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doi:10.15199/48.2025.02.20

Effect of ultrasound stimulation on the growth of Gram positive and Gram negative bacteria

***Abstract**. In the food industry, ultrasonic technology is widely used for processing, cooling and preserving food products due to its high efficiency. Ultrasound can be used as an alternative method to heat treatment to eliminate microorganisms and enzymes without destroying nutrients in food [1]. The purpose of the present study was to determine the effect of ultrasonic waves of different amplitudes and pulse durations on the cell growth of selected microorganisms. The scope of the study was to subject selected microbial strains to ultrasonic waves with different values of ultrasonic wave amplitude (2 µm and 10 µm) and pulse duration (5 min, 10 min and 15 min). For each of the above-mentioned interaction combinations, the growth rate represented by the optical density was determined. Subjecting microorganisms such as Escherichia coli and Staphylococcus aureus to ultrasound interaction affected the cell growth capacity of these microorganisms. Based on the study, it was observed that subjecting Escherichia coli to ultrasound regardless of the magnitude of the wave amplitude and regardless of the exposure time, increased the value of the optical density of this bacterium. Exposure of Staphylococcus aureus to ultrasound with a wave amplitude size of 2 µm and 10 µm, and regardless of the exposure time, increased the value of the optical density of this bacterium*

***Streszczenie.** W przemyśle spożywczym technologia ultradźwiękowa, ze względu na swoją wysoką wydajność, jest szeroko stosowana do przetwarzania, chłodzenia i konserwowania produktów spożywczych. Ultradźwięki mogą być stosowane jako metoda alternatywna do obróbki cieplnej w celu wyeliminowania mikroorganizmów i enzymów bez niszczenia składników odżywczych w żywności [1]. Celem niniejszego badania było określenie wpływu fal ultradźwiękowych o różnej amplitudzie i czasie trwania impulsu na wzrost komórek wybranych mikroorganizmów. Zakres pracy obejmował poddanie wybranych szczepów drobnoustrojów działaniu fal ultradźwiękowych o różnych wartościach amplitudy fali ultradźwiękowej (2 µm i 10 µm) oraz czasu trwania impulsu (5 min, 10 min i 15 min). Dla każdej z wyżej wymienionych kombinacji interakcji określono szybkość wzrostu reprezentowaną przez gęstość optyczną. Poddanie mikroorganizmów takich jak Escherichia coli i Staphylococcus aureus oddziaływaniu ultradźwięków wpłynęło na zdolność wzrostu komórek tych mikroorganizmów. Na podstawie przeprowadzonych badań zaobserwowano, że poddanie Escherichia coli działaniu ultradźwięków niezależnie od wielkości amplitudy fali i niezależnie od czasu ekspozycji, zwiększyło wartość gęstości optycznej tej bakterii. Poddanie Staphylococcus aureus działaniu ultradźwięków o wielkości amplitudy fali 2 µm i 10 µm oraz niezależnie od czasu ekspozycji, zwiększa wartość gęstości optycznej tej bakterii.(**Wpływ stymulacji ultradźwiękami na wzrost bakterii Gram dodatnich i Gram ujemnych**)*

Keywords: ultrasounds, bacterial growth, gram positive bacteria, gram negative bacteria
Słowa kluczowe: ultradźwięki, wzrost bakterii, bakterie gram dodatnie, bakterie gram ujemne

Introduction

Food processing refers to the intentional transformation of agricultural products, through numerous unit operations, into higher value-added, longer-lasting, portable, useful and safe products for human consumption [1]. The basic principle of most traditional food processing methods is to expend heat to reduce the growth of microorganisms and inhibit food-borne pathogens , making food safe to eat. New methods used in food processing include high-pressure processing, cold plasma, pulsed electric field, supercritical fluid extraction, ultraviolet radiation and ultrasound [2,3,4,5]. Ultrasound is defined as acoustic energy or sound waves with frequencies above 20 kHz. When acoustic waves propagate through a medium, they generate compression and decompression in the particles of the medium. This, in turn, generates a large amount of energy, due to turbulence and increased mass transfer [3,4]. Ultrasound is an emerging sustainable technology that increases the speed of several processes in the food processing industry and their efficiency. Based on intensity and frequency, ultrasonic waves used in the food industry can be divided into two categories: low- and high-intensity ultrasound [5]. Low-intensity or high-frequency ultrasonic waves have a characteristic frequency of more than 100 kHz and intensities of less than 1 W/cm^2. These waves can be used to evaluate the structure of a food product and determine the composition of fresh food products. They are employed as a tool for non-invasive and non-destructive analysis of food products during processing and storage [6]. The principle of low-energy ultrasound is that it effectively uses the interaction between matter and high-frequency sound waves to obtain detailed information in terms of the structure, dimensions and composition of the product through which it propagates [7]. High-intensity, low-frequency ultrasound waves are characterized as disruptive and therefore have significant effects on the physical, biochemical and mechanical properties of food products, unlike low-power ultrasound. Their frequency ranges from 20 to 100 kHz and their intensity ranges from 10 to 1000 W/cm^2 [8]. High-intensity ultrasound is characterized by the induction of acoustic cavitation, which results from the production, subsequent growth and sudden collapse of larger bubbles, releasing a large amount of energy [9].

Conventional thermal sterilization and pasteurization are the most commonly used microbial inactivation techniques in the food industry. However, these thermal treatments can significantly affect the nutritional and sensory properties of food. The first site of action of ultrasound for microbial inactivation is the cell wall of bacteria. Bacterial inactivation involving the cell wall is usually attributed to pore formation or mechanical damage to the cell wall and cell membranes caused by shock waves during cavitation. In addition, pore formation is also a cause of cell death, as the pores formed on the cell wall are small enough to prevent the passage of small molecules across the cell membrane, significantly affecting the osmotic balance of the cell [10]. Ultrasound is widely considered harmful to cell growth. However, cells can grow at low sonication intensities due to the following properties of ultrasound: its ability to increase the transport of small molecules in solution and its inability to completely remove cells (or even non-living particles) from the surface [11]. Ultrasound enhances the transport of small molecules in liquid solution by increasing convection in a relatively slow-moving fluid. The boundary layer of the fluid adjacent

to the solid surface creates resistance to the transport of small molecules to the surface. Increased convection reduces the thickness of this boundary layer while increasing transport to the surface. To increase the rate of cell growth at the surface, it is often desirable to increase the transport of oxygen and nutrients into the cells and to increase the transport of cellular waste products away from the cells [12,13]. In addition to the direct effects of ultrasound on microbial cells, phenomena associated with ultrasonic cavitation can contribute to biofilm destruction. The high-velocity microstreams created during bubble collapse induce localized shear forces on the biofilm, which cause it to detach from the surface of the [14]. Ultrasound can damage not only Gram-positive and Gram-negative bacteria, but also yeast, fungi, algae and even viruses. Comparing the effectiveness of different methods is hampered by significant differences between studies in the frequency, intensity and cycle of sonication pulses [15,16].

The purpose of the study was to determine the effect of ultrasonic waves of varying amplitude and pulse duration on cell growth of selected microorganisms. The experiments carried out made it possible to determine correlative and functional relationships between stimulation with varying ultrasonic wave amplitude and organic matter, and allowed to parameterize the amplitude of the ultrasonic wave and the duration of pulses causing improvement or regression of the growth of the studied bacteria.

Material and methods

The strains used in the study were *Escherichia coli* ATCC 29212 and *Staphylococcus aureus* ATCC 278596 from the strain collection of the Laboratory for Experimental Research Techniques of Raw Materials and Biological Products [17]. In order to restore vital functions, reduction cultures were performed on TSA solid medium in petri dishes. The plates were incubated for 24 hours at 37 °C. Then suspensions of microorganisms with an optical density of 0.5 on the McFarland scale were prepared.

A Hielscher UP200St sonifier was used in the study (figure 1). The complete ultrasonic system - consisting of an ultrasonic transducer and a generator - converts electrical energy into mechanical vibrations and transmits them to the sonotrode. The mechanical amplitude of the processor is adjustable from 20% to 100%. The probotrodes are impedance-matched, so they can be used without amplitude limitation. Temperature-sensitive samples can be processed in high-intensity pulse mode.

The test used an S26d26 probe with a diameter of 26mm and a maximum amplitude of 20µm. The device is equipped with a color touch screen. The amplitude/power setting and pulse mode can be adjusted using the color touchscreen slider.

Four samples each of suspensions of the test microorganisms were prepared: a control sample and three test samples. The samples were then exposed to ultrasonic waves at three amplitudes of 2 µm and 10 µm and power of 25W. Three variants of exposure time of 5, 10 and 15 minutes were used. The samples were then incubated in a hothouse at 35°C for 7 days, measuring the optical density every 24 hours.

Results

Figure 1 shows the growth curve recorded after exposure to ultrasonic waves with an amplitude of 2 µm and different exposure times. Up to 72h of culture, an increase in the optical density value of *Escherichia coli* above 4 McF was recorded regardless of the time of exposure to ultrasonic waves. After 96h of culture, an increase in optical density values up to 5.51 McF was obtained for 5 minutes of exposure of ultrasonic waves to the samples, the optical density values of the remaining samples remained below 5 McF. In the interval of *Escherichia coli* culture time from 120 to 166 hours, there was a decrease in optical density values to 4.62 McF for samples exposed to 5 minutes of ultrasonic waves. For the remaining samples in the time interval from 72 to 144 hours of culture, the optical density values increased. The highest optical density value after 144 hours of culture guidance was obtained for samples subjected to 15 minutes of exposure to ultrasonic waves, which was 5.32 McF.

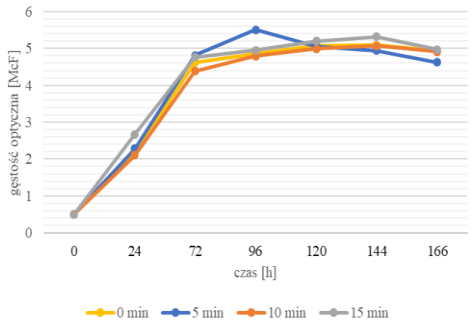

Fig. 1. Growth curve of *Escherichia coli* after exposure to ultrasonic waves with an amplitude of 2 µm and different exposure times

Figure 2 shows the growth curve recorded after exposure to ultrasonic waves with an amplitude of 10 µm and different exposure times. Up to 72h of culture, an increase in the value of optical density of *Escherichia coli* above 5 McF was recorded for samples exposed to ultrasonic waves. In the 72 to 120h interval, a decrease in optical density values to 5.01 McF was recorded for 10 minutes of ultrasonic wave exposure to samples, optical density values of the remaining samples increased and remained below 6 McF. In the interval of *Escherichia coli* culture duration from 120 to 166 hours, a decrease in optical density values was noted for all samples. The highest optical density value after 144 hours of culture was obtained for samples subjected to 15 minutes of exposure to ultrasonic waves, which was 5,22 McF.

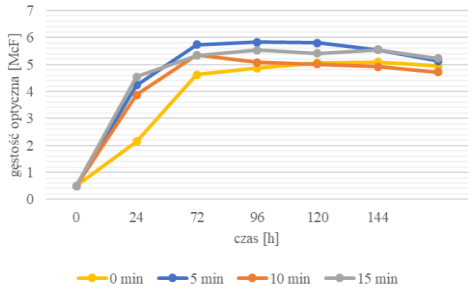

Fig. 2. Growth curve of *Escherichia coli* after exposure to ultrasonic waves with an amplitude of 10 µm and different exposure times

Figure 3 illustrates the relationship between the amplitude and exposure time of the ultrasonic wave and the optical density of *Escherichia coli*. In the case of *Escherichia coli*, in order to obtain the highest optical density, the parameters of ultrasonic wave amplitude and exposure time to bacteria should be selected so that the combination of parameters situates the system in the red area and does not exceed the yellow limit line. Using combinations of parameters of ultrasonic wave power up to an amplitude of 6 µm and exposure time not exceeding 10 minutes, optical density was registered not exceeding 2,6 McF.

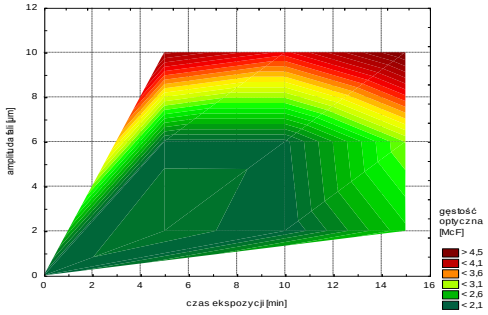

Fig. 3. Relationship between ultrasonic wave power and exposure time and optical density of *Escherichia coli*

Figure 4 shows the growth curve recorded after exposure to ultrasonic waves with an amplitude of 2 µm and different exposure times. Up to 72h of culture, an increase in the optical density value of *Staphylococcus aureus* below 2 McF was recorded for samples not exposed to ultrasonic waves, while in other cases the value was between 4 and 6 McF. The value of the control sample increased and reached its maximum value after 166 hours (2.48 McF). After 96 hours of culture, an increase in the value of optical density to 4.57 McF was obtained in the case of 10-minute exposure of ultrasonic waves to the samples, the optical density values of the other samples remained below 6 McF. In the range of *Staphylococcus aureus* culture duration from 72 to 96 hours, there was a decrease in optical density values for samples subjected to 5, 10 and 15 minutes of ultrasonic wave exposure. In the interval from 96 to 120 hours, a decrease was observed only for 15-minute exposure to ultrasonic waves. In the interval from 120 to 166 hours, a decrease in optical density values was observed for all samples exposed to ultrasonic waves. The highest value of optical density after 144 hours of culture was obtained for samples subjected to 5 minutes of exposure to ultrasonic waves, which was 6,12 McF.

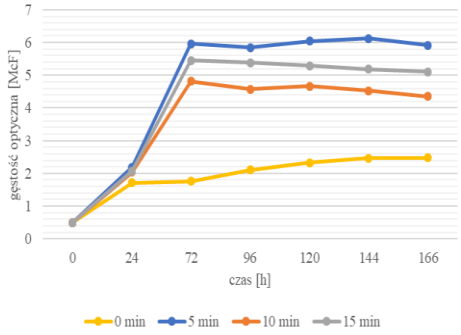

Fig. 4. Growth curve of *Staphylococcus aureus* after exposure to ultrasonic waves with an amplitude of 2 µm and different exposure times

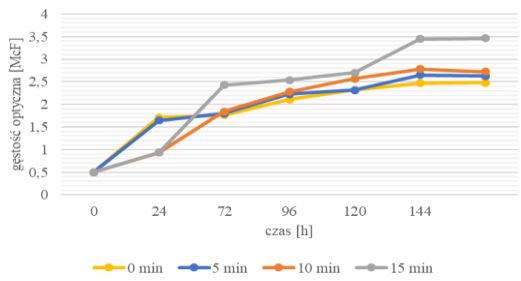

Fig. 5. Growth curve of *Staphylococcus aureus* after exposure to ultrasonic waves of 10 µm amplitude and different exposure times

Figure 5 shows the growth curve recorded after exposure to ultrasonic waves with an amplitude of 10 µm and different exposure times. A steady increase in the optical density of all samples was recorded throughout the course of the culture run. Up to 72 hours, the greatest increase in optical density values of *Staphylococcus aureus* was recorded for samples exposed to 15 minutes of ultrasonic waves (2.43 McF). Between 72 and 120 hours, the optical density values were between 1.5 and 3 McF. After 120 hours, there was a sharp increase in optical density for the 15-minute samples to 3.45 McF and remained constant, while the remaining samples after 144 hours showed a decrease in optical density not exceeding 2,5%.

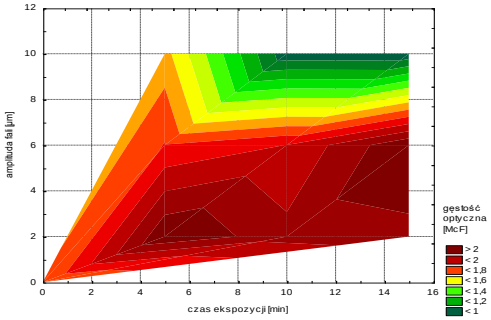

Fig. 6. Relationship between amplitude and exposure time of ultrasound wave and optical density of *Staphylococcus aureus*

Figure 6 shows the relationship between the amplitude and exposure time of the ultrasonic wave and the optical density of *Staphylococcus aureus*. In the case of *Staphylococcus aureus*, in order to obtain the highest optical density, the parameters of the ultrasonic wave amplitude and exposure time to the bacteria should be selected so that the combination of parameters situates the system in the red color region and does not exceed the yellow color limit line. Using combinations of the parameters of ultrasonic wave amplitude of 10 µm and exposure time exceeding 10 minutes, the optical density was registered not exceeding 1 McF.

An r-Pearson correlation analysis was performed to determine the interrelationships between the various parameters studied. The obtained results of the analysis were statistically significant. The correlation matrix of the individual factors is shown in Table 1.There was a significant effect of both the amplitude of the ultrasound wave and the and the time of its exposure on the optical density of *Escherichia coli*. The value of the correlation coefficient between the amplitude of the ultrasonic wave and the optical density of *Escherichia coli* was 0.76, while the value of the correlation coefficient between the exposure time of the ultrasonic wave and the optical density of *Escherichia coli* was 0.32. The values of the correlation coefficients between these parameters were positive, indicating that the optical density of *Escherichia coli* increased with the increase of the amplitude and exposure time of the ultrasonic waves.

Table 1 Correlation coefficients between amplitude and exposure time of the ultrasound wave and optical density *Escherichii coli*

	Amplitude [µm]	Exposure time [min]	Optical density [McF]
Amplitude [µm]	1		
Exposure time [min]	0,31*	1	
Optical density [McF]	0,76*	0,32*	1

- calculations performed at α=0.05 level of significance
** correlation coefficients statistically significant*

The correlation matrix of the various factors is shown in Table 2. There was a significant effect of both the amplitude of the ultrasound wave and its exposure time on the optical density of *Staphylococcus aure*us. The value of the correlation coefficient between the amplitude of the ultrasonic wave and the optical density of *Staphylococcus aureus* was -0.68. The negative value indicates that the optical density increased with a decrease in the amplitude of the ultrasonic waves. On the other hand, the value of the correlation coefficient between the ultrasonic wave exposure time and the optical density of *Staphylococcus aureus* was -0,09.

Table 2. Correlation coefficients between ultrasonic wave power and exposure time and optical density *Staphylococcus aureus*

	Amplitude [µm]	Exposure time [min]	Optical density [McF]
Amplitude [µm]	1		
Exposure time [min]	0,31*	1	
Optical density [McF]	0,68*	-0,09	1

- calculations performed at α=0.05 level of significance
** correlation coefficients statistically significant*

Conculusion

Subjecting *Escherichia coli* and *Staphylococcus aureus* to ultrasonic exposure affected their quantitative structure by increasing or decreasing the growth capacity of these microorganisms. The increase or decrease in growth capacity was dependent on the ultrasonic wave parameters used. It was observed that subjecting *Escherichia coli* to ultrasound irrespective of the magnitude of the wave amplitude and irrespective of the exposure time, increased the optical density value of this bacterium. On the other hand, subjecting *Staphylococcus aureus* to ultrasonic interaction with a wave amplitude size of 2 µm and 10 µm, and regardless of the exposure time, increases the optical density value of this bacterium. On the other hand, the application of ultrasonic interaction with a wave amplitude size of 6 µm, regardless of the exposure time, has the effect of reducing the value of optical density.

Funded by a subsidy of the Ministry of Education and Science for the Agricultural University of Hugo Kołłątaj in Krakow for 2025r.

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