Ultrasonic non invasive techniques for microbiological instrumentation

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Abstract

Non invasive techniques based on ultrasounds have advantageous features to study, characterize and monitor microbiological and enzymatic reactions. These processes may change the sound speed, viscosity or particle distribution size of the medium where they take place, which makes possible their analysis using ultrasonic techniques. In this work, two different systems for the analysis of microbiological liquid media based on ultrasounds are presented. In first place, an industrial application based on an ultrasonic monitoring technique for microbiological growth detection in milk is shown. Such a system may improve the quality control strategies in food production factories, being able to decrease the time required to detect possible contaminations in packed products. Secondly, a study about the growing of the *Escherichia coli* DH5α in different conditions is presented. It is shown that the use of ultrasonic non invasive characterization techniques in combination with other conventional measurements like optical density provides complementary information about the metabolism of these bacteria.

Keywords: *Escherichia coli* DH5α; microbiological growing; milk quality control; ultrasonic process monitoring

1. Introduction

A microbiological culture growing in liquid medium induces changes in the medium as a result of the microorganism replication and their metabolism. Different instruments like spectrophotometers, impedancimeters… use these changes to obtain information about the culture evolution. Ultrasounds have been used to monitor processes started by microorganisms like milk lactic [1] or alcoholic fermentation [2], but they can also be used to monitor the microorganism growth [3] as a consequence of the changes in sound speed and attenuation in the
Ultrasonic monitoring systems can be designed as nondestructive and noninvasive devices. This feature makes this technology advantageous over other monitoring solutions.

Two different ultrasonic applications based on microbiological growth monitoring are shown in this work: an industrial NDT system for UHT milk quality control and a study about the growing of the *Escherichia coli* DH5α in different conditions. The devices developed are adapted to the conditions required in each case. Both are based on a pitch-catch measurement configuration, with an emitting and a receiving transducer per channel. Besides the specific ultrasonic equipments (transducers and electronics) both measurement devices have other elements in common: a thermal control system and a measurement control unit.

The thermal control systems are of great importance to avoid interferences caused by temperature changes. These changes induce variations in the ultrasonic propagation speed and attenuation which could be wrongly interpreted as a microbiological process. Thermal systems are constituted by passive components like isolating materials and active components like temperature sensors, actuators, PID electronics and ventilators for homogenizing the temperature.

The measurement control is implemented by means of the software LabView. This way, the computer communicates with the electronic devices, captures the waves and extracts the phase and amplitude information from them. The evolution of these parameters along time provides the information needed to characterize the microbiological processes under study.

2. Ultrasonic milk quality control system

Milk is an ideal substrate for bacterial growth due to the large amount of nutrients it has. The occurrence of bacterial infections in the production of dairy products, which invalidate them for human consumption, is usual. Contaminations while packing or induced by heat resistant bacteria are the main cause of product spoilage in the case of UHT milk. Therefore, rapid microorganism detection gives important economic and environmental benefits resulting from an early stopping of the contaminated production.

A suitable bacterial control might include direct measurements, which involve microbial count. However, these direct measurements entail an important waste of time and money. As a result, many indirect quality tests are commonly used in the industry, for example pH, acidity, electric impedance or ATP presence measurements. Nevertheless, they all are destructive techniques, being necessary opening the pack to take a sample of the product at a given moment.

The metabolic activity associated to the bacterial growth changes the elastic and viscosity characteristics of the milk. This makes possible using ultrasounds as a non-destructive technique for detecting bacterial contamination inside milk packs [3]. In the following, DUMIC, an industrial device to achieve the milk quality control is presented.

2.1. DUMIC system description

The system is constituted by three units: the main incubation chamber, the electronic control board and a small preheating unit (Figure 1).

Inside the incubation chamber, UHT milk packs are placed to reach a constant warm temperature of 36°C, to enhance the growth of many microorganisms which are relevant to milk production. There are temperature control systems inside the chamber to maintain the temperature: a resistance to compensate thermal dissipation and temperature sensors Pt-100 to measure the temperature in the chamber. An axial fan induces air turbulences in the chamber to obtain a uniform temperature in the whole volume. There are 24 housings to test the corresponding milk packages. Each housing is equipped with an engine automatically driven through an Input/Output card to allow charge and discharge of samples. A couple of piezoelectric transducers in a through-transmission configuration, placed face to face in two sides of each housing are used to make the ultrasonic inspection. The transducers are constituted by PZT27 (Ferroperm) piezoceramic discs with a 20mm diameter. They are stuck to a Plexiglas disc coupled to the carton packs through a silicone layer. The resonant frequency of these transducers is 820kHz. The emission transducers are excited with tone-burst waves. After traveling through the milk packs the waves are detected by the reception transducers.
A metallic cupboard is placed closed to the incubating chamber, containing the most of the electronic systems, which are described in the following. First, the PID thermal controller from JUMO, which communicates with the CPU by means of a RS-485 serial port. A PXI module also placed in the cupboard is used for generating, multiplexing and digitalizing waves, opening and closing the pack holders and managing the visual and acoustic signalization. It is constituted of six main units: the NI PXI-5401 function generator, the NI PXI-5124 digitizer, two NI PXI-2503 24 channel multiplexers, the NI PXI-6515 digital input/output card and the NI PXI-PCI-8336 MXI-4 module to communicate the chassis, NI PXI-1042, with the CPU. The signals coming from the receiving transducers are amplified with a 40dB amplifier before being multiplexed and digitalized. All the measurement process is controlled by means of a program implemented using LabView. The waves are analyzed using the FFT algorithm. Variation of time of flight and amplitude are registered during the established measurement period (between 24 and 48 hours). The 24 channels are alternatively driven by means of the multiplexer. After the last channel is measured, another measurement round begins. When all the channels are active two minutes are needed to complete one measurement round. A touch panel is used to command the system, to open and close the pack holders and to give the information relevant to the measurements in course: the time elapsed from the beginning of the analysis, the state of each channel or the temperature of the chamber between others. A subroutine to translate ultrasonic data in terms of the microbiological quality of the sample and to activate the corresponding alarm when a contamination is detected is also included in the handling software. Besides alarms, a light and acoustic signaling column at the top of the cupboard provides information about the device state from some distance. The measuring system is protected against power failure. For this purpose an independent feeding system MGE Evolution 1150 is installed inside the cupboard. It is able to provide 1150 W and autonomy of about 50 minutes to the measuring device.

The small preheating unit shown in figure 1.a is used to increase the temperature of milk packs before putting them into the incubation chamber, avoiding perturbation of the measuring in course when a new sample begins the analysis. This unit has a hot aluminum plate where the packs are placed for preheating.

2.2. DUMIC detection results

Two groups of measurements were made for testing the system performance. In first place more than 50 sterile
samples were analysed to obtain the ultrasonic parameter evolution of non contaminated packs and their variation thresholds.

Table 1. DUMIC detection tests.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Inn./ml</th>
<th>Detection (hours)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. cereus</td>
<td>$10^4$</td>
<td>9-12</td>
<td>5,8</td>
</tr>
<tr>
<td>Micrococcus</td>
<td>$10^4$</td>
<td>21</td>
<td>6,5</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>$10^5$</td>
<td>10</td>
<td>6,1</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>$10^4$</td>
<td>11</td>
<td>6,5</td>
</tr>
<tr>
<td>B. Subtilis</td>
<td>$10^3$</td>
<td>21</td>
<td>6,6</td>
</tr>
<tr>
<td>Salmonella</td>
<td>$10^4$</td>
<td>10-13</td>
<td>6,3</td>
</tr>
<tr>
<td>E. coli</td>
<td>$10^4$</td>
<td>9</td>
<td>5,0</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>$10^4$</td>
<td>17</td>
<td>6,3</td>
</tr>
<tr>
<td>Lactococcus</td>
<td>$10^5$</td>
<td>9-12</td>
<td>4</td>
</tr>
<tr>
<td>S. maltophilia</td>
<td>$10^5$</td>
<td>17</td>
<td>6,6</td>
</tr>
<tr>
<td>R. terrigena</td>
<td>$10^4$</td>
<td>17</td>
<td>6,1</td>
</tr>
</tbody>
</table>

In the conventional quality control procedure, samples are incubated a minimum of 24 hours before being analysed using pH or other technique. Therefore it was important to assess the possibility of detecting the contamination growth before this period. Once the sterile behaviour was established, several packs were inoculated with different microorganisms. These results are shown in table 1. It can be seen that detection can be achieved using the DUMIC system between 9 and 21 hours with the strains and concentration inoculated. Moreover, there are contaminations which would be hardly detected by pH measurement (sterile milk has a 6,7 pH). Each hour, a modern steriliser can produce more than 15000 litres of milk, which may be contaminated. Therefore an alarm turning up 15 hours in advance, would save an important amount of money. Nowadays this is only possible using a non-destructive method and the one described here has proved a good performance.

3. Escherichia coli growing analysis.

There are several procedures to monitor the microorganism growth in the lab. Spectrophotometry is probably one of the main techniques used for that purpose. The optical measurements obtained with the spectrophotometer can be related to the concentration reached by a given microorganism in a culture. HPLC devices are a good complement of spectrophotometry because they provide information about the concentration of different substances in the medium, which can be related to the metabolic activity of the microorganisms. Both are off-line methods which require a culture medium sample for the analysis. An ultrasonic measuring technique can be used to provide real time
information about the evolution of microbiological cultures. This important feature, make this an interesting microbiologic analysis method for the laboratory.

3.1. Experimental set-up

A measurement instrument based on ultrasounds with four measurement channels was specially designed to analyze microbiological processes in the laboratory [4]. Besides the ultrasonic sensing systems and the thermal control, it incorporates magnetic stirrers to enhance the microbial growth and silicone tube connections for taking culture media samples during measurements.

3.2. Escherichia coli growing results

*Escherichia coli* is a bacterium commonly used as a microbiological model in the laboratory. The strain DH5α, which is non-pathogenic was chosen for the experiments shown in this work. It was grown in the media M-63 supplemented with glucose (10mM). Before being inoculated in that medium, the *E. coli* had been grown in L-B medium, which has a high aminoacid concentration.

When a microorganism is placed in a new medium, it has to adapt to these new conditions, changing its metabolism to the different nutrients of the medium and even changing the medium itself by means of enzymes. This stage is called latency and no growth takes place during it. The latency period depends on the microorganisms and the differences between the growing media and other conditions like temperature or amount of oxygen. After latency, the exponential stage begins. During this second stage a rapid increasing of the bacterial number takes place until one or more nutrients begin to be scarce or until pH reaches a critical value. Then, the number of bacterial births and deaths results in a constant concentration of alive bacteria: it is the steady stage.

Two different growing experiments were performed. In first place, a $2 \times 10^{7}$ c.f.u/ml (colony forming unit per ml) concentration of *E. coli* was placed in the M-63 medium. For this concentration the O.D. (Optical Density) is 0.025. To make each O.D. measurement, a 1ml sample has to be taken from the culture bottle. It is analyzed using a 600 nm wavelength with a Beckman DU-250 spectrophotometer. In a second experiment the same inoculus was placed in the medium suplemented with 0.01% v/v casaminoacids. O.D. (solid figures) and sound speed measurements (line with white figures) corresponding to these experiments are shown in figure 2.

![Graph](image-url)

Fig.2 Sound speed (open circles and squares) at 3MHz and optical density (solid circles and squares) of an E. coli culture
Circles belong to the growing run without casaminoacids. No O.D increment was registered during the first five hours. There is a 15 hours period without measurements (which belongs to the night time), and after this the culture is in the exponential stage. The ultrasonic measurement made continuously shows some more information. Sound speed is constant during the first 7-8 hours, after which a slow decreasing begins standing for the beginning of the exponential stage. After the hour 20, this stage is more evident. When casaminoacids were added to the medium, the latency stage is shortened. The O.D. (squares) shows a slow increment during the first hours and after night time they reached a constant, that is the steady stage. The ultrasonic sound speed measurements also show a rapid decreasing from hour 4, reaching stability after hour 20. This sound speed decreasing is related to the glucose consumption. From these results is evident that sound speed can be used to monitor bacterial growth in a similar way than O.D. Both parameters are complementary: while O.D. is directly related to the bacterial concentration, sound speed depends on the medium components, which is related to the metabolic activity through the nutrient decreasing and the appearing of some metabolism products.

4. Conclusions

Non invasive techniques based in ultrasounds have advantageous features to analyze microbiological growth processes. Two ultrasonic systems were designed and constructed for microbiological monitoring applications. The first of them, DUMIC, is a measuring system adapted to an industrial environment. It was made to perform the quality control of UHT milk. The results show that this device is able to provide an early detection of a contamination growing in a closed milk pack. The second is a laboratory instrument which was applied to the study of E. coli growth. The continuous measurement is easily automated and provides information about the metabolic processes taken place during the different microbiological growing stages.

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Reference