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Synergistic effect of ultrasonic waves under pressure at mild temperatures (MTS) in yeast inactivation

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ABSTRACT

Ultrasonic treatments are one of the new technologies for microbial inactivation that could serve as an alternative for food preservation. However, decimal reduction times for most of microbial species generally exceed 1 min. Therefore, combined processes have been designed, based on the simultaneous application of ultrasonic waves under pressure at moderate temperatures (mano-thermo-sonication process, MTS). The aim of this study was mathematically quantify the synergism of MTS treatments on *S. bayanus* and different microbial groups including vegetative cells and bacterial spores and compare them. Results show that the lethal effect of MTS treatments may have both, additive (*A. hydrophila*, *Y. enterocolitica*) or synergistic effect (*S. bayanus*, *L. monocytogenes*, *Salmonella* spp., *Bacillus* spp.). The synergistic effect increases with temperature until reaching a maximum and then descending. A big synergistic effect was observed in yeasts and bacterial spores while lower synergy was observed in bacterial vegetative cells. The more heat resistant bacterial species showed higher synergistic effect of the MTS treatments.

1. Introduction

In the last years non-thermal technologies have received a great deal of attention as alternatives to heat processing of foods, which occasionally may produce negative effects such as changes in the nutritional and sensorial properties of food (Aguilar et al., 2017). Ultrasound (US) is one of the new technologies that have been suggested as an alternative to current heat treatments for microbial inactivation (US FDA, 2000).

Ultrasound is defined as a sonic wave at frequencies over the threshold human hearing. Ultrasonic waves are generally classified by their frequency and their wavelength. Waves with frequencies between 20 and 40 kHz are considered as high-energy or high-power ultrasound, whereas those whose frequency ranges between 40 kHz and 1 MHz are known as low-power ultrasound (Mason et al., 1996). Ultrasonic power is defined as the energy transmitted by the wave per second (W), and ultrasonic intensity as the power per surface unit (W/cm^2) (Meullemiestre et al., 2017). When high-power ultrasound is propagated through a liquid media, it creates alternating compression and expansion cycles. These cycles produces the phenomenon of cavitation which is considered the main responsible of the effects produced by ultrasound. As consequence of cavitation, molecules violently collide with each another, giving rise to shock waves and creating spots of very high temperature and pressure (Mason et al., 1996). These extreme conditions usually also induce water sonolysis, resulting in the formation of highly reactive radicals. Today most authors (Condón et al., 2011; Lee et al., 2009; Wu et al., 2015) agree that the bactericidal effect of high power ultrasound is a consequence of cells envelope breakdown due to the shock waves. However, most published data indicate that the bactericidal efficacy of ultrasound is low (Jambrak et al., 2017; Lee et al., 2013; Meullemiestre et al., 2017). Therefore, most researchers have tried to improve the efficacy of the process by designing combined processes to enhance the overall lethal effect (Lee et al., 2013; Lopez-Malo et al., 2005).

The combined US processes proposed to date have been classified (Chemat et al., 2011; Piyasena et al., 2003) as: thermosonication (TS, combination of ultrasound and heat), manosonication (MS, combination of ultrasound and pressure), and manothermosonication (MTS, combination of ultrasound, pressure and heat). The increment of the lethal effect of ultrasound by raising the temperature of the treatment media (TS) has been observed on bacterial vegetative cells (Lee et al., 2009), yeast (Abid et al., 2014; Bermudes-Aguirre and Barbosa-Canovas, 2012), and on bacterial spores (Milly et al., 2007). However, in some cases the lethal effect of TS increases, when increasing

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temperature, until reaching a maximum beyond which the lethality decreases. This behavior has been associated with an increase of the vapor pressure of the medium (Sala et al., 1995), which should facilitate cavitation, but also reduce the intensity of implosion.

In case of MS an increase in hydrostatic pressure makes the formation and growth of bubbles more difficult and with a higher energetic consumption, but releases more energy when it implodes, increasing the lethal effect of ultrasound (Lee et al., 2009, 2013). However, some authors have observed increases on the bactericidal effectiveness of US associate with pressure until reaching a threshold, above which the effect of pressure progressively decreases until it vanishes (Guzel et al., 2014; Pagán et al., 1999b). Manothermosonication (MTS) process was designed in 1992 (Spanish Patent No. 9200686) with the aim of overcoming the limitations of TS and MS, while making use of their advantages. The authors hypothesized that pressure would increase cavitation intensity and compensate changes in the vapor pressure of the medium as a result of heating, thereby permitting an advantageous use of thermosonication even at high temperatures (Sala et al., 1995). Published data over the last 20 years confirm the validity of this hypothesis.

Although MTS is mentioned in most reviews of microbial ultrasonic inactivation (Huang et al., 2017; Piyasena et al., 2003), the number of published investigations is limited, and some important questions remain unclear. One of the most controversial aspects of the MTS process is whether the lethal efficacy achieved by combining ultrasonic waves under pressure with heat is the result of an additive (Pagán et al., 1999a; Raso et al., 1998a) or synergistic effect (Guzel et al., 2014; Lee et al., 2009). Probably the most significant problems are 1) the magnitude of the synergistic effect changes with temperature and 2) the magnitude of the effect has not been mathematically measured. This study is the first approach to clarify these questions using quantifiable parameters.

The overview of the literature showed that a relatively high number of studies had determined the efficacy of MTS process for the inactivation of vegetative cells, a few for the inactivation of bacterial spores, but none for the MTS inactivation of yeast. However, yeasts are often the limiting factor in the shelf life of derivatives such as juices and wine (Deák, 2008; Fleet, 2007), products in which MTS technology could be easily implemented. *Saccharomyces bayanus* has been isolated from fruit juices (Stratford et al., 2000), and is also considered as a contaminant of wines (Puértolas et al., 2009). So, the objectives of this investigation were: (1) To study the MTS inactivation of *Saccharomyces bayanus*, (2) to evaluate the magnitude of the synergism using the mathematical procedure described by Condón-Abanto et al. (2016), and (3) to compare the magnitude of the synergistic effect of MTS for different microbial groups analyzing previous publish data. Finally, based on the results the factors influencing the magnitude of the synergism of MTS process were analyzed.

2. Material and methods

2.1. Yeast culture and media

The strain *Saccharomyces bayanus* STCC 1969 was obtained from the Spanish Type Culture Collection (STCC), and the bacterial cultures were maintained frozen at −80 °C in cryovials. Broth subcultures were prepared by inoculating 10 mL of Sabouraud Broth (SB, Biolife, Milan, Italy) with a loopful of growth on Potato Dextrose agar (PDA, Biolife). The subcultures were incubated overnight at 25 °C in aerobic conditions (Selecta, mod Incudigit, Barcelona, Spain). With these subcultures, 250-mL Erlenmeyer flasks containing 50 mL of SB were inoculated to an initial concentration of 10^3 CFU/mL and incubated in a shaking incubator (130 rpm; Selecta, mod. Rotabit) for 48 h, which resulted in stationary-phase cultures containing approximately 1-5 × 10^9 CFU/mL.

2.2. TT, MS and MTS treatments

Thermal treatments (*TT*) were carried out in a specially designed resistometer (Condón et al., 1993). Briefly, this consists in a 400 mL vessel equipped with an electrical heater for thermostation, an agitation device, and ports for injecting the microbial suspension and for sample extraction. Once the preset temperature had attained stability (T ± 0.05 °C), 0.2 mL of an adequately diluted microbial suspension was inoculated into the thermoresistometer containing 350 mL of citrate-phosphate buffer of pH 7.0 (Dawson et al., 1974). After inoculation, 0.2 mL samples were collected at different heating times and immediately pour-plated. Survival curves to heat were obtained at different temperatures ranging from 49 to 55 °C.

MS/MTS treatments were carried out in a resistometer previously described (Raso et al., 1998a) and modified, to simplify its use by Condón-Abanto et al. (2016). In this investigation a 2000 W Branson Sonifier® ultrasonic generator (Branson Ultrasonics Corporation, Danbury, Connecticut, USA) with a constant frequency of 20 kHz was used. Survival curves to ultrasound treatments were obtained at different temperatures ranging from 20 to 55 °C, at a constant input power of 3.2 W mL^{-1}, using an overpressure of 100 kPa. Temperature control during the experiments was achieved by dissipating excess heat developed during sonication by circulating cool water through the main vessel. The temperature of the treatment medium was continuously monitored by a thermocouple (type K, NiCr-Ni sensor class 1, ref. FTA05L0100, ALMEMO®, Ahlborn, Germany). The device was switched on and when treatment conditions were attained, 0.2 mL of an appropriate dilution of the cell suspension was injected into the 23 mL MTS treatment chamber containing citrate-phosphate buffer of pH 7.0. After injection, samples of 0.1 mL were collected at preselected times and immediately pour-plated.

2.3. Incubation and enumeration of survivors

PDA (Biolife) was used as recovery medium. Pour plates were incubated at 25 °C for 48 h. Previous experiments demonstrated that longer incubation times did not modify the profile of survival curves. After incubation, colony-forming units (CFU) were enumerated with an Image Analyzer Automatic Colony Counter (Protos, Synoptics, Cambridge, UK) as described elsewhere (Condón et al., 1996).

2.4. Mathematical models and statistical analysis

Survival curves to heat/MS/MTS treatments were obtained by plotting the logarithm of the survival fraction (Log_{10} N/N_0) versus treatment times. Two different profiles in the survival curves were observed. While some displayed a log-linear profile, others showed deviations from linearity in the form of shoulders. To fit survival curves and calculate resistance parameters, the GInaFiT model-fitting tool (KU Leuven, Leuven, Belgium) was used. Survival curves showing shoulders were fitted with the log-linear regression plus shoulder model of Geeraerd et al. (2000) (Eq. (1)). The model describes the survival curves via two parameters: shoulder length (*Sl*), defined as the time before the exponential inactivation begins, and the inactivation rate (K_{max}), which corresponds to the slope of the exponential portion of the survival curves. Therefore, the traditional decimal reduction time value (*D*) can be calculated from the K_{max} parameter with the equation: $D = 2.303 / K_{max}$. The GInaFiT software also provides the 4*D* parameter, defined as the treatment time required to inactivate the 99.99% of the microbial population.

$$N_t = N_0 e^{-K_{max} \times Sl} \left(\frac{e^{K_{max} \times Sl}}{1 + (e^{-K_{max} \times Sl} - 1) e^{K_{max} \times t}} \right) \quad (1)$$

Survival curves displaying a log-linear profile were fitted to the Bigelow and Esty model (Eq. (2)) using the same software, thereby