Assessement of the effectiveness of ultrasonic cell disruption by acoustic phenomena as a function of the suspension concentration.

Attila Lőrincz and Miklós Neményi

University of West Hungary, Faculty of Agricultural and Food sciences, Mosonmagyaróvár;
Institute of Agricultural, Food and Environmental Engineering*[*]

Summary In our experiments, under the conditions of an output of 9 W/cm^2 and a frequency of 1.117 MHz, we determined the concentration, expressed in [g/l], of lyophilized *Saccharomyces cerevisiae* baker's yeast needed for stopping cavitation in the sound field. Then by using multiples of the aforementioned concentration, we monitored the acoustic phenomena occurred in the sound field and, simultaneously, we examined the survival dynamics of the cells by vitality dyeing. Examined acoustic phenomena were the following: acoustic streaming, standing wave, and cavitation. Physical parameters of the sound field had essential effect on the phenomena formed in the sound field and on the threshold levels of their formation. The phenomena affected the composition of the material in the sound field, so a phenomena – effect chain reaction took place during the radiation. This paper helps in understanding the cell destruction effect caused by the different phenomena and the role of physical conditions of the sound field in the formation of the acoustic phenomena.

Keywords: Acoustic phenomenon, cavitation, standing wave, acoustic streaming, yeast, R, D values, sound field

Introduction

According to Miller et al. (1996), the most useful approaches in analyzing the biological effects of ultrasound are examining its mechanic and physical effects. The most important phenomenon of all the effects is cavitation. It is originating from the interaction among the ultrasound, the microscopic bubbles and the liquid. Cavitation can occur in two forms: stable and transient forms. According to Miller (1987), when stable cavitation occurs, the bubbles that are present in the sound field in some stabilized form are excited and their volume oscillates around their resonance dimensions. Biological effects observed in *in vitro* systems include the lysis of cells and fragmentation of cell membranes. These effects are caused by following factors: micro streaming near the boundary layer; shearing stress around the moving bubbles; formation of jets when the bubbles collapse; and the formation of sonochemical compounds. According to Apfel (1986), in transient cavitation the bubble suddenly expands vigorously then violently collapses. If this happens near a boundary layer, asymmetric collapse occurs and a jet is formed that goes towards the boundary layer. According to Verall and Sehgall (1988), cell lysis is caused by transient cavitation and this phenomenon is responsible for the fragmentation of the cell membranes. According to Miller et al. (1996), the relative ultrasound intensity needed for the formation of transient cavitation is higher than the intensity needed for stable cavitation. Both types of cavitation cause wide range of biological effects. According to Liebeskind et al. (1979), the effects of ultrasound on surviving cells may include structural changes and effects on DNA, which is the carrier of genetic information. According to Riesz and Condo (1992), transient cavitation may led to the formation of free radicals and of other sonochemical compounds in an indirect way. According to Miller and Thompson (1994) formation of hydrogen peroxide and other sonochemical compounds in sufficient concentration causes biochemical changes in living cells. According to Braymen at al. (1994), the reason for that fact that cells of larger diameter are more sensitive to cavitation than the smaller ones is that the probability of meeting cavitation bubbles is higher in the case of larger cells. Blackshear and Blackshear (1987) observed in relation to hemolysis, that the shearing force needed for disrupting the cell membrane increased steeply when the size of the cells decreased. Carstensen et al., (1993) established that hemolysis of cow erythrocytes was inversely proportional with the cell concentration. Raso et al. (1994) compared the cell destroying effect of thermoultrasonic treatment to the effect of heat treatment. The "D" decimation values observed during the thermoultrasonic

[*] Correspondence Address: Mosonmagyaróvár, Vár 2. 9200 Hungary
Phone: 00 36-96-576-635
e-mail: aenginst@mtk.nymc.hu

treatment were significantly lower than the effect experienced in the case of heat treatment. According to Lee at al. (1989), the degree of resistance of the same organisms against ultrasound may be different in different foodstuffs. For example Mandralis and Feke (1993) applied planar ultrasound standing wave field for continuous fractionation of a suspension.

Materials and methods

Control instrument

The ultrasound signal syntheser generated sinusoidal signal of 1,117 MHz frequency. The ultrasound amplifier could produce 0 – 40 W amplification that we could correlate to W/cm^2 in the knowledge of the radiation surface. According to Göschl et al. (1999), the ultrasound resonators that are suitable for manipulating, handling and controlling small suspended particles shall consist of at least four parts. These are the following: piezoelectric transducer, the carrier glass container, the suspension and the acoustic reflector. Based on the findings of Armour and Corry (1982) and the authors referred to above, we used a resonator whose construction is shown in Figure 1. This resonator is suitable for generating both standing and propagating longitudinal waves.

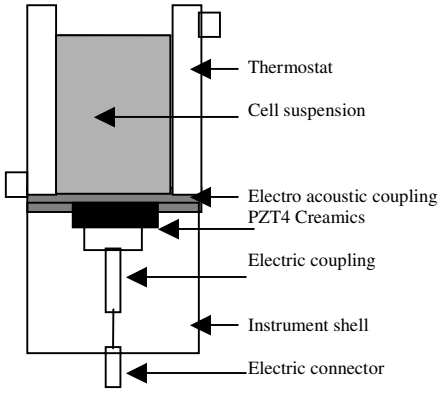


Figure 1: Resonator

The main component of the resonator is the piezoelectric ceramics made of lead zirconate. It has a planar surface and its diameter is 22 mm. Casing for the suspension, or the sound field is formed by a double walled cylindrical glass thermostat. Internal and external diameters and height of this thermostat were 30 mm, 80 mm and 200 mm, respectively. The resonator was connected to the ultrasound amplifier through an electric connector. The non-rigid reflector was the air layer situated above the suspension, facing the piezoelectric ceramics. Weight was measured by an analytical balance of Precisa 505M – 2020C DR SCS type. Its accuracy was 0,001 g.

Suspension

Water was used as a suspending agent. Its temperature was 20 ^{o}C and it was conditioned for an hour before used. Lyophilized baker's yeast Saccharomyces cerevisiae was used as suspended material.

Detection

According to Veit (1977), cavitation appears as a noise acoustically and this noise can be recorded by a microphone and it can be analyzed. Our audionic detection method is based on this finding. Components of the arrangement are the microphone, audio amplifier and the oscilloscope that was used for detecting the cavitation threshold. Saad and Williams (1985) showed that acoustic streams are formed in the liquid due to cavitation caused by the ultrasound. Wathmough et al. (1990) experienced a mixing of liquids in the sound space caused by the acoustic streaming. Chrunch and Miller (1983) observed that as a result of the standing wave the cells and the bubbles were separated into different layers, so no interaction can occur between them. An adsorber placed opposite to the transducer helps in avoiding this situation, but the air layer situated opposite to the transducer results in a near perfect reflection and a near perfect standing wave can be generated with this arrangement. With our visual detecting method we monitored the observable acoustic phenomena, the standing wave, the acoustic streaming and the formation of the jet as a function of the length of the treatment period. Changes in yeast vitality was examined by methylene blue vitality dyeing; the pictures were recorded by a computer through a CCD camera mounted on a biological microscope.

Experimental plan

When performing the experiments, first we determined the cavitation threshold concentration (as a g/25 ml and from this data, expressed as g/l concentrations) at a frequency of 1,117 MHz, at an ultrasound output of 9 W/cm^2 and at a temperature of 20 ^{o}C by applying the basic and auxiliary methods. Later, when the "point of time when the standing wave was formed", the "point of time when cavitation occurred" and the biological effects were examined simultaneously, we applied the multiplied concentrations (1; 1.5; 1.7; 2.2; 3) of the threshold concentration.

Basic method for determining threshold concentration of the cavitation

25 ml tap water conditioned for an hour was poured into the thermostat and 3 drops of 1 % methylene blue indicator solution was added by pipette.

During this operation, the temperature of the sound field was kept at a steady 20 ^{o}C. A quantity of 0.5 – 1 g lyophilized yeast was measured into a measuring spoon with an accuracy of 0.001 g on an analytical balance. We switched on the equipment detecting the acoustic phenomena in the sound field. When we switched on the resonator, cavitation occurred. Then we started to add the particles to be suspended from the measuring spoon. We added yeast to the suspension until the cavitation effect of the sound ceased to exist. This is the cavitation threshold concentration. This point was detected by an audional detector through a signal shown on the oscilloscope. For determining the threshold concentration of the cavitation, the quantity of yeast added to the suspension was determined by measuring back the quantity of yeast that remained in the measuring spoon, with an accuracy of 0.001 g. The experiments were repeated four times at this output level.

Auxiliary method for determining threshold concentration of the cavitation

More accurate results can be obtained by this method. It is based on approaching the cavitation threshold concentration from both direction (from below and from above) by measuring gradually increasing quantities of yeast into the suspension near the cavitation threshold concentration. If, in the case of a series of three different samples, which differed from each other by not more than 2 mg, respectively, cavitation occurred within 1 sec after switching on the ultrasound, it occurred immediately and it occurred much later for the middle, lower and higher concentrations, we established that we had found the cavitation threshold concentration.

Examination of vital cell number and of the acoustic phenomena in the sound space

Cell disrupting effects of the ultrasound occur immediately in the cells. We suspended a quantity of biological material as determined in the experimental plan into the sound field by artificial agitation, then the mixer was removed. The moment when the ultrasound was switched on, we started to measure the elapsed time with a stop-watch. We took samples of 0.05 ml in every 15 second from the treated suspension and determined the vital cell number in the samples. Sampling was carried out until the steady state of the acoustic wave phenomena was achieved. We measured the time elapsed from starting the experiment until the formation of the standing wave or of the cavitation (until cavitation sound was detected) with a stop-watch. We repeated the experiments several times and used the results of those experiments where the moments when the standing wave and the cavitation was formed, respectively, fell in the same respective sampling period. Walsch et al. (1999) examined the changes in vitality of cells exposed to propagating and plane ultrasound waves by methylene blue dyeing. According to Bíró (1976), vitality dyeing is suitable for determining the vitality of microorganisms, primarily of yeasts. Basis of this method is that if a suspension that consists of living and died cells is put in touch with methylene blue, the died cells become blue immediately, while the living ones will not be dyed. We determined the "D" decimation and "k" specific destruction rates with the method of Deák (1997) for the different phases of the acoustic phenomena and plotted the survival diagrams.

Results and discussion

The cavitation threshold concentrations (CTC) measured by the basic and auxiliary methods are shown in Table 1.

Table 1: Cavitation Threshold Concentrations

Cavitation Threshold Concentrations	[g/l]
1.1. Results given by the basic measurement method	
Repetition 1.	3,72
Repetition 2.	3,24
Repetition 3.	3,52
Repetition 4.	3,32
Average of the basic measurements	*3,45*
1.2. Result given by the auxiliary method	3,2

Relative change in surviving cell number

Cell concentrations applied in the examinations are shown in Table 2.

Table 2: Suspension concentrations applied in the biological examinations

Suspension	concentration	
CTC*	[g/25ml]	[g/l]
[CTC]*1	0,08	3,2
[CTC]*1,5	0,12	4,8
[[CTC]*1,7	0,136	5,44
[CTC]*2,2	0,176	7,04
[CTC]*3	0,24	9,6

Results of the examination of the relative number of the surviving cells are shown in Table 3. In case of the untreated control samples, no significant change occurred in the number of cells even after hours of preparing the suspension. In the table "*" and "**" indicate when the formation of the standing wave and cavitation occurred, respectively.

Table 3. Changes in the relative number of surviving cells during the exposition

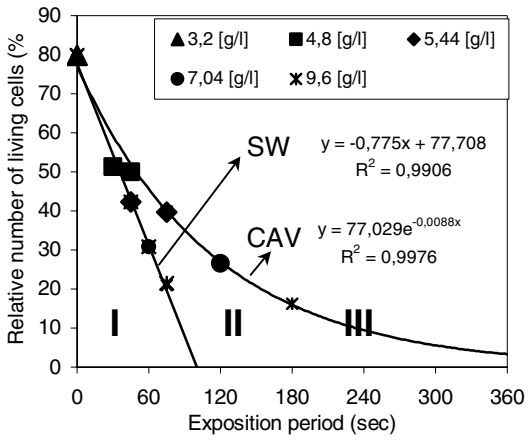
	Exposition period (sec)																
	0	15	30	45	60	75	90	105	120	135	150	180	200	240	270	280	360
Relative Number of Surviving cells (%)																	
3,2 g/l																	
1.	78*	27	13	7	3	0	0										
2.	84*	36	19	10	5	1	0										
3.	81*	31	18	6	4	2	0										
4.	78*	28	9	6	3	1	0										
Average (%)	80,25*	30,5	15	7,25	3,75	1	0										
Standard deviation	2,87	4,04	4,65	1,89	0,96	0,82	0,00										
4,8 g/l																	
1.	83	62	54*	52**	34	19	7	4	2	0							
2.	81	65	56*	54**	39	21	8	7	3	0							
3.	79	63	46*	46**	32	16	6	5	2	0							
4.	79	61	49*	48**	33	17	4	3	1	0							
Average (%)	80,5	62,8	51*	50**	34,5	18	6,25	4,75	2	0							
Standard deviation	1,91	1,71	4,57	3,65	3,11	2,22	1,71	1,71	0,82	0,00							
5,44 g/l																	
1.	76	62	50	40*	39	39**	17	11	5	1	0						
2.	79	61	52	41*	39	38**	18	12	7	2	0						
3.	82	66	56	45*	43	42**	22	14	8	4	0						
4.	80	67	53	43*	41	40**	20	13	5	2	0						
Average (%)	79,25	64	53	42,3*	40,5	40**	19,3	12,5	6,25	2,25	0						
Standard deviation	2,51	2,94	2,50	2,22	1,91	1,71	2,22	1,29	1,50	1,26	0,00						
7,04 g/l																	
1.	81	62	40	35	31*	30	30	28	26**	17	8	6	4	2	0	0	
2.	75	60	38	33	30*	30	29	27	27**	19	12	8	5	3	2	0	
3.	88	65	42	36	32*	31	29	28	26**	16	9	5	2	1	0	0	
4.	82	61	39	33	30*	30	28	28	27**	22**	13	7	4	2	1	0	
Average (%)	80	62	40	34,3	30,8*	30	29	27,8	26,5**	18,5	10,5	6,5	3,75	2	0,75	0	
Standard deviation	5,32	2,16	1,71	1,50	0,96	0,50	0,82	0,50	**0,58**	2,65	2,38	1,29	1,26	0,82	0,96	0,00	
9,6 g/l																	
1.	83	54	34	29	22	21*	20	20	20	19	17	16**	14	10	4	3	0
2.	76	56	30	27	24	23*	22	22	21	20	19	18**	17	12	5	4	0
3.	79	49	33	28	23	22*	21	20	19	18	17	16**	13	11	4	2	0
4.	80	53	31	25	21	20*	20	18	18	18	16	15**	12	8	7	5	0
Average (%)	79,5	53	32	27,3	22,5	22*	20,8	20	19,5	18,8	17,3	16,3**	14	10,3	5	3,5	0
Standard deviation	2,89	2,94	1,83	1,71	1,29	1,29	0,96	1,63	1,29	0,96	1,26	1,26	2,16	1,71	1,41	1,29	0,00

This method resulted in significant standard deviation where the relative number of the surviving cells were low, so the zero values shown at the time of the last sampling are hypothetical; they means that an equilibrium has been achieved and the decrease of the surviving cells probably continues at the same rate as before.

Acoustic phenomena formed in the sound field

In Figure 4. we connected the relative numbers of the surviving cells measured at the moments when cavitation occurred, and at the moments when standing waves were formed with lines indicating the trends of the processes. Three different zones are shown in the figure (I. Acoustic streaming; II. Standing wave; III. Cavitation). Therefore the zones show different acoustic phenomena in the sound field. Zone I. starts from the initial 80 % living cell ratio and ends at the moment when the standing wave is formed. Dominant factor in this zone is the acoustic streaming. Doida et al. (1992), examined the conditions for the formation of standing waves in a pipe. Weak standing waves were formed if the scientists applied an acoustic adsorber, and strong ones were observed if an acoustic reflector was present opposite to the transducer.

Figure 4. Zones of acoustic phenomena bordered by trend lines connecting points of time when the formation of the standing wave (SW) and the cavitation (CAV) occurred



In our case acoustic streaming was the dominant phenomenon of the sound field when no reflector was used. This streaming was formed due to the overfeed of the particles into the sound field. When clear suspension media was examined, the perfect reflection capability of air located opposite to the transducer dominated and cavitation was formed due to the increasing amplitude of the acoustic pressure. However, when solid particles were suspended in the sound field, due to their adsorption and sound dispersion features, the amplitude of the acoustic pressure was decreased below the cavitation threshold, the role of the acoustic reflector decreased significantly, and a swirling acoustic streaming was formed. This is a feature of Zone I, the zone of acoustic streaming that prevails if the particle concentration in the sound field is higher than the cavitation threshold concentration. In time, this zone lasts from the beginning of the experiment until the formation of the standing wave. Under the conditions of higher suspension concentrations, the time interval of the existence of the acoustic streaming phenomenon is longer. Brayman and Miller (1993) observed that bubble activity was suppressed near the bubbles by the cell aggregation. In case of a given sample, the acoustic streaming remains in existence until the moment when the radiation forces trap the particles in pressure modal planes and the standing wave is formed. Zone II starts at the moment when the standing wave is formed and ends when cavitation is formed. (Figure 4). In a standing wave, the cells are concentrated in the sound field in planes in a clearly visible way. Distance of these planes is equal to the half of the wavelength. An another process that takes place simultaneously is the sedimentation of the particles. In our experiments carried out with samples having different cell concentration, after starting the experiments, we drew a linear trend line on to the relative cell number values measured at the moment when the standing wave was formed. This line forms the boundary of the zones of the standing wave and the acoustic streaming. Due to the linear relationship, no incremental time period is needed for trapping the particles in the case of higher concentrations. Limaye and Coakley (1998) purified microbiological suspensions of low volume in ultrasound standing wave space. *Escherichia coli* and *Saccharomyces cerevisae* were separated. The concentrated aggregates precipitated on the bottom of the resonator due to the sedimentation. As a result of the particle agglomeration effect of the standing wave, sedimentation occurred, and as a result of this process, the number of the adsorption and scattering centers located in the sound field decreased. This resulted in increasing the amplitude of the acoustic pressure, so detectable cavitation occurred. Zone III starts from the moment when the cavitation occurs and lasts until infinity. On the relative living cell numbers measured at the very moments when the cavitation occurred, an exponential trend line was fitted. This curve forms the boundary of the zones of the standing wave and the cavitation phenomenon. Cavitation occurs later and later as the concentration increases, because the decrease of the particle concentration in the sound field forms larger and larger acoustic pressure amplitudes and this acts against the sedimentation. It can be established, that the concentration of the suspension in the sound field have decisive effect on the acoustic phenomena and on the threshold times when the individual phenomena occurred. In the experiment, where an initial concentration of 3.2 g/l was applied, no standing wave and acoustic streaming was occurred. Acoustic cavitation was formed at the beginning of the experiment and it lasted until the end of the experiment. In the case of concentrations higher than 3.2 g/l, the standing wave and the cavitation occurred later and later as the concentration increased. Reproducibility of the repeated experiments was good the calculated standard deviation values were low. Sequential occurrence of the acoustic phenomena in the sound field was named phenomenon – effect chain reaction.

Results of the examination of the survival dinamics

Based on our Bürker chamber cell counting method, it was established that in case of 1 g/l cell suspension, the number of cells: $N = 5.6 * 10^6$ units/l. This means that in the knowledge of the initial absolute cell numbers and of the measured relative cell numbers the survival dynamics of the cells was determined in all the three acoustic zones (I. Acoustic streaming; II. Standing wave; III. Cavitation). According to Deák (1997), majority of the experiments indicate that the interferences acting through environmental factors that cause the destruction of the microorganisms, show exponential relationships.

$$k = ((2.303/(t-t_o)*\log(N_o/N_t)$$

[k]: the specific destruction rate; t: time (sec) integrated between limiting values of N_o: (initial number of cells at t_o); and N_t: (number of surviving cells at t). Plotting the logarithm of the number of surviving cells as a function of time, a linear relationship is established. If the time $(t-t_o)$ that occurs in the equation is defined as the period of time during which the number of surviving cell decreases to one tenth of its original value, the concept of decimation time ("D") is established. If $t-t_o = D$ and $N_t = 0.1 * N_o$, then:

$$k = 2.303 / D, \text{ or } D = 2.303 / k$$

Decimation time is the measure of the resistance of the microorganism population expressed in minutes. In the knowledge of the initial aggregated number of cells (N), the initial number of living cells (N_o) and the final absolute number of the surviving cells (N_t), the common logarithms of the absolute number of cells belonging to the individual zones ($logN_o$, log N_t) and the initial (t_o) and final (t_t) points of time belonging to the aforementioned cell numbers ware determined. In the knowledge of these variables the "D" decimation and "k" destruction rate coefficients ware determined for all three phenomenon zones. Table 4 shows the "D" and "k" values. In Figure 5 the survival dynamics of the different phenomenon zones are shown.

Table 4: Survival dynamics values characteristic of the different acoustic phenomenon zones as observed in the individual experiments

Concentration (g/l)	Acoustic phenomenon	D (sec)	k (sec-1)
3,2	I.	-	-
3,2	II.	-	-
3,2	III.	40,3	0,057
4,8	I.	155,6	0,0148
4,8	II.	1404,2	0,00164
4,8	III.	50	0,046
5,44	I.	165,5	0,0139
5,44	II.	1112	0,002
5,44	III.	57	0,04
7,04	I.	145,75	0,0158
7,04	II.	919,3	0,0025
7,04	III.	94,77	0,0243
9,6	I.	134,91	0,0171
9,6	II.	895,7	0,00257
9,6	III.	150,5	0,0153

In the figure, the common logarithm of the cell number values and the time elapsed from the beginning of the radiation treatment were plotted in the vertical and horizontal axes, respectively. Steeper sections show quicker cell destruction. This means that the resistance of the cells against the given acoustic phenomenon is lower here than in the phases represented by less steep sections. It can be seen that quicker cell number decreases (smaller "D" values) is observed during the acoustic streaming and cavitation than during the standing wave phenomenon. Doida et al. (1992) explained, that in case of weak standing wave, or where propagating waves dominated, strong cell destruction was observed while in the case of a strong standing wave, the cell destruction was negligible. According to Walsch et al. (1999), the most significant effects of the propagating ultrasonic waves on yeast physiology are the decease of the number of living cells and the decrease of the capability of the cells for division. These authors did not experience significant cell destruction effect in the standing wave space. Radel et al. (2000) measured the changes in the vitality of yeast in planar propagating and standing wave fields. In the standing wave field they did not experience significant change in the vitality. Brayman and Miller (1992) also experienced that only small cell destruction occurred in the standing wave field. In our case, as the initial cell numbers were higher and higher, the length of the standing wave phase were longer and this made possible longer manipulation of the cells in the sound field. In Figure 5. it is clearly seen that definite survival dynamics are prevailing in the different phases of the different phenomena. Under the conditions of low cell concentrations, the quickest cell destruction is caused by cavitation, the "D" decimation time is the shortest in this phase. In the range of the acoustic streaming and of the standing wave the decimation time slightly longer, and much

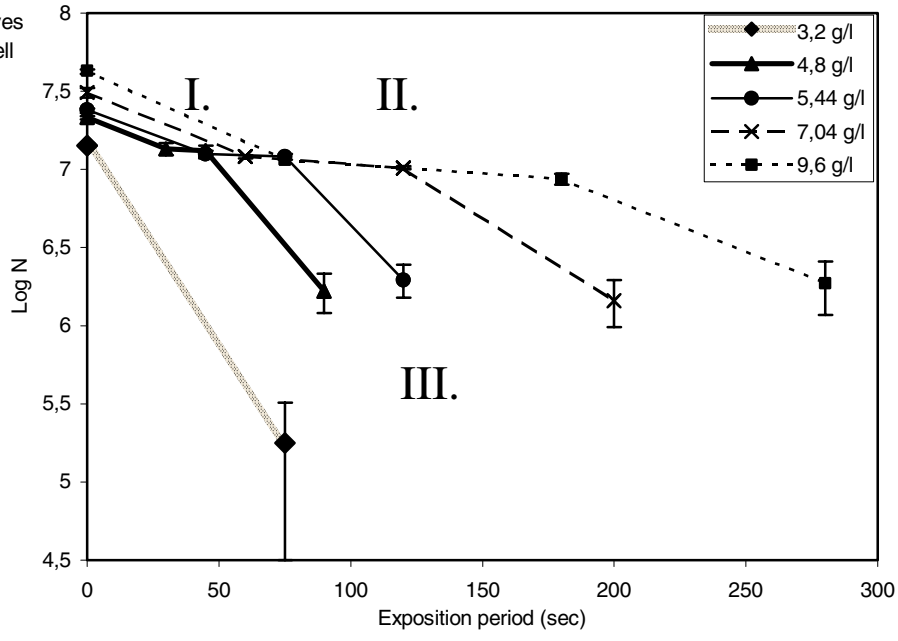
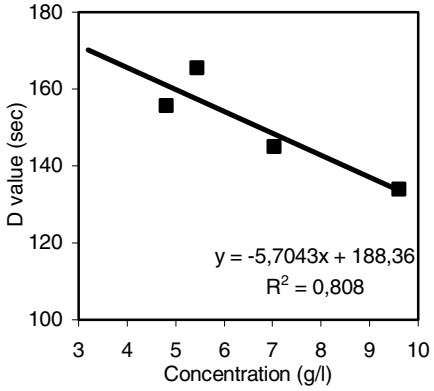


Figure 5. Calculated survival curves belonging to the individual initial cell concentrations by the different acoustic phenomena

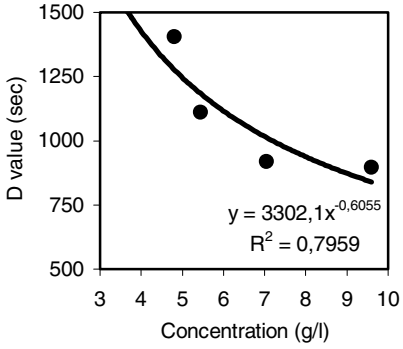
longer, respectively. When the cell concentration is high, the aforementioned facts are modified to some extent. In order to understand these changes, the results obtained shall be interpreted in a wider framework. Veress and Vincze (1977) showed the importance of cell concentration in the *in vitro* sonolysis. Sonolysis, which can be detected easily and clearly if the cell concentration is low, takes place in a sluggish way if the cell concentration is high. When considering the survival changes in the ranges of the different acoustic phenomena, it can be observed that in the range of acoustic streaming the survival dynamics produces very similar "D" values, almost regardless to the initial cell concentrations; our calculations show that the "D" values may even be slightly lower at higher initial cell concentrations (see fig. 6.).

Figure 6. Survival times measured under the conditions of acoustic streaming at different cell concentrations



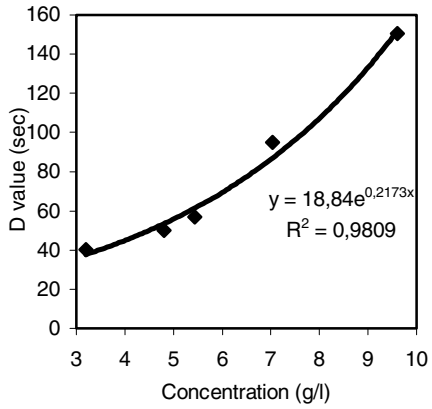
When examining the effect of the standing waves on the survival of the cells, it can be established that there is little difference in the survival features of the suspensions having different initial cell concentration. When the initial cell concentrations are higher, the observed "D" values are slightly lower. This may be caused by the fact that in this situation the cells spend longer time in the sound field (fig. 7.).

Figure 7. Survival times measured under the conditions of standing wave at different cell concentrations



The results of Carstensen et al. (1993) also show that in the standing wave field small but significant cell destruction occurs, but if the cells are moving from their spaces in the modal planes, the rate of their destruction suddenly increases. Based on out examination results, it can be established that the effect of the cavitation on the decimation time of the surviving cells significantly differs from the effect of the acoustic streaming and of the standing wave on the same variable if the initial cell concentrations are different. In the case of samples having higher cell concentrations, the decimation time is longer, while if the initial cell concentration is lower, the decimation time is shorter (fig. 8.).

Figure 8. Survival times measured under the conditions of cavitation at different cell concentrations



This phenomenon is caused by more than one factors. According to some experts, one of these factors is that if the same number of cavitation nuclei is formed, they inactivate larger number of cells during proportionally longer period of time; the other is that the cells that are precipitated in the standing wave phase are continuously stirred up due to the intensive currents induced by the cavitation and this ensures their continuous meeting with the cavitation bubbles. Another reason may be that cell rosettes are formed around the cavitation bubbles and this suppresses the cavitation activity. In our experiments the equilibrium phase is characterized by the fact that the cavitation becomes stable after the standing wave phase, so the effects of this phase prevails until the number of the living cells decreases to zero. In the individual experiments, if we lengthened the sections of lines indicating the cell number decreases until the ordinate, this line intersects the axis at the starting point of the experiment carried out at a cell concentration of 3.2 g/l, that is the cavitation threshold concentration, which means a cell number of log 7.14. Survival dynamics in the standing wave range, as a continuous virtual line separates the survival dynamics prevailing in the acoustic

streaming and cavitation ranges that are located above and below this line, respectively.

Conclusions

Phenomena formed in the ultrasonic space clearly affect the survival dynamics of the cells present in the sound field. Formation of these phenomena can be affected deliberately and this makes possible to affect the survival dynamics of the cells. There is an interaction between the suspension concentration in the sound field and the formation of the acoustic phenomena and through this, between the survival dynamics of the cell suspension and the suspension concentration.

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