-intensity ultrasound (UIP 500, Hielscher, Germany). A titanium sonotrode with a frontal diameter of 22 mm (BS2d22, Hielscher, Germany) was inserted into the bioreactor. The distance between the ultrasonic probe and the bottom of bioreactor was maintained at 40 mm. Cooling water was kept pumping through a cooling water jacket on the outside of bioreactor, which maintained the inner temperature of bioreactor at 30, 40, and 50 °C. The output power of ultrasound into the media was 60, 80, and 100 W; the intensity of ultrasound was calculated to be 15.79, 21.06, and 26.32 W/cm$^2$, respectively. Duration of each US-TM treatment remained constant at 15 min.

2.3. COMSOL simulation

Acoustic pressure and temperature distributions in the ultrasonic bioreactor were simulated in silico by COMSOL Multiphysics (V5.2, Stockholm, Sweden) conjugating pressure acoustics in frequency domain (ACPR) model, non-isothermal flow, turbulent flow, k-ε (NITF) physical model, and heat transfer model. A 3D model of the ultrasonic bioreactor was first established using computer-aided design (CAD)
import module kernel as shown in Fig. 1A. The diameter and height of bioreactor and its inner container were 142 and 130 mm, respectively. In addition, the diameter and height of inner container were 94 and 107 mm, respectively. A cooling water jacket was covered on the outside of inner container with an inlet and outlet tube (diameter: 5 mm; length: 26 mm) that connected to a cooling water bath (XODC-1006, Atpio, China). The glass slide cultivated *P. fluorescens* biofilm was located at the center of inner container with a diameter of 20 mm and a thickness of 0.2 mm. During the ultrasonication, 500 mL distilled water was transferred into the inner container, which had a height of 70 mm. The ultrasonic probe (diameter: 22 mm) was immerced in the water with a depth of 23 mm. Furthermore, water-probe, water-air, and water-glass were assigned as three acoustic impedance boundaries. The mesh was automatically generated in the 3D model and subsequently refined the mesh until no observation of predictions with noticeable changes (Fig. 1B).

The Helmholtz equation in Eq. (1) was used to simulate acoustic pressure based on the method described in Cao, Hao, Ma, Yang and Sun [19] with modifications.

$$\nabla \cdot \left( \frac{\rho}{\rho} \nabla p \right) - \left( \frac{\rho}{\rho} \right)^2 = 0 \tag{1}$$

$$\nabla = \frac{\partial p}{\partial x} \nabla \frac{\partial p}{\partial y} + \frac{\partial p}{\partial z} \nabla \tag{2}$$

$$\omega = 2 \pi f \tag{3}$$

where $p$ (N/m$^2$) is pressure field; $\rho$ (kg/m$^3$) is material density; $c$ (m/s) is sound velocity in different materials; $f$ (Hz) refers to the frequency of ultrasound.

The input of ultrasonication was determined as normal acceleration ($a_n$) on the surface of ultrasonic probe, which is derived from Eq. (4):

$$a_n = \alpha_n \sqrt{\frac{2 I}{\pi r^2}} \tag{4}$$

Table 1

| Parameters | Description | Value |
|------------|-------------|-------|
| $f$ | Frequency of ultrasound | 20 Hz |
| $\omega$ | Angular frequency of ultrasound | 1.256 $\times$ $10^7$ rad/s |
| $l_1$ | Intensity of ultrasound | 15.79 W/cm$^2$ |
| $l_2$ | Intensity of ultrasound | 21.06 W/cm$^2$ |
| $l_3$ | Intensity of ultrasound | 26.32 W/cm$^2$ |
| $P_1$ | Power of ultrasound | 60 W |
| $P_2$ | Power of ultrasound | 80 W |
| $P_3$ | Power of ultrasound | 100 W |
| $r_1$ | Radius of ultrasonic probe | 22 mm |
| $\alpha$ | Absorption coefficient | 0.025 m$^{-1}$ |
| $T_{SO}$ | Temperature of cooling water | 28.5 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 38.5 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 48.0 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 28.0 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 35.7 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 47.0 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 27.5 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 36.5 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 46.5 $\text{°C}$ |
| $T_{SO}$ | Temperature of cooling water | 24.0 $\text{°C}$ |
| $\rho_{ws}$ | Density of water | 1.2 kg/m$^3$ |
| $\rho_{ws}$ | Density of water | 998 kg/m$^3$ |
| $\beta_{glass}$ | Density of silica glass | 2,500 kg/m$^3$ |
| $\beta_{glass}$ | Density of silica glass | 4,510 kg/m$^3$ |
| $C_{glass}$ | Speed of sound in water | 1,500 m/s |
| $C_{glass}$ | Speed of sound in water | 343 m/s |
| $C_{glass}$ | Speed of sound in water | 5,639 m/s |
| $C_{glass}$ | Speed of sound in water | 6,100 m/s |

$$I = \frac{P}{\pi r^2} \tag{5}$$

where $I$ (W/cm$^2$) is intensity of ultrasound; $P$ (W) is the input power of ultrasound; $r$ (cm) is the radius of ultrasonic probe.

The fluid flow in both inner reactor and cooling water jacket was governed by incompressible Navier-Stokes equation with mass continuity equation [20].

$$\rho \frac{\partial u}{\partial t} + \rho (u \cdot \nabla) u = \nabla \left[ - p I + (\mu_1 + \mu_2) \left( \nabla u + (\nabla u)^T \right) \right] + F_A + F_B \tag{6}$$

$$\nabla u = 0 \tag{7}$$

$$|F_A| = \frac{2 \pi^2 \rho I^2}{\rho c^3} \tag{8}$$

$$|F_B| = \rho g R (T - T_{ref}) \tag{9}$$

where $F_A$ and $F_B$ refer to radiation and buoyancy forces, respectively; $\beta$ refers to thermal expansion of water; $T$ and $T_{ref}$ refer to the water temperature and reference temperature, respectively.

As long as the high-intensity ultrasound was applied in the sample solution with a high input power (>60 W), the acoustic steaming was induced. A modified k-ε model was adopted to simulate the turbulent flow during the ultrasonication as shown in Eqs. (10) and (11). The heat transfer in the liquid is followed by Eq. (12). Above-mentioned parameters in the equations are summarized in Table 1.

$$\rho \frac{\partial k}{\partial t} + \rho (u \cdot \nabla) k = \nabla \left[ \left( \mu_1 + \mu_2 \right) \left( \nabla k \right) \right] + P_k - \rho \varepsilon \tag{10}$$

$$\rho \frac{\partial \varepsilon}{\partial t} + \rho (u \cdot \nabla) \varepsilon = \nabla \left[ \left( \mu_1 + \mu_2 \right) \left( \nabla \varepsilon \right) \right] + C_1 \frac{\varepsilon}{k} P_k - C_2 \rho \frac{\varepsilon^2}{k} \tag{11}$$

$$\rho c_p \frac{\partial T}{\partial t} + \rho (u \cdot \nabla) T = \nabla \left[ \left( k_1 + C_\nu H_T \right) \left( \nabla T \right) \right] + Q_{US} \tag{12}$$

$$Q_{US} = \frac{\alpha}{\rho c_p} \frac{I^2}{\pi r^2} \tag{13}$$

2.4. Enumeration of viable cells

At the end of US-TM treatment, a cell scraper was completely dispersed the biofilm cells from the glass slides to the sterile saline in the culture dish. Both cells and sterile saline in the culture dish were transferred to a sterile beaker followed by adding 100 mL sterile saline. One mL of the sample solution was collected and then properly diluted. The 1 mL of diluted sample solution was transferred to a culture dish followed by pouring 15–20 mL of plate counting agar and incubating at 28 °C for 24 h. The total number of colonies was counted lastly.

2.5. Determination of biofilm removal

The crystal violet semi-quantitative method was used to determine the biofilm removal. At the end of US-TM treatment, the glass slide cultivated biofilm was carefully rinsed by sterile water to remove loosely attached cells followed by drying at room temperature for 10 min. Two mL of 0.2% crystal violet solution was added to the surface of glass slide to stain the cells for 15 min. Exceed dye was washed by sterile water. The glass slide with the stained cells was then transferred to a 12-well plate and added 2 mL of 33% acetic acid to dissolve the biofilm for 10 min. Lastly, a microplate reader was used to measure the absorbance value at 595 nm.
2.6. Confocal laser scanning microscopy (CLSM) analysis

At the end of US-TM treatment, the biofilm formed on the glass slide was subjected to CLSM analysis. A mixture of SYTO 9 and PI was prepared at the ratio of 1:1 followed by an instruction from the manufacturer (Live/Dead BacLight bacterial viability kit, L7012, Thermo Fisher Scientific Co., Ltd., USA). In brief, SYTO 9 and PI kits were separately mixed with distilled water (weight ratio 1:30) to make dyeing solutions. Three hundred μL of each staining solution was added to the glass slides and maintained in darkness for 15 min. The biofilm stained by SYTO 9 and PI was visualized under CLSM (LSM 710, Carl Zeiss Microscopy GmbH, Germany). The parameters of CLSM were followed by the method as described in [7].

2.7. Scanning electron microscopy (SEM) analysis

At the end of US-TM treatment, the treated biofilm was subjected to SEM analysis. The P. fluorescens biofilm was fixed by 2.5% glutaraldehyde for 4 h at 4°C. Exceed chemical was gently washed two times by distilled water. The fixed biofilm was dehydrated by ethanol at concentrations of 30%, 50%, 70%, 90%, and 100%, respectively, for 15 min each. The glass slide was immersed into a mixture of isoamyl acetate and ethanol solution (volume ratio 1:1) for 30 min followed and then transferred into isoamyl acetate for 12 h. The glass slide was then dried in air and coated by gold–palladium. The biofilm on glass slides was visualized under SEM (SU1510, Hitachi Co., Ltd., Japan) at 5.00 kV with 4,000× magnification.

2.8. Statistical analysis

All data presented in this study was a mean of triplicate. Significant difference test among the data was tested by SPSS statistical software (version 22, IBM, USA) using one-way analysis of variance (ANOVA) with Duncan’s multiple range test (p < 0.05).
3. Results and discussion

3.1. Acoustic pressure distribution in the bioreactor

The acoustic pressure distribution in the bioreactor during US-TM treatment is shown in Fig. 2. The regions of high acoustic pressure were gathered around the surface of ultrasonic probe. The core acoustic pressure reached $1.19 \times 10^6$, $1.38 \times 10^6$, and $1.55 \times 10^6$ Pa, respectively, with the ultrasound power of 60, 80, and 100 W. With the increased distance between target place and ultrasonic probe, the acoustic pressure was rapidly dropped. The acoustic pressure on the center of glass slide cultivated *P. fluorescens* biofilm ($Z = 23.2$ mm) was only $1.67 \times 10^4$, $1.93 \times 10^4$, and $2.15 \times 10^4$ Pa when the input power of ultrasound was 60, 80, and 100 W, respectively. It can be seen that the value of acoustic pressure on the surface of glass side cultivated *P. fluorescens* biofilm was reduced by about two orders of magnitude.

Fig. 3. Simulated results of temperature distribution in the 3D model of bioreactor during US-TM treatment at Y-Z plane and X-Y plane ($Z = 23.2$ mm where is the surface of *P. fluorescens* biofilm on the glass slide). The power of ultrasound was 100 W. The processing temperature was (A, B) 30 °C, (C, D) 40 °C, and (E, F) 50 °C. Unit in the colored labels is °C.
when compared with the core acoustic pressure region around the ultrasonic probe. This phenomenon is mainly attributed to the attenuation of ultrasound in the water solution. It is noticeable that the absolute value of acoustic pressure was slightly increased (X-Y plane, Z = 20.0 to 0 mm) where the position was close to the water–glass boundary (Fig. 2C). It is mainly attributed to the reflectivity of the sound waves.

Since the biofilm is normally grown on the surface of material, it is necessary to further investigate acoustic pressure distribution on the bottom layer (X = 0, Y = 46.8, Z = 23.2) and inner wall (X = 0, Y = 46.8, Z = 23.2 ~ 70.0) of bioreactor. As shown in Fig. 2F, the highest absolute value of acoustic pressure was \(3.14 \times 10^4\), \(3.63 \times 10^4\), and \(4.06 \times 10^4\) Pa at the position (X = 0, Y = 36 and + 36, Z = 23.2) with the ultrasound power of 60, 80, and 100 W, respectively. As elucidated previously, the increase in acoustic pressure at this position is mainly attributed to the reflectivity of sound waves. Boundary condition of the bottom layer might subvert a traditional understanding that the highest acoustic pressure on the bottom layer of inner bioreactor was right below the ultrasonic probe. In addition, it is noticeable that there is a contradiction between simulation and experimental findings. The simulated value of acoustic pressure was about 0 Pa at the position (X = 0, Y = 14.2 and + 14.2, Z = 23.2). However, the following experimental studies also observed a bactericidal effect and a biofilm removal at this position when treating the \(P.\) \(fluorescens\) biofilm using ultrasound.

Acoustic pressure can be further converted to intensity distribution as an indicator of sono-energy density. Although the value of acoustic pressure at this position is 0 Pa, cavitation, free radicals, and mechanical actions of ultrasound could mainly contribute to the bactericidal effect and biofilm removal [21,22].

Fig. 2J indicates the acoustic pressure distribution on the inner wall of bioreactor. The overall trend of acoustic pressure was first increased and then decreased with the increase of Z-axis value. The highest absolute value of acoustic pressure where is the closest to the ultrasonic probe was \(6.84 \times 10^4\), \(7.89 \times 10^4\), and \(8.82 \times 10^4\) Pa at the position (X = 0, Y = 46.8 and + 46.8, Z = 43.2) with the ultrasound power of 60, 80, and 100 W, respectively.

Although power/intensity and frequency of ultrasound are determining factors that have great impacts on the ultrasonic treatment, many other factors, such as shape and position of ultrasonic probe, sample material, temperature, etc., would also affect the distribution of acoustic pressure in the bioreactor and therefore, have impacts on control efficacy of \(P.\) \(fluorescens\) biofilm [3,23]. The above-mentioned factors which influenced the ultrasonication have been fully considered during the COMSOL simulation [24]. Results indicated high/low regions of acoustic pressure on the surface of inner bioreactor where the potential “dead ends” in this study referred to about 0 Pa of simulated acoustic pressure. The low regions of acoustic pressure could become potential “dead ends” where the biofilm might not be effectively controlled. The “dead ends” present real problems leading to unacceptable and sudden high numbers of taint and microorganisms [25]. The subsequent study still observed the removal of \(P.\) \(fluorescens\) biofilm at these “dead ends”, which could be attributed to shear force, shock waves, micro-streams, and micro-jets generated from ultrasound as mechanical actions. The overall energy on these spots were still lower than the others based on the simulated results that may lead to a relatively poor bactericidal effect.

The current setup of ultrasound system (i.e. insert the ultrasonic probe into the reactor) could be utilized to clean reactors, storage tanks, etc. as a potential upgrade of conventional fill-boil-dump cleaning in place (CIP) system in food factories. The ultrasonic generator can also be located on the outside of pipes and tunnels. This type of setup needs another simulation to acquire acoustic pressure and temperature distribution. Furthermore, the improved design of ultrasonic cleaning equipment is recommended to avoid the “dead ends” and thereby promoting the control efficacy of \(P.\) \(fluorescens\) biofilm.

### 3.2. Temperature distribution in the bioreactor

The temperature distribution in the bioreactor during US-TM treatment is shown in Fig. 3. The regions of high temperature were distributed around the surface of ultrasonic probe. The input power of ultrasound was constant at 100 W. The temperature of cooling water was determined as 27.5, 36.5, and 46.5 °C through experiments according to the measured temperature of 30.0, 40.0, and 50.0 °C in the inner reactor, respectively. In these conditions, results of simulation showed that the core temperature reached 43.96, 53.89, and 64.53 °C. With the increased distance between target position and ultrasonic probe, the temperature was rapidly dropped to the temperature of external cooling water. Furthermore, the temperature distribution on the bottom layer and the inner wall of bioreactor was simulated; the temperature in these places was also very similar with that of cooling water since heat the transfer coefficient of silica glass was 1.38 [W/(m × K)].

Thermal effects of ultrasound are normally generated when a substantial amount of energy is transferred to media [26]. As long as the intensity of ultrasound is high enough, transient/collapse cavitation can be largely produced. The cavities are normally generated during the expansion cycle. The radius of bubble keeps increasing through absorbing acoustic energy until reaching a critical size. The sudden reversal in the motion of the bubble wall leads to implosion followed by producing a shock wave, releasing energy, and heating the surrounding
area to a temperature near 5,500 K at peak compression [27]. Under such an extraordinarily high pressure and temperature, water and other molecules can be fragmented into free radicals leading to bactericidal effects [28,29]. The current simulation results only showed a temperature distribution at a stationary phase during the US-TM treatment. An extremely high, albeit momentary temperature generated by the implosion of cavities is possibly brought a better thermal effect in the *P. fluorescens* biofilm. Results of a preliminary experiment showed that the processing temperature could not reach 50 °C without the assistance of external heating water since the thermal effect of ultrasound highly depends on the size of media and input power of ultrasound. Therefore, an external heating source is necessary to maintain a certain temperature level during the US-TM treatment. In addition, the simulated temperature on the surface of inner boundaries was normally lower than the measured value in the sample solution during the US-TM treatment. The temperature difference between the boundaries and sample solution was about 3 to 4 °C. These results again emphasize that determination of boundary conditions is critical to biofilm control.

Fig. 5. CLSM images of *P. fluorescens* biofilm after the US-TM treatment for 15 min at the combined (I-IV) four ultrasound power levels (0, 60, 80, and 100 W) and (A-C and a-c) three temperature levels (30, 40, and 50 °C). The first and the second row at the same temperature represented (A-C) 2D and (a-c) 3D CLSM images, respectively.
3.3. Biofilm removal and inactivation of *P. fluorescens* at different US-TM conditions

Results of *P. fluorescens* biofilm residue are shown in Fig. 4A after the US-TM treatment at different combinations. Without the assistance of ultrasound, thermal treatment could not peel off *P. fluorescens* biofilm from the surface of glass slide. In general, the residue of biofilm was significantly decreased with the increased ultrasound power from 60 to 100 W. Furthermore, the biofilm removal was largely improved with the increased temperature level when the ultrasound power remained unchanged. For instance, the lowest residue of biofilm was only 21.76 ± 0.01 % when treated by US-TM at 100 W and 50 °C, which was significantly lower than those at 100 W and 40 °C (38.46 ± 0.01 %), as well as at 100 W and 30 °C (41.85 ± 0.01%). Therefore, *P. fluorescens* biofilm removal was less influenced by the thermal treatment, the synergistic cleaning effect of biofilm was significantly improved in combination with ultrasound and thermal treatment. Similar results on antimicrobial effects of US-TM are shown in Fig. 4B. When *P. fluorescens* biofilm underwent thermal treatment at 30, 40, and 50 °C, the viable cell count was only decreased from 10.30 ± 0.04 to 9.34 ± 0.02 log_{10}CFU/mL. This phenomenon indicates that bacteria in the biofilm structure become insensitive to environmental stresses. Thermal resistance of cells in biofilm would become up to thousands of times higher than their planktonic counterparts [30]. With the combination of ultrasound, the bactericidal effect was significantly improved. For example, the viable cell count was decreased from 7.04 ± 0.02 to 5.37 ± 0.02 log_{10}CFU/mL with the increased ultrasound power from 60 to 100 W at 50 °C. At the same power level of ultrasound (e.g. 80 W), the viable cell count was significantly decreased from 8.99 ± 0.09 to 6.48 ± 0.01 log_{10}CFU/mL with the increased temperature from 30 to 50 °C during the US-TM treatment. These results again indicate that a synergistic antimicrobial effect was significantly improved in US-TM treatment compared with the individual counterparts [31].

The biofilm removal and bactericidal action are conducted simultaneously during the US-TM treatment. The biofilm removal is mainly attributed to the ultrasound treatment, and the bactericidal action can be caused by both ultrasound and thermal treatment. The shear force, shock waves, micro-streams, and micro-jets generated from ultrasound are believed to effectively disrupt the biofilm structure and fragment the biofilm on the surface [32]. The liquid jets normally occur when the cavities implore near the boundary of container. Meanwhile, the localized high-shear force and the cavitation clouds along with microjets at a high velocity are exerted onto the surface of inner container to detach biofilm and release inner bacteria [33]. Besides these factors, acoustic streaming can be generated during the momentum transfer from the acoustic wave to the liquid. The thickness of boundary layer can be reduced for mass transfer and therefore, be helpful in removing the biofilm [34]. Furthermore, acoustic streaming can also increase the permeability of cell membrane, which also contributes to the bactericidal effect of ultrasound. As mentioned before, inner bacteria are released from the biofilm and dispersed to the environment when treated by ultrasound. As long as the bacteria released into the environment, they would be much easier to be inactivated by thermal treatment compared with those in the biofilm [35]. Several studies have reported a relatively high inactivation rate of microorganisms using sono- and thermal treatment as hurdle technologies, such as Neosartorya fischeri ascospores in apple juice, Salmonella and Escherichia coli O157:H7 on alfalfa seeds, etc. [36,37]. Results of CLSM analysis in the following section indicate a good bactericidal performance of US-TM treatment on the *P. fluorescens* biofilm [38].

### Table 2

| Temperature (°C) | Ultrasound power (W) | Average thickness (μm) | Roughness coefficient | Biomass (μm²/μm²) |
|------------------|----------------------|------------------------|-----------------------|-------------------|
|                  | 0                    | 23.01 ± 1.20           | 0.13 ± 0.00           | 10.42 ± 0.01     |
|                  | 60                   | 19.45 ± 0.88           | 0.16 ± 0.01           | 10.11 ± 0.06     |
|                  | 80                   | 17.71 ± 0.84           | 0.28 ± 0.02           | 8.49 ± 0.89      |
|                  | 100                  | 10.69 ± 1.01           | 0.37 ± 0.04           | 6.72 ± 0.77      |
|                  | 40                   | 20.99 ± 0.92           | 0.16 ± 0.02           | 9.99 ± 0.80      |
|                  | 60                   | 16.72 ± 0.76           | 0.30 ± 0.02           | 8.39 ± 1.02      |
|                  | 80                   | 12.03 ± 0.59           | 0.43 ± 0.05           | 6.35 ± 0.51      |
|                  | 100                  | 8.18 ± 0.47           | 0.67 ± 0.09           | 4.47 ± 0.43      |
|                  | 50                   | 19.86 ± 0.79           | 0.19 ± 0.02           | 7.02 ± 0.58      |
|                  | 60                   | 15.61 ± 0.92           | 0.45 ± 0.04           | 5.32 ± 0.49      |
|                  | 80                   | 7.50 ± 0.75           | 0.62 ± 0.07           | 3.62 ± 0.25      |
|                  | 100                  | 4.88 ± 0.69           | 0.80 ± 0.10           | 1.87 ± 0.23      |

Significant differences of values within each temperature level are indicated by lowercase letters (p < 0.05). Significant differences of values within each power level of the ultrasound are indicated by capital letters (p < 0.05).

**3.4. Effects of US-TM treatment on ultrastructure of *P. fluorescens* biofilm and cells**

Fig. 5 shows CLSM images of the biofilm labeled with SYTO 9/PI when treated by US-TM at different conditions. The green fluorescent color indicated live bacteria since the SYTO 9 would penetrate through both intact and damaged membranes of bacteria and stain nucleic acids. The red fluorescent color indicated dead bacteria instead since the PI would not permeate intact membrane but damaged one. Meanwhile, the PI has a higher affinity to nucleic acids compared with SYTO 9. When the temperature was maintained at 30 °C for 15 min without the ultrasound treatment, the surface of biofilm remained smooth and almost all the bacteria emitted a green fluorescence (Fig. 5A I). With the increased power of ultrasound at the same temperature level (Fig. 5A II to 5A IV), the surface of biofilm became rough and a higher proportion of red fluorescence was observed. The biofilm removal and antimicrobial effect of US-TM were synergistically improved when the temperature was raised to 40 °C more than half of the area showed a red fluorescence in Fig. 5B III when treated by 80 W ultrasound. With the increased power of ultrasound (100 W), a basic structure of biofilm almost disappeared and a large proportion in Fig. 5B IV showed the red fluorescence. Looking at the highest condition of US-TM treatment (100 W, 50 °C) in Fig. 5C IV, the biofilm was severely damaged and all the bacteria virtually emitted red fluorescence. Results of average thickness, roughness, and biomass of biofilm in Table 2 also supported these findings. The average thickness and biomass were significantly decreased from 8.99 ± 0.01 to 6.48 ± 0.01 μm²/μm² with the increased power of ultrasound at the same temperature level (Fig. 5).
and thermal treatment. These results are in accordance with the biofilm removal as reported in the previous section.

Fig. 6 shows the morphological change of *P. fluorescens* after the US-TM treatment at different power and temperature levels. Without the assistance of ultrasound, *P. fluorescens* cells were gathered together and maintained the biofilm structure. Most bacteria remained intact shapes and only a few leaked inner contents. With the assistance of ultrasound, *P. fluorescens* cells were significantly dispersed as single cells. In addition, combined ultrasound and mild heat treatment led to a significant change of morphological appearance. A high proportion of cells showed a shrink shape, integration of cell membrane, and leakage of cytoplasmic materials.

Previous studies have proved that the cells are not instantaneously inactivated but undergo a sublethal injury process when the bacteria are exposed to mild heat [9,42]. The presence of sublethal injured cells is a big concern during heat treatment. Although the sublethal injured cells cannot be cultured, membrane integrity, metabolic activity, and even production of enterotoxins remained in the cells [43,44]. Considering the thermal resistance of *P. fluorescens* biofilm, it is speculated that the proportion of sublethal injured cells in the biofilm could be even higher than that of planktonic counterparts when treated by mild heat. After a certain time, the sublethal injured cells that remain in the biofilm could be retrieved and continuously cause the spoilage of food products [3].

Different from thermally induced lethal mechanisms, ultrasound treatment can be regarded as “all-or-nothing processing”, which is an advantage over the thermal process. The ultrasound treatment is capable of disrupting bacteria morphology, forming large pores on the cell membrane and cell wall, and lastly defragmenting cellular structure [45]. A similar result was observed by transmission electron microscope (TEM) in which a 20-min sonication resulted in a leakage of cytoplasmic materials from *Staphylococcus aureus* and *E. coli* cells [42]. Visualized results of *P. fluorescens* biofilm and cells in this study also supported these findings. The US-TM treatment not only significantly detached the *P. fluorescens* biofilm from the glass slide, but also led to the bactericidal effect. Compared with their mono-counterparts, both biofilm detachment and bactericidal efficacy were significantly improved when adopted the US-TM treatment.

4. Conclusions

This study indicated combined US-TM treatment as an effective mean of removing *P. fluorescens* biofilm and inactivating viable cells. *In vitro* studies showed that ultrasound played a key role in the biofilm removal. The ultrasound treatment can release the bacteria in the biofilm to the environment and simultaneously expose inner bacteria at the deep layer of biofilm. These actions are mainly attributed to shear force, shock waves, acoustic streaming, etc. generated by ultrasound. As long as the biofilm structure is destroyed, both ultrasound and thermal treatment would synergistically inactivate *P. fluorescens* cells. The US-TM treatment only requires a mild heat (up to 50 °C) along with a moderate ultrasound treatment (>80 W) to achieve the efficient control of biofilm and the reasonably high bactericidal effect of *P. fluorescens*. *In silico* studies provided simulated results of acoustic pressure and temperature distribution in the sample container. It is emphasized that boundary conditions are important to be concerned since the biofilm is grown on the surface of container. The COMSOL simulation may become a powerful tool to consider all factors together to figure out potential “dead ends” due to the reflectivity of the sound waves and the place where ultrasound cannot be effectively transduced. Therefore, it is necessary to design proper ultrasound and thermal conditions for the biofilm control. Furthermore, the US-TM treatment as a hurdle technology would be further adopted to cleaning food raw materials and

![Fig. 6. SEM images of *P. fluorescens* biofilm (4,000 and 8,000 × magnification) after the US-TM treatment for 15 min at the combined (I-IV) four ultrasound power levels (0, 60, 80, and 100 W) and (A-C) three temperature levels (30, 40, and 50 °C).](image-url)
food contact surfaces. It may inspire the food industry to effectively achieve the bactericidal effects and biofilm control.

**CRediT authorship contribution statement**

Ying Su: Investigation, Data curation. Lin Jiang: Data curation. Danying Chen: Visualization. Hang Yu: Conceptualization, Writing – original draft, Writing – review & editing, Supervision. Fangwei Yang: Formal analysis. Yuhui Guo: Resources. Yunfei Xie: Supervision. Weirong Yao: Validation, Project administration.

**Declaration of Competing Interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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