Investigation of Ultrasonic Parameters for Ultrasound Exposure of 36 kHz on E.coli Culture

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***Abstract*: The extensive research work has been carried out on kill rate of E.coli with increased time of ultrasound exposure that depending upon the power, frequency and intensity. In contrast, the knowledge of ultrasonic parameters induced by ultrasound is rather limited, especially when it comes to the measurement of ultrasonic parameters with kill rate of E.coli. The present paper therefore, aimed to measure the ultrasonic velocity, attenuation coefficient and acoustical impedance with disruption of E.coli as a function of ultrasound exposure time at 36 kHz frequency. The ultrasonic velocity and attenuation coefficient were carried out by using novel Pulse Echo Overlap (PEO) technique. Spread Plate Techniques were used as a measure of microbial activity. It was observed that ultrasonic velocity and attenuation coefficient of E.coli were increased with initial exposure of ultrasound. This may be due to result of increased convection in E.coli culture by onset of stable cavitation. The onset of transient cavitations disrupts cells in suspensions with increased ultrasound exposure. Therefore, it was reported that ultrasonic velocity and attenuation coefficient decreases almost linearly with disruption of E.coli. The results of ultrasonic velocity and attenuation coefficient of E.coi were found in the right order of magnitude and confirmed with A Zips and U Fast.**

***Key Words*: E.coli, Ultrasound, Pulse Echo Overlap (PEO) technique, Ultrasonic velocity.**

I. INTRODUCTION

Ultrasound is defined as acoustic energy or sound waves with frequencies above 20 kHz. Ultrasound is able to defuse bacteria, make them more vulnerable to biocides and disintegrate bacterial clusters or flocks. It is depending upon the power and frequency applied through a number of physical, mechanical and chemical effects that are arising from acoustic cavitations. The effects of a range of ultrasonic frequencies (20 kHz – 10 MHz), acoustic power and exposure time on bacterial kill have been reported [1-3]. The results showed a significant increase in kill rate for E.coli species with increasing duration of exposure and intensity of ultrasound in the low-kilohertz range (20 – 38 kHz). The results obtained at higher frequencies (above 850 kHz) indicated significant bacterial declumping. In assessing the bacterial kill with time under different sonication regimes three different types of behavior were characterized.

The high power ultrasound (lower frequencies) in low volumes of bacterial suspension results in a continuous reduction in bacterial cell numbers i.e. the kill rate predominates. The high power ultrasound (lower frequencies) in larger volumes results in effective declumping of the bacteria giving an initial rise in cell numbers, but this initial rise then falls as the declumping finishes and the kill rate becomes more important. Low intensity ultrasound (higher frequencies) gives an initial rise in cell numbers as a result of declumping. The kill rate is low, and so there is no significant decrease in bacterial cell numbers.

Ultrasound increases convection in liquid by at least two mechanisms. The first is acoustic streaming flow in which momentum from directed propagating sound waves is transferred to the liquid, causing the liquid to flow in the direction of the sound propagation. Acoustic streaming increases with insonation intensity, and there are reports of acoustic streaming flow at velocities as high as 14 cm/sec [4]. Thus any amount of ultrasound in a liquid produces additional convective transport from acoustic streaming.

The second and more notable mechanism of enhancing convection is known as microstreaming, and is produced by cavitating gas bubbles in the liquid [5-7]. The cycles of low and high acoustic pressure cause the gas bubbles to expand and shrink, which in turn creates shear flow around the oscillating bubbles causes rise in cell numbers as a result of declumping. Stable cavitation results when the acoustic intensity is sufficiently low that the bubbles do not collapse completely during their contraction cycle. The onset of stable cavitation greatly increases convective transport.

The transient cavitations occur if the acoustic pressure amplitude is sufficiently high and above a threshold level. Under this condition the encapsulated microbubbles (EMB's) will first grow in volume and then implode violently. This cavitation can affect a biological system by virtue of the localized temperature rise and mechanical stress [8-9]. Moreover, the dissociation of water molecules into H and OH free radicals, as a consequence of the very high temperature and pressures produced by cavitation, may induce adverse chemical changes such as DNA or protein denaturation [10]. However, the ultimate reason for the lethality of ultrasound on micro-organisms is still unknown.

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In the present paper, the ultrasonic parameters like ultrasonic velocity, attenuation coefficient and acoustical impedance were measured with increased ultrasound exposure time at 36 kHz. The colony forming units (cfu) per ml and % of survivals were measured and used to correlate acoustical parameters.

II. MATERIALS AND METHODS

A. Bacteria and Growth Conditions

E.coli strain HB 101 (kindly supplied by Hi-media, Hyd.) was used throughout the study. Stock cultures were stored on nutrient agar slants in the dark at 0-4 ^{0}C. The cultures were either grown in nutrient Broth as 100 ml volumes in 250 ml Erlenmeyer flasks at 37^{0}C, 200 rpm or as solid cultures on Nutrient Agar plates (37 ^{0}C)

B. Ultrasonic Studies

Bacterial cultures were harvested by centrifugation (13000g, 20 min) and the resulting pellet re-suspended in sterile to a concentration of '10^8' cfu/ml. This corresponded to an optical density absorbance value of 0.72 at a wavelength of E_{600}. The re-suspended cells were placed in the 11 number of sonication vessels of 10 ml each (15 ml glass bottles, internal diameter 21mm, flat base, 2.5 mm wall thickness) to a depth of 25 mm and exposed to ultrasound for time period of 0 min (control), 1 min, 2 min, ….10 min respectively using ultrasonic-generator SG-25-500 Series with an operating frequency of 36 kHz and an output power of 500 W (kindly supplied by Roop Telsonic Ultrasonix Ltd., Bombay). A 12 mm diameter (Sonotrode) titanium probe set at 5 mm below the surface of the culture was used throughout. After sonication, samples (0.1 ml) were removed to solutions of sterile saline (9.9 ml) and serially diluted. The viable counts were made in triplicate on the surfaces of pre-dried Nutrient Agar plates. All plates were subsequently incubated at 37 ^{0}C for 24 h. The viable cells were counted by using spread plate technique. The results were expressed as percentage reductions in viability relative to appropriate unexposed controls.

C. Measuring the Ultrasonic Velocity

The ultrasonic velocities were measured with the help of microprocessor based Pulse Echo Overlap (PEO) system at same frequency (kindly supplied by Roop Telsonic Ultrasonix Ltd., Bombay). The internal circuit of pulse echo overlap system is designed with fully solid state version, which allows immediate measurement of the ultrasonic velocity as given in the following equation [11]

$$v = \frac{2l}{t}$$

Where '*l*' is the column length of the culture and 't' is the time interval to travel column length.

The error in measurement of ultrasonic velocity is ± 1 m/sec.

D. Attenuation Coefficient

The transducer is immersed into the sonication vessel then ultrasonic waves are allowed to pass through the bacteria culture under study. They are reflected from the opposite face and received by the same transducer, which now acts as a receiver. The Pulse Echo Wave train pattern is observed on the screen. Gate pulse was made to coincide with the bottom of the secondary echo pulse and amplitude is adjusted to 80% of its maximum value. The attenuation coefficients of the bacteria cultures are carried out by measuring the amplitudes of transmitted pulses of selected two successive echoes on CRO screen. The ultrasonic attenuation coefficient is calculated by using the following formula.

$$\alpha = \frac{1}{2l}\ln\left(\frac{A_n}{A_{n+1}}\right) \quad \text{nep/cm}$$

Where '2*l*' is distance travelled and 'A_n / A_{n+1}' is the ratio between two successive echoes of 'A_n' and 'A_{n+1}'. The uncertainty in the calculation of ultrasonic attenuation coefficient is ± 0.001 nep /cm.

E. Calculation of Density

The density of bacteria cultures are carried out for 100 samples under study by using bicapillary pyknometer of 10 ml volume. The transfer of bacteria cultures is made by using micro pipette in the laminar flow chamber to avoid contamination with air and body.

The density of bacteria cultures is measured by using the following procedure

Mass of the empty bicapillary pyknometer = w_1 gm
Mass of the bacteria culture + pyknometer = w_2 gm
Mass of the culture (m) = $w_2 - w_1$ gm
Volume of the culture = V cm^3
Density of the bacteria culture (ρ) =

$$\frac{Mass\ \ of\ \ the\ culture\ (m)}{Volume\ \ of\ the\ \ culture\ (V)} \quad gm/cm^3$$

F. Calculation of Adiabatic Compressibility

The Adiabatic compressibility can be calculated as

$$\beta_s = 1/\rho v^2 \quad cm^2/dynes$$

Where 'ρ' is the density and 'ν' is the ultrasonic velocity

G. Calculation of Intermolecular Free Length

The equation to calculate Intermolecular free length is

$$L_f = K\beta_s^{\frac{1}{2}} \quad A.U.$$

Where K = Jacobson's temperature constant

= 631 x 10^{-6} at 303 K
= 642 x 10^{-6} at 313 K
= 651 x 10^{-6} at 323 K

H. Acoustical Impedance

Acoustical impedance is calculated by using the following formula

$Z = \rho v$

Where 'ρ' is the density and 'ν' is the ultrasonic velocity.

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III. Result And Discussion

The results ultrasonic velocity, % survivors, attenuation coefficient and acoustic impedance for exposure of ultrasound (500 W) with an operating frequency of 36 kHz on E.coli taken in 11 different sonication vessels for the time period of 0 min (control), 1 min, 2 min, ….10 min are tabulated in Table 1. Figure (1) shows the initial increase of ultrasonic velocity and then exponential decrease with increase of ultrasound exposure time. Figure (2) shows that attenuation coefficient and acoustic impedance also increases with ultrasound exposure time and then exponentially decreases with further increase of exposure time. Figure (3) shows that adiabatic compressibility and intermolecular free length decreases with sonication time upto one minute after that they increase linearly with sonication time.

The initial rise in ultrasonic velocity and attenuation coefficient of E.coli with ultrasound exposure time for all growth periods of E.coli may be due to convective transport by two mechanisms namely acoustic streaming and micro streaming. The onset of stable cavitation for the small period (around one minute) of ultrasound exposure will enhance the convective transportation. The high convective transport initiates the declumping process. The declumping decreases inter cellular distance and intern the compressibility of E.coli culture and hence the ultrasonic velocity increases. The declumping also increases attenuation coefficient because in addition to the absorption the attenuation due to scattering is increased. Further increase of ultrasound exposure time on E.coli causes onset of transient cavitation which is responsible for disruption of cells in suspension. The exponential decrease of E.coli cells in suspension intern decreases compressibility of E.coli suspension. Therefore, it was observed that the ultrasonic velocity and attenuation coefficient linearly decrease with increased ultrasound sound exposure. It was observed that the acoustic impedance within the suspension increases in the period of declumping may be increased stiffness of E.coli suspension.

TABLE I
Ultrasonic Velocity (v), Attenuation Coefficient (α), Colony Forming Units, (%) Survivors and Acoustical Impedance (z) of E.coli as a function of sonication time at 27^0C.

Sonicat iontime (t) min	Ultrasonic Velocity (v) × 10^2 cm/sec	Attenuatio n Coefficien t (α) nep/cm	Colony Forming Units (CFU/ml)	(%) Surviv ors	Acoustical Impedance (z) × 10^5 gm/cm^2-sec
0	1592	0.5271	1566	100	1.806
1	1610	0.5302	1560	99.6	1.828
2	1568	0.4523	1118	71.3	1.781
3	1553	0.3923	756	48.2	1.764
4	1531	0.3201	343	21.9	1.739
5	1510	0.3002	95	0.06	1.715
6	1501	0.2890	57	0.036	1.705
7	1493	0.2602	21	0.013	1.696
8	1486	0.2505	12	0.007	1.688
9	1481	0.2409	05	0.003	1.682
10	1480	0.2408	04	0.002	1.681

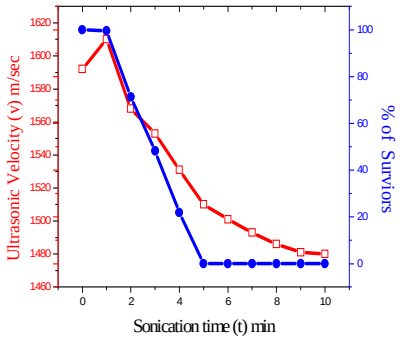

Figure 1. Sonication time Vs Ultrasonic velocity and % of Survivors

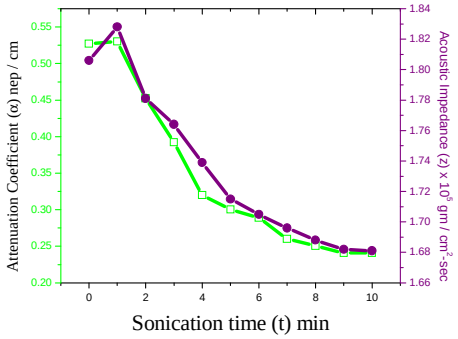

Figure 2. Sonication time Vs Attenuation Coefficient and Acoustic Impedance

TABLE II
Density (ρ), Inter molecular free length (L_f) and Adiabatic compressibility (β_s) of E.coli as a function of Sonication time at 27^0C.

Sonication time (t) min	Density (ρ) gm/cm^3	Adiabatic Compressibility (β_s)X 10^{-11} dyn/cm^2	Inter Molecular Free length (L_f)X 10^{-9} cm
0	1.134	3.479	1.176
1	1.135	3.399	1.163
2	1.135	3.583	1.194
3	1.135	3.653	1.206
4	1.135	3.758	1.223
5	1.135	3.864	1.24
6	1.135	3.910	1.247
7	1.135	3.953	1.254
8	1.135	3.989	1.26
9	1.135	4.016	1.264
10	1.135	4.022	1.265

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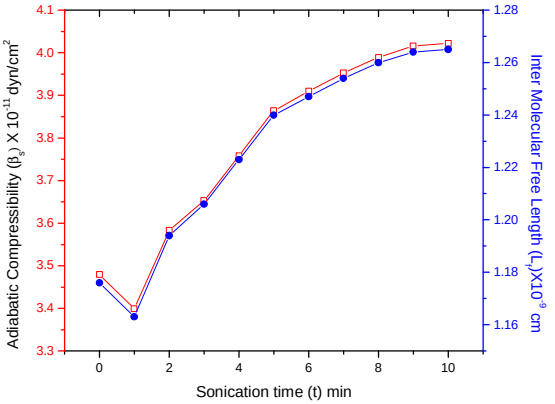

Figure 3. Sonication time Vs Adiabatic compressibility and Intermolecular free length

CONCLUSIONS

The considerable variation of ultrasonic parameters with cell destruction was noted in this present research and may be applicable to other bacteria cultures. The possibility is that the variation of ultrasonic parameters could be used to assess the cell viability and non-linear growth of bacteria cultures. Moreover, it is hypothesized that the detailed study of this research work could be used non-destructively to find the presence of bacteria in packaged food etc.

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