EXPERIMENTAL STUDY OF THE MICROBIAL GROWTH IN LIQUID CULTURES USING QUALITATIVE CHANGES OF THE LINEAR AND NON-LINEAR ULTRASONIC PROPAGATION PARAMETERS.

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ABSTRACT

Detection of the growth of bacteria colonies is a matter of prime interest in clinical analysis. In this work, a transmission ultrasonic measurement technique for microbiological detection is presented. Linear and non-linear propagation parameters in liquid cultures are affected by the appearance of metabolism products and bacteria colonies. This technique allows a non-invasive, real time control of biological processes avoiding the disadvantages arising from the manipulation of samples (contamination, time waste).

INTRODUCTION

Microbiological infection is still a sanitary world problem; therefore, the improvement of detection in terms of rapidity and microorganism identification is essential in diagnostics. An ultrasonic technique for microbial growth detection is presented in this paper. It is based on the well known fact that the elastic and acoustic propagation parameters of liquids can be accurately measured by using ultrasounds [1].

Some of the authors of this paper presented an innovative work [2] related to the non-invasive ultrasonic detection of bacteria colonies growing in glass recipients having liquid nutritive media. These recipients are commonly used in medical practise for microbiological analysis, being inoculated with blood. The presence of bacteria into the inoculated recipient gives rise to the growing of bacteria colonies which can be detected by different methods: pressure measurement, light dispersion, light absorption, change of electric impedance of the liquid, … In the cited work, the change on the delay of an ultrasonic pulse travelling through the culture liquid was used to detect the presence of bacteria in the liquid.

The ultrasonic techniques have the important advantage of being non-invasive, which prevent the sample from infection during the analysis. The main objective of this work is the improvement of the ultrasonic techniques for microbial detection. Usually, amplitude and velocity measurement are used to characterise materials. Recently, there is an increasing amount of literature devoted to the characterisation of biological materials using nonlinear propagation measurements [3-4]. One of the main nonlinear characterisation techniques is based on the detection of the second harmonic of the propagating signal. A high amplitude ultrasonic tone-burst is distorted as it propagates through a medium. Considering the frequency spectrum of the signal, the distortion of the time-domain signal gives rise to the appearance of the harmonics of the fundamental frequency. In this work, the amplitude and the phase of the fundamental and the second harmonic components of the signal

were obtained from the time-domain signals. All of these parameters change with the growth of micro-organisms in the culture liquid.

A new ultrasonic transducer based on a four channel array structure has been developed to carry out the microbial growth characterisation. This transducer is placed at the bottom of the culture bottles. An ultrasonic pulse is sent from one array element towards the gas-liquid interface inside the bottle; this pulse is detected by another element of the array after being reflected at that surface. The segmented array consist of two 2MHz sector individual transducers together with other two 4Mhz transducers with the same geometry and sector aperture. The total array aperture is focalised to improve the reception signal. This configuration allows an enhanced detection of the second harmonic of an emitting pulse centred at 2MHz. The growth of an *Escherichia coli* culture was ultrasonically detected using this transducer.

EXPERIMENTAL METHOD

Commercial culture bottles of Hemoline (BioMérieux) were used as a nutritive media suitable for microbial growth. These bottles are commonly used in medical practise for microbiological analysis, being inoculated with blood. In the tests presented here, the recipients were aseptically inoculated with an *Escherichia coli* controlled suspension. This microorganism produces an important amount of O_2C which makes it easily detectable by ultrasonic measurements. The microbial concentration at the beginning of the ultrasonic detection test was 1000 UFC/ml.

Montero and Maestre [2] showed the suitability of the measurement of the time of flight of an ultrasonic transmitting burst to detect the growth of *Escherichia coli* inside these recipients. For the measurements, these bottles were place inside a water bath to achieve a stable temperature of 35ºC in the samples. A through transmission technique was used and two transducers (the emitter and the receiver) were placed in the opposite sides of the bottles (Figure 1). This configuration will be referred as “S”. The specific array-type transducer presented in this paper (Figure 2) was developed to make the measurement from the bottom of the bottles. This configuration enlarges the ultrasonic path length and will be referred as “B”. The longer the ultrasonic path, the higher the differences of the ultrasonic parameters measured as the culture liquid is being colonized by the microorganisms.

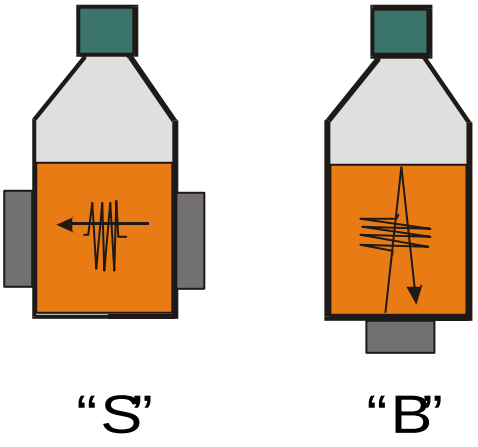

Figure 1. “S” configuration: two transducer placed in the opposite sides of the culture bottle. “B” configuration: One transducer array placed at the bottom of the culture bottle, with the ultrasonic tone-burst being reflected at the liquid-gas interface.

The active part of the developed transducer consists of 4 different PZ27 (Ferroperm) piezoceramic sector elements. Two elements have a resonant frequency of 2MHz and the other two have a resonant frequency of 4 MHz. Each element can be independently used as emitter

or receiver. A backing of Araldite D (Ciba&Geigy) mixed layer were implemented to enhance the efficiency of the transducer. All these elements are embedded into a plastic tube.

Figure 2. Photograph of the 2-4MHz transducer array.

The emitting elements were electrically excited by a MATEC MBS 8000 high power ultrasonic generator that gives 400V P-P tone-burses. The transducer is able to work at one of the following three modes: Emitting at a central frequency of 2MHz and receiving with the same frequency, emitting at a central frequency of 4MHz and receiving with the same frequency and, finally, emitting at a central frequency of 2MHz and receiving with the double frequency (4MHz). This was the operation mode selected for the measurements presented in this paper. It must be noted that although the receiving transducer element works as a band-pass filter centred at 4MHz, an important amount of energy of the emitting signal fundamental component (2 MHz) is detected. Therefore, a multi-parameter characterisation of the changes in the liquid culture can be achieved using this measurement configuration.

EXPERIMENTAL RESULTS

Fundamental Frequency Component (2MHz)

Figure 3 shows a plot of the time of flight delay registered during the growth of *Scherichia coli* using the “S” configuration (transducers placed at two opposite sides of the bottle). There is a decreasing trend on the delay time related to an increase of the propagation velocity in the liquid caused by the microbial growth. It can be seen that the maximum negative slope is reached near the fifth hour, although the decreasing trend begins before. Nevertheless, determining the time when the velocity starts to increase is not easy because of the measurement fluctuations caused by temperature variations in the water bath. The same plots are shown in Figure 4 with the time of flight variation measured with the new developed transducer at the bottom of a test bottle (“B” configuration). The positioning of the transducer respect to the reflecting surface is critical. The changes registered by this second configuration are bigger due to the increase of the ultrasonic path length. Nevertheless, the amplitude of the time of flight fluctuation cannot be explained only by the increasing of this length, which is multiplied by a factor of 2.6. Furthermore, there is a quite different behaviour of the delay time after five and a half hours: when this delay begins to increase in the “S” configuration, the delay slope continues slightly decreasing. The increase of the delay time in the “B” configuration is caused by the precipitation of bacteria colonies at the bottom of the bottle and the appearing of supernatant colonies at the reflecting surface.

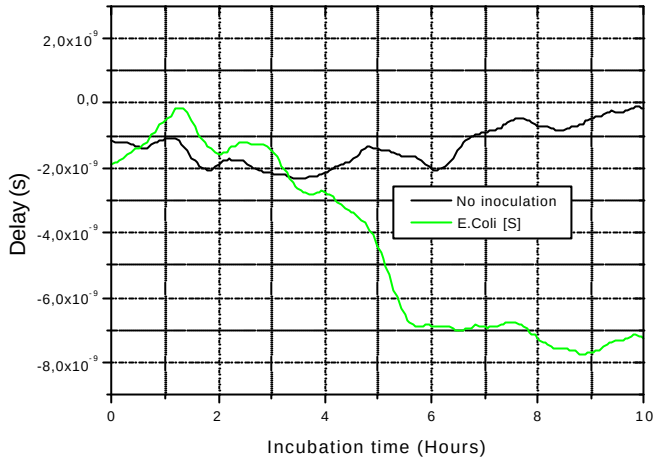

Figure 3. Time of flight delay (s) as a function of the incubation time. The green line represents the delay of the inoculated sample measured with the transducers at opposite sides of the bottle ("S" configuration). The black line is the delay time registered at a reference bottle.

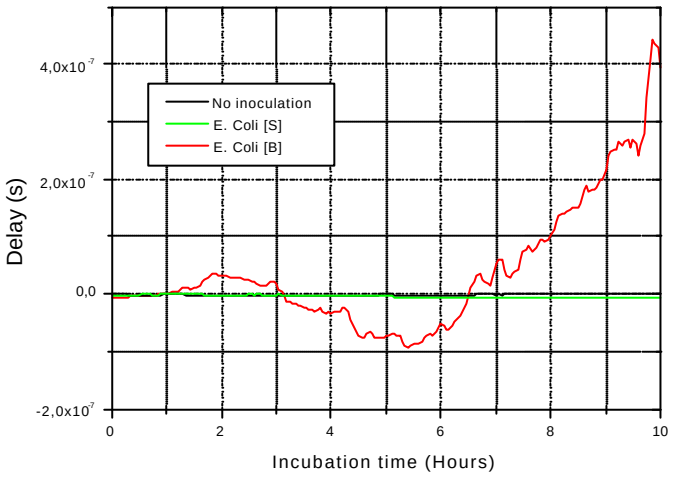

Figure 4. Time of flight delay (s) as a function of the incubation time. The green and red lines represent the delay of the inoculated samples measured with "S" and "B" configurations, respectively. The black line is the delay time registered at a reference bottle.

The amplitude measurement is plotted in Figure 5 and describes the same phenomena. First, there is an increasing of the amplitude related to the microbial growth beginning before the second hour. The amplitude reaches a maximum near the third hour and, after that, a sudden decrease of the amplitude takes place. This can be related to the precipitation or/and the flotation of the bacteria colonies. After this decrease, there is an oscillating behaviour of the amplitude which is consequent with the chaotic characteristic of the reflection and transmission phenomena taking place at the surfaces were depositions and supernatants develop. Finally, a stabilization is reached at the eighth hour.

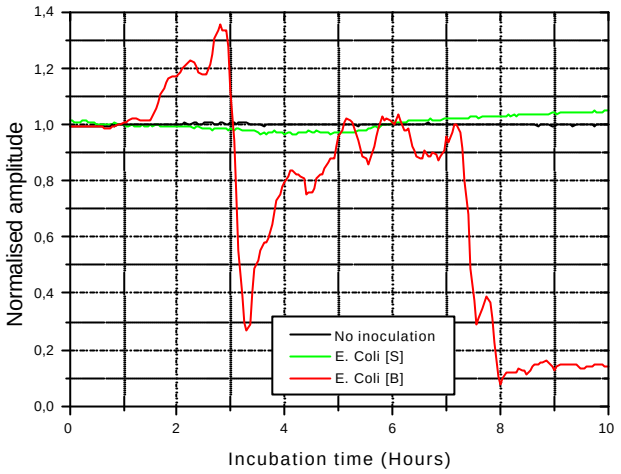

Figure 5. Normalised amplitude as a function of the incubation time. The green and red lines represent the amplitude of the inoculated samples measured with “S” and “B” configurations, respectively. The black line is the amplitude registered at a reference bottle.

Second Harmonic Component (4MHz)

The second harmonic amplitudes are plotted on Figure 6. Comparing this figure to Figure 5 it can be seen that there is not a positive slope at the beginning of the bacterial growth which maybe related to an initial appearance of small microbial depositions that can be seen by this higher frequency but could not be detected by the fundamental. This also explains the fastest decreasing slope which is shifted forward almost half of an hour compared to the fundamental component case, showing the more sensitiveness of the second harmonic.

The difference between the time of flight of the second and the fundamental component of the frequency is plotted in Figure 7 for the first three hours. It can be seen that initial fluctuations of time of flight of fundamental and second harmonic component, which presumably belong to instabilities of the bath, cancel. Therefore, an initial flat behaviour is achieved, which suddenly changes abruptly some minutes before the second hour showing the *Escherichia coli* growth.

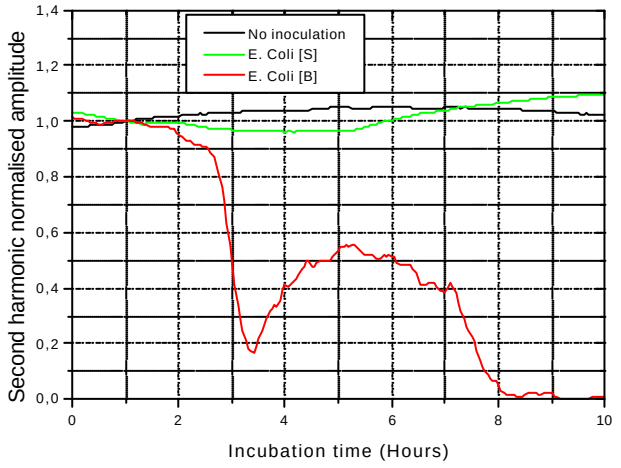

Figure 6. Second harmonic amplitude as a function of the incubation time. The green and red lines represent the second harmonic amplitude of the inoculated samples measured with “S” and “B” configurations, respectively. The black line is the second harmonic amplitude registered at a reference bottle.

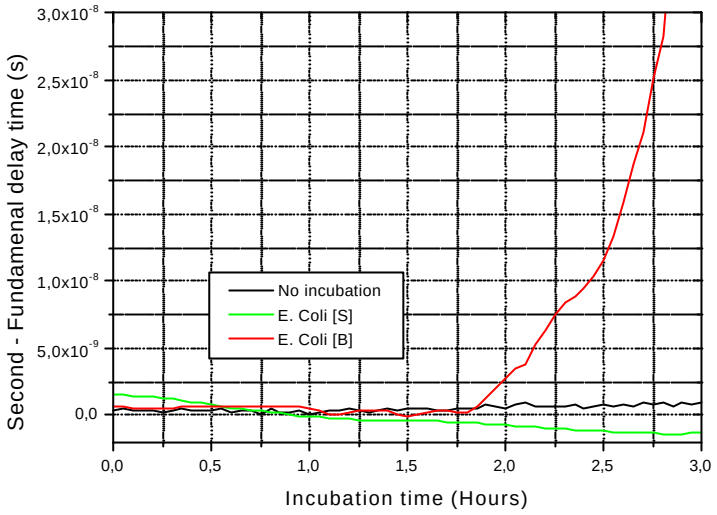

Figure 7. Difference between Second harmonic and fundamental time of flight delay as a function of the incubation time. The green and red lines belong to the inoculated samples measured with "S" and "B" configurations, respectively. The black line is the second harmonic amplitude registered at a reference bottle.

CONCLUSIONS

A new ultrasonic transducer based on an array structure was designed and constructed for non invasive, real time detection of microbial growth in bottles with a culture liquid. This transducer can be used to measure linear and non linear ultrasonic propagation parameters.

The fundamental and second harmonic amplitude and time of flight of an ultrasonic propagating burst was registered, while propagating through a culture bottle inoculated *with Escherichia coli*. Promising detection results were obtained showing the interest of nonlinear parameter measurement to characterise the microbial growth. Future work will be done to analyse the suitability of the method to detect other kind of microorganisms.

ACKNOWLEDGEMENTS

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