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Concentration measurement of yeast suspensions using high frequency ultrasound backscattering

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ABSTRACT

This work proposes the use of an ultrasound based technique to measure the concentration of yeasts in liquid suspension. This measurement was achieved by the detection and quantification of ultrasonic echoes backscattered by the cells. More specifically, the technique was applied to the detection and quantification of *Saccharomyces cerevisiae*. A theoretical approach was proposed to get the average density and sound speed of the yeasts, which were found to be 1116 kg/m^3 and 1679 m/s, respectively. These parameters were needed to model the waves backscattered by each single cell. A pulse-echo arrangement working around 50 MHz, being able to detect echoes from single yeasts was used to characterize experimentally yeast solutions from 10^2 to 10^7 cells/ml. The Non-negative Matrix Factorization denoising technique was applied for data analysis. This technique required a previous learning of the spectral patterns of the echoes reflected from yeasts in solution and the base noise from the liquid medium. Comparison between pulse correlation (without denoising) and theoretical and experimental pattern learning was made to select the best signal processing. A linear relation between ultrasound output and concentration was obtained with correlation coefficient $R^2 = 0.996$ for the experimental learning. Concentrations from 10^4 to 10^7 cells/ml were detected above the base noise. These results show the viability of using the ultrasound backscattering technique to detect yeasts and measure their concentration in liquid cultures, improving the sensitivity obtained using spectrophotometric methods by one order of magnitude.

1. Introduction

The assessment of cell concentration constitutes a fundamental measurement for the analysis of microbiological systems. Cell population provides information about the effect of internal and external conditions on the cell biology. For this, instruments developed to measure cell concentration have become essential tools for biological and clinical research laboratories. This interest has been extended to the industry, due to the increasing number of processes driven by microorganisms like bacteria and yeasts. In such processes, the number of cells per unit volume has a direct impact on the production efficiency and quality.

Many techniques were developed for the measurement of microorganism concentration (plate counting, spectrophotometry, flow cytometry, electrical resistance...). Among them, those methods achieving a real-time measurement and requiring minimal sample handling have an especial relevance. Innocuous and non-invasive ultrasound has long been applied for the characterization of biological tissues and presents as a suitable tool for the measurement of cell concentration. Nevertheless, up to date, there is no ultrasound based instrumentation designed and constructed for providing cell counting in laboratories or biotechnological plants.

The objective of this work was, in first place, to propose an ultrasound measuring technique to evaluate the concentration of microorganisms in liquid suspensions. In second place, the sensitivity of the technique was evaluated at low cell concentrations (below 10^5 cells/ml). Sensitivity improvement beyond this range is attractive because this is the limit of the standard use of spectrophotometry in microbiology. Monitoring the positive or negative growth evolution of cell cultures at the earlier stages may provide information about the cell biology which can be used to improve the bioprocess control from the beginning of reactions in an industrial setting. These early growing stages are the most poorly understood phases of microorganism growth.

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Different ultrasonic methods to detect microorganisms growing in liquid media were reported. Piezoelectric sensors based on quartz crystal microbalance (QCM) devices were able to detect microbial biofilms settling over and attached to resonant piezoelectric discs. Bandwidth and resonance frequency changes were induced by the biomass added to the sensor. In addition, these devices were sensitive to viscoelastic changes in the media induced by the microorganism growth. It was also proved that culture growth-related changes could be detected by measuring changes of sound speed and attenuation of elastic waves [1]. This makes detection of microbial presence possible by means of non-invasive transmission or pulse-echo ultrasound configurations. In this case, detection was not directly due to microorganisms but to the changes they produced in the liquid medium. Consequently, these ultrasound techniques provided no direct quantitative relation between the cell concentration and the parameters measured.

An increasing use of ultrasound high frequencies (above 15 MHz) for clinical applications, with wavelengths under 0.1 mm, opened the possibility of obtaining information at microscopic ranges (1–100 μm). With such techniques, echoes from single cells could be detected and studies of animal cells in liquid suspension at low concentrations (10^3–10^6 cells/ml), with typical sizes in the range 10–30 μm appeared [2–4]. Extending these technologies to cell quantification in microbiology constitutes a challenge because of the low intensity waves backscattered by microorganisms, with typical sizes even smaller (1–10 μm). As an example, at a frequency of 50 MHz, the energy of the acoustic wave backscattered by a 7 μm diameter cell would be approximately 15 times less than it would be for the same kind of cell but having double diameter.

In this work, the yeast *Saccharomyces cerevisiae* was chosen as the detection target for being a microorganism with a high industrial and scientific impact. This yeast is involved in the production of alcoholic beverages, bakery and bioethanol, between others. Real time monitoring of microbial dynamics during fermentation by real-time yeast quantification in fuel and food processors constitutes a critical issue to improve the production yield and quality. Along such processes, yeasts take carbohydrates such as glucose, fructose and sucrose to obtain energy for their living functions and transform them into ethanol and carbon dioxide. Therefore, there is a direct relation between production and yeast concentration. In addition, *S. cerevisiae* constitutes a reference microorganism for microbiologists and the concentration measurement of yeast colonies can be related to their response to culture factors such as nutrients, toxic agents or, environmental physical conditions.

Among the different methods used for yeast quantification, optic cytometry has shown to reach a detection level of 10^3 cells/ml with the help of dyes [5]. However, techniques as spectrophotometry, requiring minimal sample handling, with no need of chemical reactions nor dying, are commonly preferred for measuring yeast concentration [6–7]. This technique provides measurements in real-time and is able to detect yeast concentrations down to the order of 10^5 cells/ml. For concentrations lower than this, light absorption due to cells is hardly detected in conventional spectrophotometers. In such scenario, cell concentration assessment based on ultrasound scattering measurement appears as an attractive method for different reasons: first, this technique does not need any sample preparation (dyes or even dilution) either and thus, an automatic sample extraction can be implemented either in reaction tanks or growing culture recipients, to pump a sample directly into an ultrasound based measuring device as the one proposed in this work. Moreover, the technique could be implemented by placing the ultrasound transducers attached to production tanks and pipes, in direct contact to the liquid or through a layer of a solid material (which could be the same tank material, to make maintenance easier), and with no limitation in relation to medium opacity.

Non-invasive direct detection of microorganisms requires using high frequency ultrasound (>15 MHz), as it was done with animal cells. It is shown in this paper that acoustic scattering from *S. cerevisiae* yeasts can be detected using ultrasound waves around 50 MHz. Three main factors limit the sensitivity of detection using ultrasound scattering: the small target size (7 μm diameter), the low mechanical impedance mismatch between the target and the surrounding fluid (0.01 intensity reflection coefficient) and the sound attenuation of the media for high ultrasound frequencies (close to 5 dB/cm for an aqueous medium at 50 MHz). In addition, for yeast concentrations below 10^6 cells/ml, the probability of "illuminating" or not a single target with the ultrasound beam must be also considered.

To obtain the theoretical ultrasound waves backscattered by *S. cerevisiae* in solution, cells were acoustically modeled as fluid-filled particles surrounded in a liquid medium. The ultrasound scattering theory proposed by Anderson [8] and reviewed by Feuillade et al. [9], describes the waves scattered by such particles based on the knowledge of their size, density and sound speed. The values of these parameters were derived from a physical model of cells proposed in Section 2. This model represents a cell as a mixture of the four main types of biomolecules: proteins, lipids, nucleic acids and carbohydrates, with an inorganic solvent (water and salts). A similar approach was used previously to obtain the average physical properties of foods [10]. Averaged values of density and sound speed of these biomolecules were obtained from the literature. From the model developed average density of 1116 kg/m^3 and sound speed of 1679 m/s, was obtained for *S. cerevisiae*. These values were put into the theoretical model to calculate the pressure spectrum backscattered by yeasts, which was compared to experimental results in Section 4.

A pulse-echo arrangement working around 50 MHz was used to experimentally characterize yeast solutions at different concentrations. This experimental set-up requires only a 500 μl sample volume for the analysis. Data obtained in these experiments were discussed with the help of the aforementioned backscattering theory. The combined influence of sound attenuation and backscattering amplitude at relevant frequencies (1–100 MHz) was also reviewed.

Given the low signal to noise ratio (SNR) presented by ultrasound echoes coming from yeast cells, from 15 dB to negative values, a denoising technique was proposed to analyze the experimental data. Among existing techniques, Non-negative Matrix Factorization (NMF) [11] was applied in this problem because NMF is able to separate a target source (in this work, echoes from yeasts) from non-target source (in this work, the base noise obtained when the buffer medium, with no yeasts, is analyzed) when the spectral patterns to separate can be classified into narrowband and broadband spectra. Specifically, echoes from yeasts could be classified as narrowband spectra while the base noise from the buffer medium could be classified as broadband spectra. NMF has also been successfully applied to separate sources in the field of image [12] and audio [13] processing. This method allowed detection of echoes even in very low (<0 dB) SNR scenarios. For this, it required a previous learning of the spectral patterns of the echoes reflected from yeasts in solution and the base noise from the liquid medium.

The results obtained for different yeast concentrations showed that yeast detection and quantification could be achieved using ultrasound for concentrations down to 10^4 cells/ml. The performance of pulse correlation (no denoising) and the theoretical and experimental pattern learning was compared. Results presented a linear relationship between the ultrasound measurement and yeast concentration with experimental learning, yielding correlation