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Inactivation of microorganisms by low-frequency high-power ultrasound: 1. Effect of growth phase and capsule properties of the bacteria

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ABSTRACT

The aim of this study was to determine the effects of high-intensity low-frequency (20 kHz) ultrasound treatment on the viability of bacteria suspension. More specifically, we have investigated the relationship between the deactivation efficiency and the physical (size, hydrophobicity) and biological (gram-status, growth phase) properties of the microbes. *Enterobacter aerogenes*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. epidermidis* SK and *Staphylococcus pseudintermedius* were chosen for this study owing to their varying physical and biological properties. The survival ratio of the bacteria suspension was measured as a function of the ultrasound power (up to 13 W) for a constant sonication time of 20 min. Transmission electron microscopy was used to evaluate the ultrasound-induced damages to the microbes. Ultrasound treatment resulted in lethal damage to *E. aerogenes* and *B. subtilis* (up to 4.5-log reduction), whereas *Staphylococcus* spp. were not affected noticeably. Further, *E. aerogenes* suspensions were more sensitive to ultrasonication in exponential growth phase than when they were in stationary phase. The results of this study demonstrate that the main reason for bacterial resistance to ultrasonic deactivation is due to the properties of the bacterial capsule. Microbes with a thicker and "soft" capsule are highly resistant to ultrasonic deactivation process.

1. Introduction

Thermal processing, such as pasteurization and ultra-high temperature treatment (UHT), have long been involved in bacterial inactivation in the manufacture of food products [1]. However, they are also known to cause loss of vitamins, nutrients and flavors [2]. As a result, non-thermal processing technologies, including ultrasound, ultra high pressure, dense phase CO_2, ozonation, high voltage pulsed electric fields and magnetic fields treatment, have attracted a lot of interest as they offer an alternative to thermal processing methods. Specifically, ultrasound treatment is known to have a reduced impact on nutritional content and on the overall food quality [3], and has been considered as an alternative method for microbial pasteurization and sterilization. Ultrasound, a kind of vibrational energy which normally has a frequency of 20 kHz or more [4]. Low frequency (20–100 kHz) ultrasound is also termed high-power ultrasound, while low-power ultrasound generally corresponds to a frequency range in excess of 100 kHz [1,4–6].

Bacteria inactivation using ultrasound treatment was first reported in 1920s [7] and the investigation on the mechanism of microbial inactivation began in 1960s [8]. There are numerous theories available on the mechanism of biocidal effects of ultrasound. Researchers believe that it is due to acoustic cavitation which causes mechanical effects and sonochemical reactions such as the generation of highly reactive radicals and molecular products such as H_2O_2 [2,4,5,8–15]. Acoustic cavitation refers to the growth and collapse of micro-bubbles in liquid media. In a liquid environment, cavitation bubbles will be produced throughout the liquid due to the pressure fluctuations generated by the ultrasound wave. The collapse of these cavitation bubbles will generate mechanical effects (shock waves, shear forces and micro-jettings) which can damage microorganisms [14,16]. For low-frequency high-power ultrasound, it is generally speculated that the main effect of bacterial inactivation is the result of acoustic cavitation [11,17–20].

Although the inactivation of bacteria by high power ultrasound is well known and extensively studied, the relationship between the effectiveness of ultrasound to inactivate bacteria and their physico-chemical properties is not yet well understood. For instance, some reports showed that gram-negative bacteria were

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more sensitive to ultrasonic inactivation that gram-positive bacteria [10,21–24], while other researchers reported no significant relationship between the gram-status of bacteria and ultrasonic inactivation [25,26]. It was reported that the larger cells are more susceptible to ultrasound than smaller ones [22,27,28] and rod-shaped bacteria were more sensitive than coccus-shaped cells [22], while it was also reported that there was no direct relationship between the size and shape on the effect of ulrasonication [26]. In summary, based on the information available in the literature, it is obvious that there are mixed opinions and different speculations on the mechanism of ultrasonic inactivation of pathogens with regard to their properties.

Hence, the aim of this study was to investigate how ultrasound inactivation of bacteria might be affected by some physical and biological properties of five different microorganisms, namely *Enterobacter aerogenes*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *S. epidermidis* SK and *Staphylococcus pseudintermedius*. These bacteria were chosen owing to their different sizes and gram-status. *E. aerogenes* is a gram-negative rod-shaped bacterium and it belongs to the family of *Enterobacteriaceae*, which have a size range of 0.3–1.0 × 1.0–6.0 μm [29]. *B. subtilis* is a gram-positive rod-shaped bacterium, normal size range is about 0.7–0.8 × 2.0–3.0 μm [30]. *S. epidermidis* is a gram-positive coccus and the size range is 0.8–1.0 μm in diameter [30]. These bacteria were sonicated using high power lower frequency (20 kHz) at different phases of their growth. Note, that ultrasound inactivation of *B. subtilis* and *S. epidermidis* were previously reported in the literature [31,32]. However in this study the ultrasound inactivation at different stages of their growth phase is considred, in addition two other species/strain of *Staphylococcus* have been used. To the best of our knowledge, ultrasound treatment of *E. aerogenes* is for the first time reported in this present study. In addition, a direct comparision of the ultrasonic deactivation of these bacteria in relation to their physico-chemical properties has never been studied.

2. Material and methods

2.1. Preparation of bacterial suspensions and determination of their growth phases

E. aerogenes, *B. subtilis*, *S. epidermidis*, and two more species/strain of *Staphylococcus*, *S. epidermidis* SK (from human skin) and *S. pseudintermedius* were obtained from the Microbiology Lab of the School of Biological Sciences at the University of Auckland, New Zealand. These bacteria were kept as stocks at −80 °C. For each bacterium, 1 ml stock was spread on a Nutrient Agar (Difco™) plate and incubated overnight at 37 °C. Following this process, a loop of the bacterial colony was transferred into 50 ml Nutrient Broth (Difco™) and then incubated at 37 °C overnight in shaking room (200 rev min^{-1}) to produce a working stock bacterial culture. 4 ml working culture was then added into 100 ml fresh Nutrient Broth and incubated with shaking (200 rev min^{-1}) at 37 °C to make a final bacterial suspensions. The incubation time for this last step is fixed in such a way that the bacteria culture is at the chosen growth phase. These bacteria suspensions are the one used for the ultrasonication experiments. The growth curves of the microorganisms were monitored by measuring the optical density change of the microorganism suspensions with time using a Spectrophotometer (HeλIOS β UV Visible, Thermo Electron Corporation, UK) at a wavelength of 600 nm.

When required, the bacteria suspensions were washed prior to ultrasonication. Two washing media were used, fresh nutrient broth or physiological salt solution (0.9% NaCl, PSS). Fresh nutrient broth is used to get rid of the waste produced during the bacteria growth while ensuring enough nutrients for the survival of the bacteria. PSS is used to ensure that there is no excess nutrient and that the bacteria are suspended in a medium similar to water. The washing procedure is carried out as follow: bacterial suspensions at stationary phase were transferred into 50 ml tubes, and were centrifuged using Biofuge Stratos (Heraeus, Thermo Electron Corporation, Germany) at 10,000 g for 10 min at 4 °C to obtain a bacterial cell pellet. The supernatant was discarded and replaced by an exact amount of either fresh nutrient broth or PSS. The centrifuge tube was thoroughly vortexed to resuspend the bacteria. This centrifugation followed by resuspension step was repeated three times.

During this work, to ensure that the bacteria cultures were not contaminated by other microorganisms, their purities were confirmed by using Gram staining [33] and catalase test [34].

2.2. Ultrasound treatment

15 ml bacterial suspensions were placed in 20 ml columniform glass vials and ultrasonicated by ultrasound homogenizer (Sonic Ruptor 250, Omni International, USA) at 20 kHz. The processing tip (diameter 12.7 mm) of the ultrasonic horn was always positioned 1 cm below the surface of the bacterial suspensions. The bacterial suspensions were surrounded by an ice bath to keep the solution temperature below 30 °C. The ultrasound power delivered, P, was determined using the calorimetric method [35]:

$$P = mC_p\left(\frac{\Delta T}{\Delta t}\right) \quad (1)$$

where C_p (=4.18 J/(g K)) is the specific heat capacity of water, m (=15 g) is the mass of sonicated water, ΔT is the increase in the temperature, and Δt is the applied sonication time.

All bacteria suspensions were ultrasonicated at different powers for a constant time of 20 min. All sonication treatments were performed at least in duplicate.

2.3. Bacterial enumeration

A slightly modified Miles–Misra method [36] was used to count the viable bacterial cells. Serial 1:10 dilutions from 10^0 to 10^{-8} times were made in 96 Well Tissue Culture plate (Cellstar, Greiner bio-one, Germany) by mixing sterile Nutrient Broth or PSS with bacterial samples. Dried sterile Nutrient Agar (Difco™) plates were sectioned into 6 parts in advance by a permanent marker. Each diluted sample (50 μl × 3) was dropped onto 3 sectors and then the plates were incubated at 37 °C overnight. The number of colonies for the sectors where the colonies were countable (15–150) and was enumerated under a light microscope, and the CFU per milliliter for the original bacterial suspensions was calculated using:

$$N = \bar{n} \times d_i \times 20 \quad (2)$$

Where N is the number of total colonies (CFU/ml), $\bar{n}$ is the average number of colonies for a dilution and d_i is dilution factor.

2.4. Size measurement

The Malvern Mastersizer 2000 (Malvern Instruments, UK) was used for the particle size measurement of bacterial suspensions. Milli-Q water with a refractive index of 1.33 was used as the dispersant medium, and the value of the refractive index of bacteria was 1.38 [37,38]. Note that the Mastersizer 2000 software used to obtain the particle size values assumes that the bacteria suspension is made of spherical particles.

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