Publikálva:

M. NEMÉNYI – A. LŐRINCZ (2002): Appreciation of an complex ultrasound system according to survival cell count. Hungarian Agricultural Engineering.

APPRECIATION OF AN COMPLEX ULTRASOUND SYSTEM ACCORDING TO SURVIVAL CELL COUNT

Summary

The decreasing of micro-organism cell count the most important task of the food industry. The killing of microbe no too difficult thing rather the preserve of essential food components is the heavey work. The modern microbe destructor technologyes its effects goals only the pointed objects and dont touchs the other essential food components. Our experimental work goals this philosophy with ultrasound. We examinated the surviving cell count of *Saccharomyces cerevisiae* suspension. This cell suspension was of an closed liquid circuit system streaming with peristaltic pump. The cell count with manual and automatical detection system was examinated and the experimental method based on vital staining with methylen blue and on the cell counting. The results gives possibility any cell phase analytical and continuous cell decreation system developing. This work is fundamental research.

Introduction

There is no cavitation in the ultrasound field until the amplitude of the acoustic pressure exceeds a certain level, the cavitation threshold [1]. Cavitation threshold is proportional with the frequency of ultrasound, with the hydrostatic pressure in the liquid, and with the viscosity of the sample and it is inversely proportional with the gas content and temperature of the sample [2]. There are two types of cavitation that are stable and transient cavitation [3]. Basically two reactions take place when ultrasound and a media interact with each other. One of them is the absorption the other one is the scattering, which changes e.g., the speed of propagation of the sound in the subject media [4]. Due to the absorption, the intensity of ultrasound decreases exponentially with distance and the absorption coefficient primarily depends on the speed of propagation of the sound in the subject media, on the wave type, on the material situated in the ultrasound field and on the frequency. The absorption always characterizes a media, a structure or an environment that determines the parameters of propagation [5]. When absorption coefficients were measured in oxo- and és methemoglobin, it was observed that the absorption is proportional with the concentration of hemoglobin in the concentration range between 0 and 15 [g/100ml] [6]. It was clearly established that the profile of the ultrasound propagation speed depends on the concentration profile of the suspension [7]. Effects of the size and concentration of the suspended particles on the propagation speed of ultrasound was examined in water based suspensions. It was established that the speed of sound largely depended on the particle size and concentration [8]. In vitro cavitation threshold measurements were carried out in human blood. In the fresh blood that contained every blood component, the amplitude of the acoustic pressure belonging to the cavitation threshold was higher than in diluted blood [9]. Due to cavitation caused by ultrasound, acoustic streaming was formed in the liquid [10]. Acoustic streaming is a movement of the liquid that is caused by intensive ultrasound [11]. Mixing of liquid was experienced in the ultrasound field due to acoustic streaming [12]. An acoustic reflector placed opposite to the transducer causes a standing wave to be formed. In a standing wave the materials whose density are lower and higher than of the liquid drift to propagation cluster planes (pressure antinodes), and pressure nodes, respectively [2]. The ultrasonic separation is used in analytical biotechnology

applications. This procedure is based on the fact that in a standing wave field, where there is no cavitation, the cells are arranged in bands distances of which are smaller than a millimeter and they can be separated from these bands [13]. Yeast (*Saccharomyces cerevisiae)* and rubber particles were manipulated in a standing wave ultrasound field at frequencies of 1 and 3 [MHz]. The particles formed bands in pressure nodes whose distance from each other was equal to half of the wavelength. In the direction of the radiation the bands formed column like structures. Stability of the bands, the conditions under which they are broken and the formation of the acoustic streaming were investigated in [14]. Effectiveness of the cell separation of *Escherichia coli* bacteria and *Saccharomyces cerevisiae* yeast cells from a yeast suspension was examined at frequencies of 1 and 3 [MHz] [16].

Materials and methods

As experimental marker microorganism we suspended 1,8 gram yeast (Saccaromyces cerevisiae) in 200 cm³ distilled water with a magnetic mixer until it became clod free and the cell concentration reached the level of $9x10^7$/ml. For the sake of a better detection we put 5 drops of methylene blue into the solution, which did not influence the vitality of the microorganism.

We put the suspension into a fluid flow system (Fig. 1.) of 116 cm^3 of inner volume with a peristaltic pump. After the filling and short-circuit of the system the suspension was circulated by a peristaltic pump between the different structural units. The suspension was then not directly treated but isolated from its environment by material flow through ultrasonic flow cuvettes (Fig. 2.) especially made for this purpose. The ultrasonic cuvettes allow the suspension to flow with a surface of 1 cm^2 and a thickness of 0.5 mm. There were two cuvettes placed 1 cm apart at right angles to the flow direction. The reason for this arrangement was that the effects on the liquid film are much easier to observe than inside the material. In order to avoid cell sedimentation an efficiently high rate of flow was applied: 50-70 cm/sec (4-5 cm^3/sec). The suspension flowing in the ultrasonic cuvettes were exposed at a frequency of 0.8 MHz and at a capacity of 10 W/cm^2. The suspension flowing in the system gets into an optical detection cell placed in a biological microscope. The picture gets then from here through a CCD camera into a computer system, where it will be saved according to time units. Which will allow evaluating the cell disruption effect of the treatment based on calibration. After short-circuit the flow system and turning on the ultrasonic system there were drop samples taken at time units through a built-in tap. The samples were immediately analyzed under a Bürker chamber. A thermostat unit also belongs to the system, which ensures a constant temperature for the reproduction of the tests.

The survive cell analysis is based on a vital stain, which means that under microscope with Bürker-chamber. The dead cells are stained blue owing to methylene blue but the living organisms remain clear. We can establish the curve of deteriorated and survived cells owing to ultrasonic treatment as blue stained and clear cells are counted at regular time units. Organisms are regarded as cells, which have intact cell walls and reflect vitality. We want to mention this fact because after a certain time of treatment cell lysis will happen.

Treatment definition

The treatment means a certain period of time during which the amount of liquid circulating in a flow system is exposed to a physical (ultrasonic) treatment of a given capacity during a given period.
The time of treatment means in the flow system the period between the turning on and turning off the ultrasound. This period was taken into account during the evaluation of the results.
As for the amount of liquid the time of treatment had to be corrected in the flow

system concerning the total amount of liquid flowing in the system and the total time of treatment. Therefore the total liquid treatment time of „A" is required to reduce the original cell count to its hundredth where „B"=116cm^3 is the total amount of flowing liquid in our system. So 1 cm^3 of cell suspension has to be treated at „A/B" minute.

Results and discussion

Treatments were carried out at 0,8 MHz and with a capacity of 7,5 W/cm^2; 9,6 W/cm^2; 10,5 W/cm^2 and 12 W/cm^2 by taking samples from the suspension at defined times. These samples were then evaluated in Bürker chambers based on the average living and dead cell numbers to be observed. Figure 3 showed the relative percentage of survive cell counts in the samples. According our examinations the relative surviving cell counts of one milliliter treated suspension showed on table 1. On fig. 3. the ♦ = 7,5 W/cm^2, ■ = 9,6 W/cm^2, Δ = 10,5 W/cm^2, X = 12 W/cm^2 are the points belonging to the ultrasonic treatments and the functions fitted to them (Fig 4.). The relative living cell counts signified by the different symbols show that more drastic and faster cell destruction is to be observed depending on the time passed if we applied higher capacities. In table 1 the relative cell counts refer to 22 cells per Bürker chamber owing to creating the model function, which indicate an initial cell count of 88 million/millilitre. In order to set up a model formula we set different trend functions onto the points. The logarithmic trend functions showed the highest correlation with the measured points. Setting trend functions on to the additive and multiplying factors of the logarithmic functions resulted in the formula shown in table 2. where „a" is the trend function referring to the additive factor and „b" is the trend function referring to the multiplying factor. Cell count can be omitted from the model formula as it refers to the initial cell count of 22 cells/Bürker chamber (cfu), so it has to be corrected by the actual cell counts respectively. This model function helps to calculate the ultrasonic capacity that is required for treating the material at a given initial cell count, so that it would reduce to the required cell count after a certain period of time. Or it allows determining the time needed to reach the required cell count at constant ultrasonic capacity. Of course these values refer to a given system. Therefore a correction factor is to be used for a general usage adapted to the actual conditions. Figure 5. shows the replacement of the treatment of 7.5 W/cm^2.

Conclusion

The model function allows modelling the effects of the ultrasonic treatment at a frequency of around 0,8 MHz and different capacities.
The ultrasonic treatment can be adapted for use for treating agricultural products if high quality is required.

Acknowledgement

We express out thanks for the help of Prof. Dr. Pál Greguss from the Technical and Economic University of Budapest.

References

[1] F. J. Fry, Ultrasound: Its Applications in Medicine and Biology, Elsevier Scientific Publishing Company, Amsterdam-Oxford-New York. 1978

[2] G.R. Ter Haar, Biological Effects of Ultrasound in Clinical Applications. In: S.K. Suslick, Ultrasound, Its Chemical, Phisical, and Biological Effets. VCH Verlagsgesellschaft mbH, Weinheim. 1988 pp 305-319.

[3] L.A Frizzel, Biological Effects of Acoustical Cavitation. In.: S. K. Suslick, Ultrasound, Its Chemical, Phisical, and Biological Effets. VCH Verlagsgesellschaft mbH, Weinheim. 1988 pp 287-301.

[4] C.R. Hill,– R.C. Chivers,– R.W. Huggins,– D. Nicolas, Scattering of Ultrasound by Human Tissue. In.: F. J. Fry,

Ultrasound: Its Applications in Medicine and Biology. Elsevier Scientific Publishing Company, Amsterdam-Oxford-New York 1978 pp 441-493.
[5] H. Kurtuff, Ultrasonics Fundamentals and Applications, Elsevier Applied Science, London and New York. 1991
[6] E.L. Carstensen, H.P. Schwann, Acoustic Properties of Hemoglobin Solutions, J. Acoust. Soc. Amer. 31 1959 p 305.
[7] D.J. Wedlock, C.J. McConaghy, S. Hawksworth, Automation of ultrasound velocity scanning for concentrated dispersions, Colloisd and Surfaces A: Physiochemical and Engineering Aspects. 77 1993 pp 49-54.
[8] P. Sayan, J. Ulrich, The effect of particle size and suspension density on the measurement of ultrasonyc velocity in aqueous solutions, Chemical Engineering and Processing. 41 2002 pp 281-287.
[9] C.X. Deng, X. Quihong, R.E. Apfel, C.K. Holland, In vitro measurements of inertial cavitation thresholds in human blood, Ultrasound in Med. and Biol. 22 1996 pp 939-948.
[10] A.H. Saad, A.R. Williams, Possible mechanisms for the agitationinduced enhancement of acoustic cavitation in vitro, J. Acoust. Soc. Am. 78 1985 pp 429-434.
[11] H. Mitome, The mechanism of generation of acoustic streaming, Electronics and Communications in Japan, Part 3. 81 1998 pp 1-8.
[12] D.J. Watmough, K.M. Quan, M.B. Shiran, Possible explanation for the unexpected absence of gross biological damage to membranes of cells insonated in susupension and in surface culture in chambers exposed to standing and progressive wave fields, Ultrasonics. 28 1990 pp 142-148.
[13] W.T. Coakley, Ultrasonic separations in analitical biotechnology, Trends in Biotechnology. 15 1997 pp 506-511.
[14] J.J. Hawkes, J.J. Cefai, D.A. Barrow, W.T. Coakley, L.G. Briarty, Ultrasonic manipulation of particles in microgravity, J. Rhys. D: Appl. Phys. 31 1998 pp 1673-1680.
[15] J.J. Hawkes, M.S. Limaye, W.T. Coakley, Filtration of bacteria and yeast by ultrasound enhanced sedimentation, Journal of Applied Microbiology. 82 1997 pp 39-47.

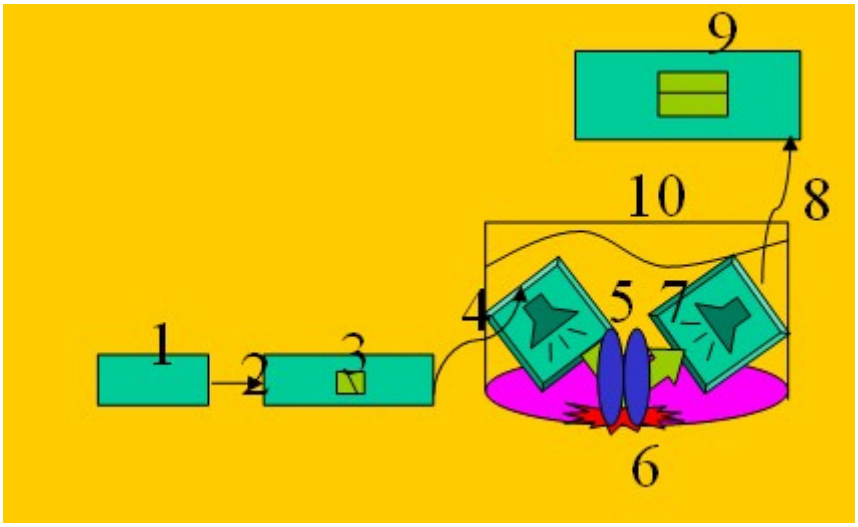

Figure 1. *The complete flow injection system. Key: 1. Peristartic pump, 2. Lead out tube, 3. Ultrasonic treating cuvettes, 4. Tube, 5. Tap 6. Optical flow cuvette, 7. Lead in tube, 8. Stereomicroscope, 9. CCD camera, 10. Ultrasound transmitter, 11. Ultrasound receiver, 12. Echoless waterbath.*

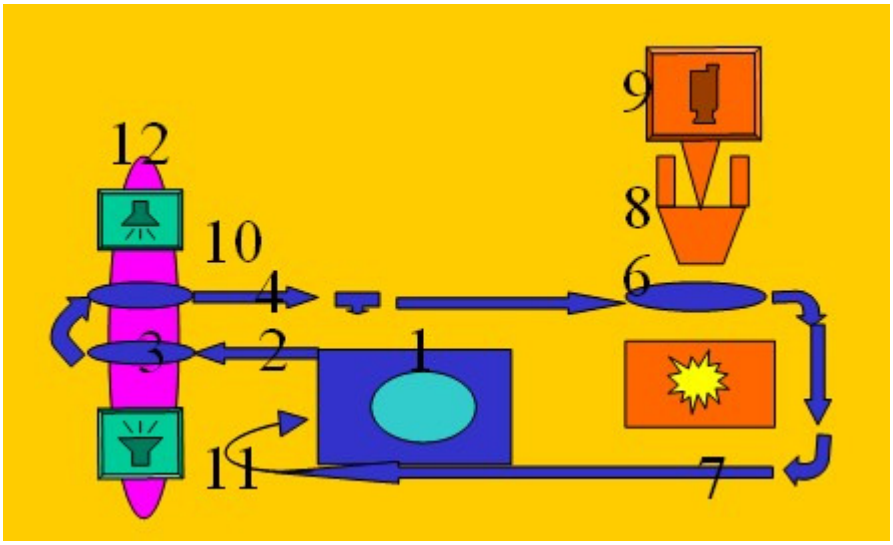

Figure 2. *The ultrasound system. Key: 1. Signal generator, 2. Electronic cable, 3. Ultrasound amplifier, 4. Electronic cable, 5. Ultrasound transmitter, 6. Reflection surface, 7. Ultrasound receiver, 9. Electronic cable, 9. Oscilloscope, 10. Echoless watertank.*

Table 1. *Relative surviver cell cell counts of one millilitre treated cell suspension after different exposition times.*

Ultrasound power **Exposition time**	7,5 [W/cm^2] 4 min 3 sec	9,6 [W/cm^2] 2 min 51 sec	10,5 [W/cm^2] 2 min 39 sec	12 [W/cm^2] 2 min 30 sec
Rel surv. Cell count	1,7%	0,9%	0%	0,8%

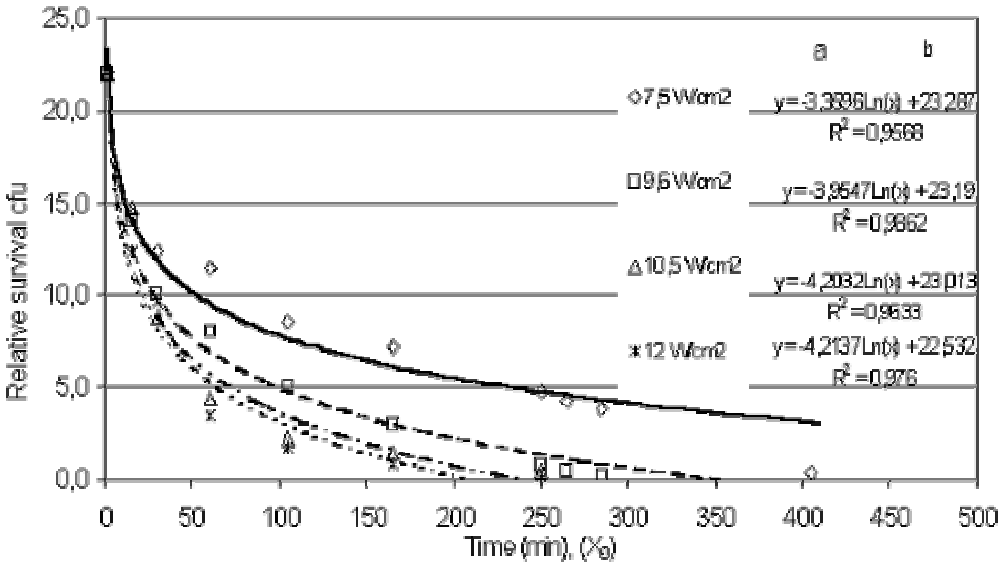

Figure 3. Relative survival cell count (%)

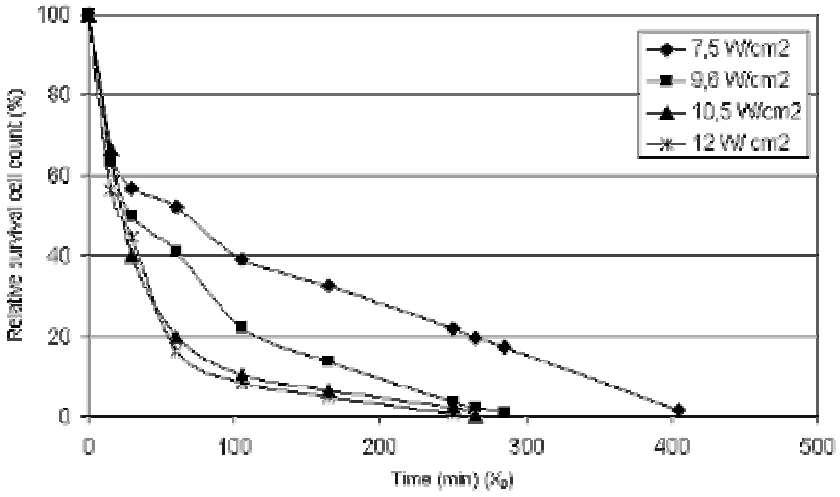

Figure 4. *Relative survival cfu*

Table 2. *Complete model function modelling the ultrasonic treatment of liquid suspensions*

$y=a*\ln x_0+b$ (x_0=time, [min])
$a=-1.9376*\ln x_1+0.4753$ (x_1= Ultrasound power, [W/cm^2])
$b=-1.5277*\ln x_1+26.567$ (X_1= Ultrasound power, [W/cm^2])

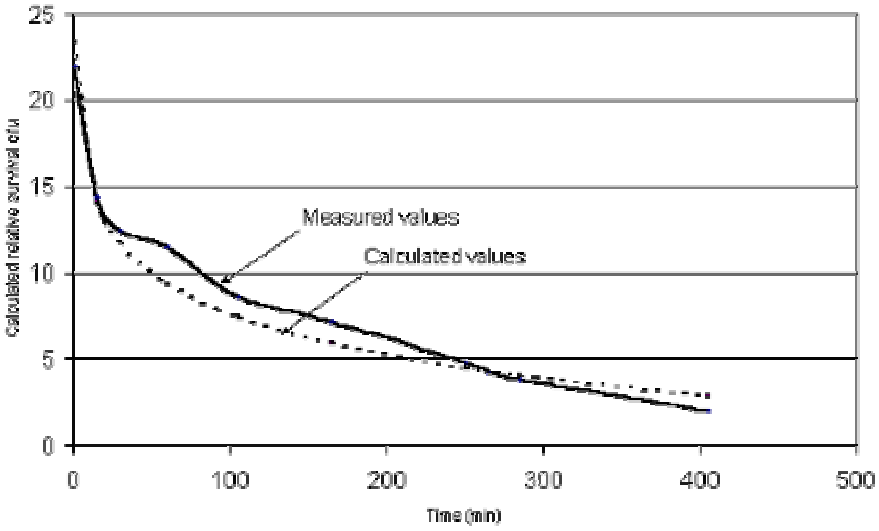

Figure 5. *Model function in relation to the real values*