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Research Report

OPTIMATION OF 48 kHz ULTRASONIC WAVE DOSE FOR THE INACTIVATION OF *Salmonella typhi*

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ABSTRACT

This study was aimed to determine the effect of ultrasonic dose exposure which could decrease the viability of Salmonella typhi by using the variation of exposure time (15, 20, 25, and 30 minutes) and volume of bacterial suspension (2, 4, 6, and 8 ml) at constant power. The sample used was Salmonella typhi. Ultrasonic wave transmitter was a piezoelectric tweeter with 0,191 watts of power and 48 kHz frequency generated by the signal generator. Piezoelectric tweeter was a kind of transducer which converted electrical energy into ultrasonic energy. This research was an experimental laboratory with a completely randomized design. The decrease of bacterial percentage was calculated by using TPC (Total Plate Count). Data were analyzed by using One Way Anova. The results showed that the variation of exposure time and volume of bacterial suspension gave significant effect on the percentage of Salmonella typhi kill. The most optimal of ultrasonic dose exposure to kill Salmonella typhi was 281.87 J/ml with 100% bacterial kill.

Key words: Ultrasonic dose exposure, ultrasonic wave, piezoelectric tweeter, Salmonella typhi, total plate count

ABSTRAK

Penelitian ini bertujuan untuk menentukan efek dosis paparan ultrasonik yang dapat mengurangi viabilitas Salmonella typhi dengan menggunakan variasi paparan waktu (15, 20, 25, and 30 menit) dan volume suspense bakteri (2, 4, 6, and 8 ml) pada kekuatan konstan. Sampel yang digunakan ialah Salmonella typhi. Transmiter gelombang ultrasonik ialah tweeter piezoelectric dengan daya 0,191 watt dan frekuensi 48 kHz yang dihasilkan oleh signal generator. Tweeter piezoelectric ialah sejenis tranduser yang mengubah energi listrik menjadi energi ultrasonik. Penelitian ini ialah percobaan laboratorium dengan desain random lengkap. Pengurangan persentase bakteri dihitung dengan menggunakan teknik pengujian total bakteri. Data dianalisis menggunakan Anova satu arah. Hasil menunjukkan bahwa variasi paparan waktu dan volume suspensi bakteri memberikan efek yang signifikan pada persentase Salmonella typhi yang mati. Dosis paparan ultrasonik untuk membunuh Salmonella typhi yang optimal ialah 281.87 J/ml dengan 100% bakteri yang mati.

Kata kunci: *Dosis paparan ultrasonik, gelombang ultrasonik, tweeter piezoelectrik, Salmonella typhi, pengujian total bakteri*

INTRODUCTION

Food is an important requirement for organisms because food serves as a source of carbohydrates, proteins, fats, vitamins, minerals, and other essential substances needed by organisms for growing process, developing process, and repairing damaged cells. Food and beverages consumed by humans must have good quality and free from pathogenic bacterial.

Pathogenic bacterial which often contaminate water, food, eggs and meat, fish and meat, milk and its processed products is Salmonella typhi.[1] Salmonella typhi is very dangerous because it is pathogenic to humans and causes fever.[2]

Most effort to obtain sterile food and beverage is using sterilization process. The method is used on sterilization process is heating. However, this method has the disadvantage because it reduces some nutrients contained

in the food during the sterilization process. Besides heating, another sterilization method which is often used is ultraviolet radiation that can cause mutagenic damage to DNA. Ultraviolet radiation is very harmful for humans when exposed directly.[3] Therefore, other alternatives are needed in the sterilization process that is ultrasonic wave's exposure.

Ultrasonic waves are very effective on materials sterilization process from bacterial,[4,5,6,7,8,9] This method is very safe because it is free from chemical substances and selectively to reduce bacterial viability without giving bad effects to humans and environment.

Ultrasonic wave exposure on bacterial suspensions showed that exposure time is proportional to the decrease of the number of Salmonella typhi colonies. The bacterial kill after ultrasonic wave's exposure occurs due to cavitation effects. Cavitation is the formation of bubble collapse which is a continuous stretch and eventually will be destroyed when it reaches the limit of its elasticity.[9] Ultrasonic wave's exposure on bacterial with causes mechanical stress on the bacterial cell wall so the cell wall stretches beyond the limits of its elasticity. Stretching of cell wall can lead to rupture of the cell wall, lyse, and ended in the death of the bacterial.[7] Cavitation occurs due to local pressure in the sound wave drops to a low enough pressure. It causes rupture of the cell as indicated by the following relationship:

$$\rho = P - P_0 \quad (1)$$

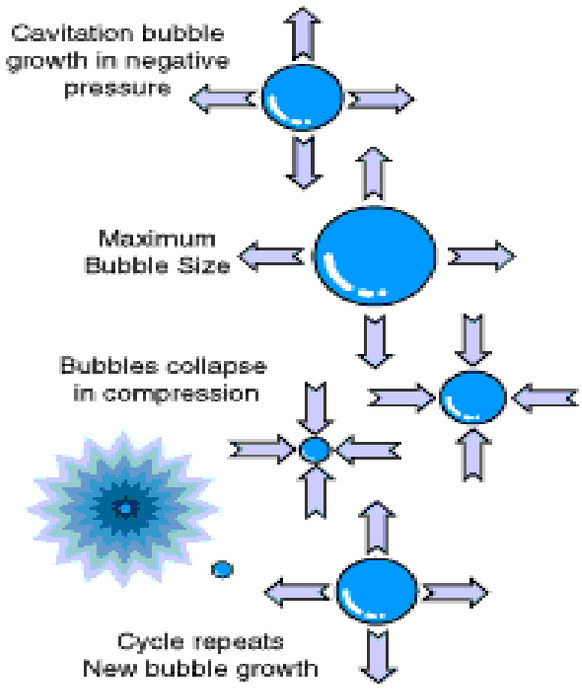

Figure 1. The mechanism of cavitation.[3]

ρ is the acoustic pressure, P is the total pressure, and P0 is the average local pressure. P value is always positive in the gas medium so that the amplitude of the acoustic pressure must be less than atmospheric pressure. While the liquid has a specific volume and can withstand the negative pressure. When the pressure is very low, the liquid will break up and form small cavities such as the ball is called cavity.[11]

Based on the characteristic of its formation, cavitation is distinguished on acoustic cavitation caused by ultrasonic wave and hydrodynamic cavitation caused by variations in fluid pressure.[10] The mechanisms of cavitation started by the formation of bubbles which get pressure from the outside so that the bubbles are unstable and eventually rupture.

Cavitation causes free radicals because of molecular bonds damage. For example H_2O molecule breaks into H^-, OH^-, and HO_2^- and eventually form H_2O_2 which can damage the chemical structure of the bacterial cell wall so the cell wall is weak and broken and the liquid from the outside enter the cell and lyse is occurred resulting in death of the bacterial.[8,11]

Besides cavitation, ultrasonic wave exposure can also increase temperature of the fluid due to acoustic energy imposed on a medium will be released back into heat. It causes temperature rising.

EXPERIMENTAL

Research Sample

The sample of *Salmonella typhi* was obtained from Institute of Tropical Desease, Airlangga University, Surabaya. The sample was grown in Nutrient Broth sterile medium for treatment and Salmonella Shigella Agar for TPC.

Expossure Equipment

Ultrasonic wave generator was a piezoelectric tweeter with 2 cm of diameter were fitted with 10 ohm resistor and generated by function generator (FG-350 IWATSU). Voltage and frequency issued by function generator were detected by using an oscilloscope type Protex 20 MHz (Oscilloscope 6502A). Scheme of the ultrasonic wave instrument is shown in Figure 2.

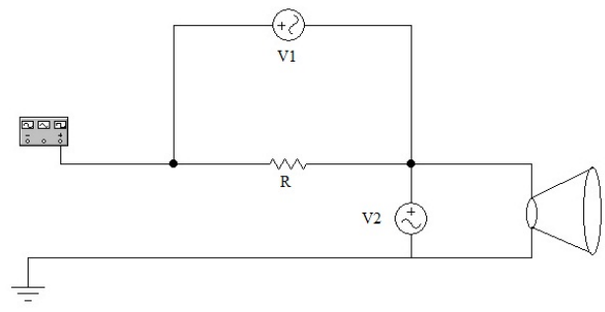

Figure 2. Scheme of ultrasonic wave instrument.

This research used square wave with 48 kHz of frequency and AC current.[4] The circuit of V_2 in Figure 1 is RC integrator circuit. RC integrator circuit is shown in Figure 3 below:

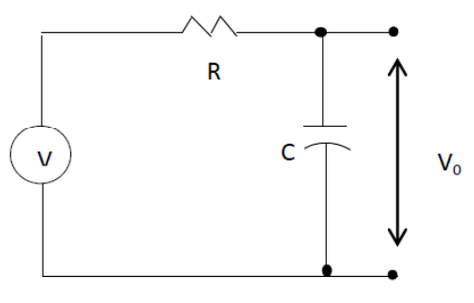

Figure 3. RC integrator circuit.[12]

Performance test which consist of calibration and measurement of fluid temperature rising were done before giving treatment on bacterial suspension. Calibration was done to calculate the voltage. That voltage was used to determine the electrical power which was converted into ultrasonic power by the transducer. The power output was calculated by Equation 2.

$$P = \frac{V_{rms_1} \times V_{rms_2}}{R} \quad (2)$$

Measurement of liquid temperature rising was performed to determine the effect of rising temperatures on the viability of Salmonella typhi because the bacterial could be killed at certain temperature. This method was used to determine whether the bacterial actually killed caused by the mechanical vibrations of ultrasonic waves or due to the heating effect. If the number of live bacterial colonies derived from ultrasonic wave exposure is fewer than heating, it means that bacterial kill caused by mechanical vibrations of ultrasonic waves.

The exposure of ultrasonic waves in liquids can increase the liquid temperature because acoustic energy imposed on a medium will be released back into heat, causing an increase in temperature of the fluid.

Besides heating, the exposure of ultrasonic waves in the sample can also cause mechanical vibration as the effects of cavitation. Ultrasonic wave's exposure on bacterial with causes mechanical stress on the bacterial cell wall so the cell wall stretches beyond the limits of its elasticity. Stretching of cell wall can lead to rupture of the cell wall, lyse, and ended in the death of the bacterial.[7]

Research Methods

This research conducted using completely randomized design. The first experiment was conducted using exposure time variation, these were 15 minutes, 20 minutes, 25 minutes, and 30 minutes[12] with a fixed volume was 2 ml to determine the optimum time with 100% of bacterial kill[7] at a fixed frequency that was 48 kHz.[4,7,13] Each treatment was accompanied by the control group using 5 times replication.

The percentage of bacterial kill was plotted in a graph to obtain the optimum time with 100% of bacterial kill by using linear regression equation (Equation 3).

$$y = m\,x + c \quad (3)$$

Where y was the percentage of bacterial kill up to 100%, x was the optimum time (minutes) required to kill the bacterial up to 100%.

This optimum time used to do the second experiment to determine the optimum dose for the 100% of bacterial kill. The second experiments were performed using a variation of the volume; these were 2 ml, 4 ml, 6 ml, and 8 ml with a fixed exposure time.

The Bacterial Growth

Salmonella typhi were cultured at Luria Bertani Broth sterile (Miller M1245-500 G). The bacterial cultures were incubated at 37°C of temperature for 18 hours. The dilution factor was qualify if the number of bacterial colonies that grew as much as 30–300 colonies, so this culture was incubated until OD600nm = 0.7 and the value of dilution up to 10^{-6} dilution (30–300 colonies). Bacterial dilution which was exposed by ultrasonic waves was cultured on sterile agar medium called Salmonella Shigella Agar (OXOID CM 0099) and incubated at 37°C of temperature for 18–24 hours.

Ultrasonic Wave Exposure on Bacterial

2 ml of bacterial suspension with 10^{-6} bacterial concentration was poured into a glass with 3 cm diameter and 4 cm of height and exposed by ultrasonic waves with a variation of exposure time 15, 20, 25, 30 minutes. The height of that suspension was approximately 3 mm. Exposure was done by dipping the piezoelectric tweeter into the bacterial suspension. The second experiments were performed using a variation of the volume, these were 2 ml, 4 ml, 6 ml, and 8 ml with a fixed exposure time which giving lethal dose 100% on bacterial. The bacterial in the treatment group and the control were grown on Salmonella Shigella Agar medium.

Counting the number of bacterial colonies

The bacterial colonies were counted by Total Plate Count Method using a Quebec Colony Counter. The next was calculating the percentage of bacterial kill by using Equation 4.

$$\%\ of\ bacterial\ kill = \left| \frac{\overline{control\ colony} - \overline{treatment\ colony}}{\overline{control\ colony}} \right| \times 100 \quad (4)$$

Statistical Analysis

This research data were analyzed by using SPSS (Statistical Package For Social Science) 20 that was one

way ANOVA for determining the effect of each factor. Multiple Comparison Post Hoc was used to determine the factors that most influence the percentage of bacterial kill.

RESULTS AND DISCUSSION

Design and Assembly Tool

The set up of experiment tool in this research is an integrator circuit which processes of charging and discharging capacitor were happened. The circuit has a time constant $\tau = RC \gg T$ so that when the capacitor was not fully charged, the voltage Vs has changed the sign to be negative. That leads to discharge the capacitor. The capacitor was charged by using negative charge up to -Vp. Before it was fully charged, the voltage Vs changed the sign. The process occurred repeatedly and forms a triangular output signal.[12] The results of electrical voltage measurements which were converted into an ultrasonic voltage by each of Piezoelectric Tweeter tabulated in a table (Appendix 1). The next step was combining the entire of Piezoelectric Tweeters into one so that the distribution of the power supplied by each transducer was the same. The results tabulated in a table (Appendix 2).

Based on the results of electrical voltage measurements which were converted into ultrasonic voltage by the entire of Piezoelectric Tweeters obtained an average power value in Appendix 2 was 0.191 Watt.

Measurement of liquid temperature rising

These measurements were performed by means of ultrasonic wave's exposure to the bacterial suspension and the rising of temperature occurred were measured. The ultrasonic exposure in 8 ml of bacterial suspension for 30 minutes increased temperature from 25°C to 27°C. Results of liquid temperature rising was plotted in a graph (Figure 4).

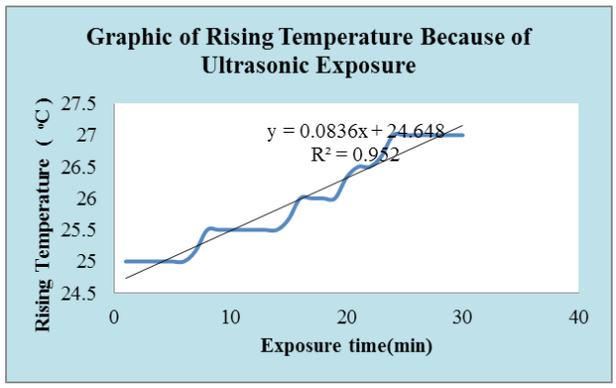

Figure 4. Graphic of Rising Temperature Because of Ultrasonic Exposure.

Bacterial exposure process using ultrasonic waves in that process has the potential to kill the bacterial *Salmonella typhi* up to 55.9%. The next step was to warm the bacterial suspension up to 27°C and obtained 0% of percentage killing of bacterial (didn't cause killing effect on bacterial). Based on these results, it was certain that the death of the bacterial was not due to the effect of rising temperature as a result of ultrasonic wave's exposure but due to the cavitation effect caused by the ultrasonic waves.

Ultrasonic Wave Exposure on *Salmonella typhi*

The results of this study is the decrease of *Salmonella typhi* colony due to exposure time variations of ultrasonic waves (15, 20, 25, and 30) min, volume variations (2, 4, 6, 8) ml, fixed frequency (48 kHz), and fixed power (0.191 W). The results of the study are shown in Figure 5 below.

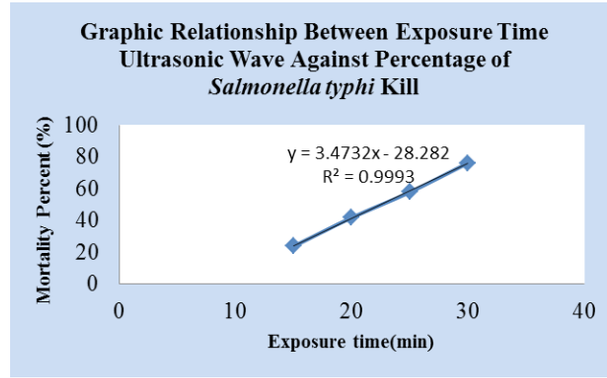

Figure 5. Graphic Relationship of Exposure Time Ultrasonic Wave against Percentage of *Salmonella typhi* Kill.

Equation 5 below is the linear regression equation obtained from Figure 5:

$$y = 3{,}4732\,x - 28{,}282 \qquad (5)$$

Percentage of bacterial kill (y%) obtained for exposure time x minutes with a gradient of 3.4732 and a constant of 28.282. Based on the linear regression equation, Lethal Dose 100% could be obtained by exposing for 36.94 minutes or 36 minutes 56 seconds. This time variation was the time that would be used in all subsequent experiments. The next experiment used variations of volume (2 ml, 4 ml, 6 ml, and 8 ml) with a fixed time (36 minutes 56 seconds). The results of the study are shown in Figure 6.

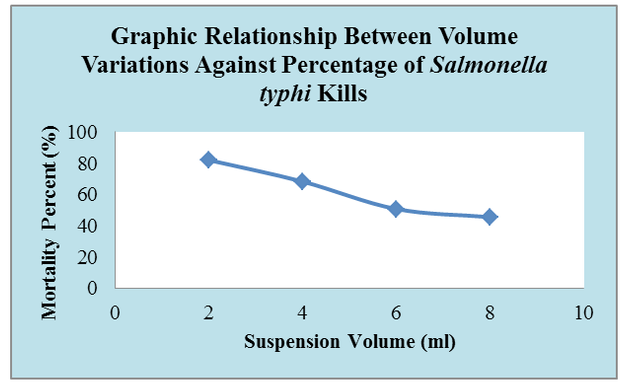

Figure 6. Graphic Relationship between Volume Variations against Percentage of *Salmonella typhi* kills.

The results were analyzed by using One Way ANOVA test to determine the effect of each factor. Terms of ANOVA test is interval and ratio scale data and normally distributed. Normality test performed using the Kolmogorov-Smirnov 1 sample. The test is used to compare the distribution of the data of the study sample with a theoretical distribution.

Results of Kolmogorov-Smirnov test showed a significance value P = 0.809 for time variation and P = 1.14 for volume variations. The test results showed that the data were normally distributed as P > α (0.05). Levene test results generated significant value for P = 0.101 and P = the time variation of 0.309 for variations in the volume so that it could be concluded that the variance of the data was homogeny, which means that the population had the same variance (uniform).

Summary of ANOVA test in Table 1 indicate that the time factor and the volume has a significance level of P = 0.000 is < 0.05, which means that the time factor and the volume effect on the decrease in the number of bacterial colonies.

$$D = \frac{E}{v} = \frac{P\ x\ t}{v} \quad (6)$$

Explanations:

D = dose (J/ml)
E = energy (J)
v = volume (ml)
p = power (W)
t = exposure time (s)

Based on *Salmonella typhi* research data obtained the percentage of bacterial kill at various energy doses which are tabulated in Table 2.

Table 1. Summary of One Way Anova test of ultrasonic exposure to percentage of *Salmonella typhi* kill

Factor	Group	N	Mortality Percent (%)		Anova	
			Average	SD	Significance	Summary
Time	15 min[a]	5	24	2.58135	0.000	There is a significant difference
	20 min[b]	5	42	6.47927		
	25 min[c]	5	59	7.46598		
	30 min[d]	5	76	1.11437		
Volume	8 ml[a]	5	46	0.61409	0.000	There is a significant difference
	6 ml[b]	5	51	0.24234		
	4 ml[c]	5	70	0.83264		
	2 ml[d]	5	82	0.76731		

(6)

Dose of Energy

Dose of energy is the energy of ultrasonic waves exposure which absorbed by the bacterial suspension. Basically, the emitted energy is electrical energy which is converted into mechanical vibration by the transducer. But there is proportionality between the electrical energy emitted by the ultrasonic energy received by a medium with a constant of proportionality *k*. Thus, ultrasonic energy received by the medium approaches the electrical energy emitted.

Mathematically, dose of energy is the result of power (P) times exposure time (t) divided by the volume of the bacterial suspension (v). The optimum dose exposure of ultrasonic wave on *Salmonella typhi* inactivation obtained from Equation 6 below.

Table 2. Results of the percentage of *Salmonella typhi* kills on the variation of ultrasonic wave exposure time and volume, frequency of 48 kHz, and a power of 0.191 W

Time (min)	Volume (ml)	Energy (J)	Dose (J/ml)	Percentage of bacterial kill (%)
15,00	2	171,90	85,95	23,68
20,00	2	229,20	114,60	41,76
25,00	2	286,50	143,25	57,80
30,00	2	343,80	171,90	76,22
36,94	2	423,33	211,67	81,98
36,94	4	423,33	105,83	68,27
36,94	6	423,33	70,56	50,72
36,94	8	423,33	52,92	45,52

The relationship between ultrasonic wave's doses exposures with the percentage of *Salmonella typhi* kill clarified by Figures 7 and 8 below.

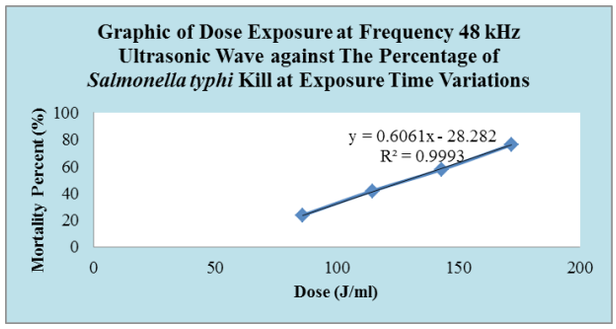

Figure 7. Graphic of Dose Exposure at Frequency 48 kHz Ultrasonic Wave against The Percentage of *Salmonella typhi* kill at Exposure Time Variations.

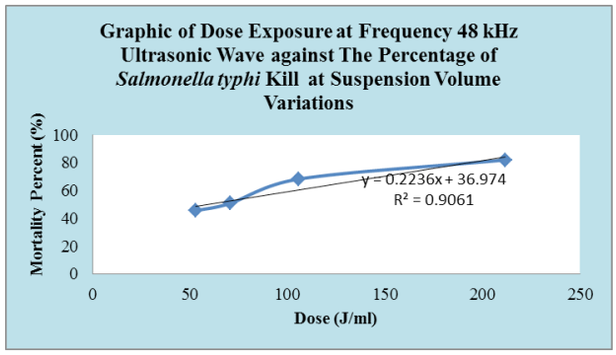

Figure 8. Graphic of Dose Exposure at Frequency 48 kHz Ultrasonic Wave against The Percentage of *Salmonella typhi* Kill at Suspension Volume Variations.

The percentage of *Salmonella typhi* kill at different doses ultrasonic waves with the volume variation plotted in linear regression to determine the dose with 100% bacterial kill. Figure 8 gives the linear regression equation on Equation 7 below.

$$y = 0{,}2236\,x + 36{,}974 \qquad (7)$$

If *y* is the percentage of bacterial kill up to 100% and x is the desired dose, the dose can be used to kill bacterial up to 100% is 281.87 J/ml.

CONCLUSION

The research results show that piezoelectric tweeter produces ultrasonic waves and the voltage generated by the signal generator. The optimum time exposure of ultrasonic waves which effectively decrease the viability of *Salmonella typhi* up to 100% is 36.94 minutes. The optimum volume of bacterial suspension is 2 ml with 81.78% of bacterial kill. The optimum dose exposure of ultrasonic waves which effectively decrease the viability of the bacterial *Salmonella typhi* is 281.87 J/ml with 100% of bacterial kill. The relationship between the dose of ultrasonic energy to the ultrasonic energy which produces mechanical vibration; ultrasonic power; and ultrasonic voltage are proportional. Dose of ultrasonic energy is proportional to ultrasonic energy where ultrasonic energy is a product of ultrasonic power with long time exposure. Ultrasonic power itself is the product of voltages generated by the transducer with long time exposure.

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