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Ultrasound-assisted supercritical CO_2 treatment in continuous regime: application in *S. cerevisiae* inactivation

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Abstract

Laboratory continuous regime equipment was designed and built for supercritical CO_2 microbial inactivation assisted by high power ultrasound (SC-CO_2-HPU). Apple juice, previously inoculated with 1-10x10^7 CFU/ml of *Saccharomyces cerevisiae*, was treated in the equipment at different juice residence times (3.06-9.2 min), temperatures (31-41 °C) and pressures (100-300 bars). Inactivation ratios were fitted to a hybrid (boolean-real) model in order to study the effect of the process variables. The maximum inactivation achieved by the system was 7.8 log-cycles. The hybrid model demonstrated that HPU has a significant effect on inactivation after shorter residence times. A multi-objective optimization performed with the hybrid model showed that 6.8 log cycles of inactivation could be obtained after a minimum residence time (3.1 min) with HPU application, whereas under the same conditions but without HPU, the inactivation would be 4.3 log-cycles. Therefore, the ultrasound assisted continuous system has shown a great potential for microbial inactivation using SC-CO_2 under mild process conditions.

Keywords: Non-thermal process; supercritical CO_2; ultrasound; continuous regime; *S. cerevisiae* inactivation.

1. Introduction

Non-thermal food preservation techniques, such as pulsed light (Ramos-Villarroel et al., 2012, Maftei et al., 2014), ozone (Patil et al., 2010; Torlak, 2014), high hydrostatic pressure (Buzrul, 2014; Baptista et al., 2015), pulsed electric fields (Boulaabaa et al., 2014; Raso et al., 2014), ultrasound (Gabriel, 2014; Khandpur and Gogate, 2016) or ultraviolet radiation (Baysal et al., 2013; Gabriel et al., 2015) have been developed in response to an increasing consumer demand for natural, fresh food which is free from chemical preservatives. These non-thermal technologies have demonstrated their capacity to preserve nutrients and functionality in food, extending its shelf-life and minimizing the changes in natural color, taste, flavor and texture. One of these technologies, supercritical carbon dioxide (SC-CO_2) processing, has been applied in the inactivation of enzymes and both pathogen and spoilage microorganisms (Choi et al., 2008). SC-CO_2 treatment involves food contact with SC-CO_2 for a certain period of time in a batch, semi-batch or continuous equipment.

SC-CO_2 treatments have been applied to inactivate gram-negative bacteria, such as *Salmonella enterica* serovar Typhimurium, *Escherichia coli* or *Yersinia enterocolitica*, and gram-positive bacteria or yeast, such as *Listeria innocua*, *Listeria monocytogenes* or *Saccharomyces cerevisiae* (Bermúdez- Aguirre and Corradini, 2012; Garcia-González et al., 2007). The studies dealing with inactivation techniques including SC-CO_2, have demonstrated that gram-positive cells are more resistant than gram-negative ones owing to the fact that their cell wall is thicker (Villas-Boas et al., 2006). Ortuño et al. (2012b and 2013) showed that when using SC-CO_2 under 225 bar and 36 °C, 50 min were necessary to reach a reduction of 7 log-cycles of *E. coli*, compared to the 150 min needed to reach a 3 log-cycle reduction for *S. cerevisiae*, under the same process conditions. These results support the connection between the wall thickness and the resistance to SC-CO_2 inactivation. In addition to the wall thickness, the cell wall composition and the expression of stress-response genes, such as the heat shock

proteins, are factors that determine the resistance of microorganisms to the process conditions (Ortuño el at., 2012a).

Most of the studies found in the literature use batch SC-CO_2 systems to inactivate microorganisms in liquid media. In order to obtain the required lethality after shorter processing times or when using a lower treatment intensity and to accelerate the CO_2 inactivation mechanisms in batch systems, previous studies analyzed the advantages of coupling SC-CO_2 with high power ultrasound (SC-CO_2-HPU) for microbial inactivation purposes (Ortuño et al., 2012b; Ortuño et al., 2013; Spilimbergo et al., 2014). Ortuño et al. (2012b) showed that the SC-CO_2-HPU treatment drastically reduced the time required for *E. coli* inactivation in LB broth with respect to SC-CO_2 processing; at 225 bar and 36 °C, an inactivation of 7 log-cycles was obtained in 2 min, instead of the 50 min required using only SC-CO_2. In the case of *S. cerevisiae* inoculated in YPD broth, Ortuño et al. (2013) showed that 7 log-cycles of inactivation were achieved after 2 minutes of SC-CO_2-HPU at 225 bar and 36 °C, while no inactivation was reached using only SC-CO_2. Therefore, with this system (batch SC-CO_2-HPU), an increase in the solubilization rate of SC-CO_2 in the liquid is produced, as well as an enhancement in the mass transfer of the SC-CO_2 into the microbial cells, due to the vigorous stirring produced by the ultrasonic field. Also cavitation can damage the microbial cell walls causing the loss of intracellular vital components.

In order to improve the efficiency of batch SC-CO_2 treatments, continuous systems have been developed. Several authors have studied the effect of continuous SC-CO_2 systems on the inactivation of different microorganisms (*E. coli, Lactobacillus plantarum, L. monocytogenes,* aerobic plate count, *S. enterica* serovar Typhimurium and *S. cerevisiae*), using different mediums (orange juice, carrot juice, watermelon juice, coconut water, beer) (Kincal et al., 2005; Gunes et al., 2005; Dagan et al., 2006; Damar et al., 2009; Fabroni et al., 2010; Zhenghui et al., 2011). These authors concluded that continuous systems require much shorter inactivation times compared with batch ones, due to the improvement in the CO_2 mass transfer produced by the

agitation, which permits both a quick saturation of CO_2 into the medium and the acceleration of the inactivation mechanisms. However, no work has been found in the literature combining a continuous SC-CO_2 system with the use of HPU.

Therefore, considering the effect of SC-CO_2-HPU on the microbial inactivation and the productivity increase of the continuous regime processes, continuous regime SC-CO_2–HPU laboratory equipment was designed and built. The aim of this paper was to study the effect of pressure, temperature and product residence time on yeast inactivation using the continuous flow SC-CO_2-HPU system constructed for this application and to model and optimize the process operation.

2. Materials and methods

2.1. Microbial preparation

The microbial strain used in this study was *Saccharomyces cerevisiae* T73 (*S. cerevisiae*). It is a natural strain isolated from wine fermentation in Alicante (Spain) (Querol, Barrio, & Ramon, 1992), and it is commercialized as Lalvin T73 (Lallemand Inc., Montreal, Canada).

2.2. Sample preparation and growth conditions

A single colony of *Saccharomyces cerevisiae T73 was* inoculated in Yeast Peptone Dextrose Broth (YPD Broth, Sigma-Aldrich, USA) and grown overnight at 30 °C, using an incubation chamber (J.P. SELECTA, Model 3000957, Barcelona, Spain) and an orbital shaker at 120 rpm (J.P. SELECTA, Rotabit Model 3000974, Barcelona, Spain). For each experiment, a subculture was prepared by inoculating 100 µL from the starter in 100 mL of sterilized medium and incubated at 30 °C for 24 h to obtain cells in the stationary phase. Growth curves were determined in advance by both plating and the measurement of absorbance at 625 nm (data not shown). The culture was inoculated

140 in 1 liter of pasteurized commercial apple juice (Apple juice, Hacendado, Spain), to a
141 cell concentration of 1-10x10^7 CFU/mL and then the juice was immediately subjected to
142 the treatment.
143
144 *2.3. Supercritical fluid processing*
145 A continuous SC-CO_2+HPU equipment was designed as a continuous stirred tank
146 reactor (CSTR) in which the HPU probe was submerged in the liquid phase (product),
147 followed by a holding tube designed to increase the contact time between the product
148 and the SC-CO_2, analog to a plug flow reactor (PFR). The plant also included a pump
149 for the CO_2 and another for the juice, a separation vessel and different auxiliary
150 elements depicted in Fig. 1.
151 The volume of the liquid phase during the experiments in the sonication vessel was
152 40 mL. The holding tube, analog to a PFR, had a volume of 52 mL. The HPU system
153 (9-12, Fig. 1) has been patented (Benedito et al., 2011) in conjunction with the
154 inactivation procedure and consists of a high power piezoelectric transducer, an
155 insulation system and a power generator unit. The transducer (>1 W/cm^2) was inserted
156 inside the inactivation vessel and included two commercial ring-shaped ceramics (11,
157 Fig. 1; 35 mm external diameter; 12.5 mm internal diameter; 5 mm thickness;
158 resonance frequency of 30 kHz) and a sonotrode (9, Fig. 1), which was specially built
159 to concentrate the highest amount of acoustic energy on the application point. The
160 sonotrode was powered with constant energy by the power generator unit (12, Fig. 1)
161 during the SC-CO_2 process.
162 The SC-CO_2-HPU process applied to the juice was as follows: first, liquid carbon
163 dioxide was supplied from the bottom of the chiller reservoir (which stores it at −18 °C)
164 to the pump where it was compressed at the targeted pressure. For start-up, the
165 equipment was stabilized at the treatment pressure (P) and temperature (T) only with
166 SC-CO_2 at a constant flow rate of 5 mL/min. Thereafter, the ultrasound equipment was
167 connected, and once the process conditions (P, T) were fulfilled, the sample to be

168 treated was pumped to the mixing point (7, Fig.1) where it mixed with the SC-CO_2. The
169 mixture went into the sonication vessel (8, Fig. 1), where the HPU was applied. For the
170 experiments with HPU, the power applied during the whole experiment was 40 W±5W
171 (I=250 ±10mA; U=220 ±5 V, measured with a Digital Power Meter, Yokogawa, Model
172 WT210) and the frequency 30.7±1.8 kHz. Pressure and temperature were kept
173 constant during the experiment. The mixture of juice/SC-CO_2 exiting the treatment
174 vessel went into the holding tube (14, Fig. 1) and, finally, into the separation vessel (15,
175 Fig. 1). Prior to each experiment, the different sections of the equipment which the
176 product flows through were cleaned and sanitized with disinfectant solution (Delladet
177 VS2, Diversey, Spain), and distilled and autoclaved water. For each process condition,
178 3 treated juice samples (3 mL) were extracted in sterile plastic test tubes at different
179 times (4 min time interval) through the sonication vessel output (18, Fig. 1) and another
180 3 samples through the separation vessel output (19, Fig. 1). The first sample was taken
181 after 125 mL of juice was treated, to ensure the steady state was reached. The
182 microbial analyses were performed on the three samples and averaged for each
183 process condition. Sampling output tubes were cleaned and disinfected with 3 mL
184 ethanol (96%v/v) after every sample extraction.
185
186 *2.4. Enumeration of viable microorganisms*
187 The viability of *S. cerevisiae* in the samples was determined by the plate count
188 method before and after every treatment. Samples were serially diluted and 100 µL of
189 the appropriate dilutions were plated on Yeast Peptone Dextrose Agar (YPD Agar,
190 Sigma-Aldrich, USA) in triplicate. The plates were incubated at 30 °C for 24 h before
191 counting. The experimental results shown are the arithmetic mean and the standard
192 deviation of –ln (N/N_0) for at least three plates, where N_0 is the initial number of cells in
193 the control sample and N is the number of cells in the sample after the different
194 treatment times.
195

196 *2.5. Experimental design*

197 Four process variables were considered: pressure (P), temperature (T), juice flow (q) and type of treatment (with or without HPU); keeping the SC-CO_2 flow (q_{CO_2}) constant at 5 mL/min. Initially, the effect of juice flow and pressure was studied in a 3x3 complete factorial design at constant temperature. The product flow levels were 5, 15 and 25 mL/min and the pressures were 100, 200 and 300 bar; every treatment was carried out at a temperature of 31 °C. In order to limit the SC-CO_2 consumption, the ratio between the SC-CO_2 and juice flows was limited to 1.The effect of temperature was studied from a 3x2x2 complete factorial design. The temperatures were 31, 36, and 41 ˚C, the pressures 100 and 200 bar, and there were 15 and 25 mL/min of product flow. All of the treatments were carried out with and without ultrasound and were run in triplicate. Taking into account that the liquid phase volume in the sonication vessel (V_{SoV}) was 40 mL and the holding tube volume (V_{SeV}) 52 mL, two residence time values were considered: the residence time in the sonication vessel (τ_{SoV}) and the total residence time (τ_{ToT}). These values were calculated using Eqs. (1) and (2).

211

212 $$\tau_{SoV} = \frac{V_{SoV}}{q + q_{CO_2}} \quad (1)$$

213 $$\tau_{ToT} = \frac{V_{SeV} + V_{SoV}}{q + q_{CO_2}} \quad (2)$$

214

215 Applying Eqs. (1) and (2) and considering the juice flow range (5-25 mL/min), 216 the residence time limits resulted in $1.333 < \tau_{SoV} < 4$ and $3.0667 < \tau_{ToT} < 9.2$ minutes. 217 The experimental design is described in Table 1.

218

219 *2.6. Statistical modeling*

A boolean-real hybrid model was assessed in order to analyze the effect of the process variables on the *S. cerevisiae* inactivation. The model was a function of the type $f:\mathcal{A}^3\times\mathcal{B}\to\mathcal{R}_+$ where $\mathcal{A}=[-1,1]$, $\mathcal{B}=\{0 \quad 1\}$ and $\mathcal{R}_+$ is the positive field of real numbers. This means that the three process variables were linearly codified as: $x_1 = f_{11}(\tau_{SoV})$ or $x_1 = f_{11}(\tau_{ToT})$, $x_2 = f_2(T)$ and $x_3 = f_3(P)$ where f_1, f_2 and f_3 are linear functions, in such a way that each combination $(x_1, x_2, x_3)\in\mathcal{A}$; and a Boolean variable $x_4\in\mathcal{B}$ is used to define the application or not of ultrasound. The codified variables were defined in Eqs. 3-5.

$$x_1 = \frac{\tau_{SoV} - 2.667}{1.333} \in \mathcal{A} \quad \text{for the sonication vessel output} \tag{3a}$$

$$x_1 = \frac{\tau_{ToT} - 6.133}{3.033} \in \mathcal{A} \quad \text{for the separation vessel output} \tag{3b}$$

$$x_2 = \frac{T - 36}{5} \in \mathcal{A} \tag{4}$$

$$x_3 = \frac{P - 200}{100} \in \mathcal{A} \tag{5}$$

where the constant values in Eqs. 3 to 5 were calculated so that any codified variable must be in the range -1 to 1.

The Boolean variable is defined as,

$$x_4 = \begin{cases} 0 & \text{for the procces without ultrasound} \\ 1 & \text{for the procces with ultrasound} \end{cases} \tag{6}$$

In order to ensure that the response was always positive, it was defined as Eq. 7.

$$y = -\ln\left(\frac{N}{N_0}\right) \in \mathcal{R}_+ \quad (7)$$

Then, the hybrid model was defined by Eq. 8.

$$y = (\beta_0 + \gamma_0 x_4) + (\beta_1 + \gamma_1 x_4)x_1 + (\beta_2 + \gamma_2 x_4)x_2 + (\beta_3 + \gamma_3 x_4)x_3 + (\beta_{11} + \gamma_{11} x_4)x_1^2$$
$$+ (\beta_{12} + \gamma_{12} x_4)x_1 x_2 + (\beta_{13} + \gamma_{13} x_4)x_1 x_3 + (\beta_{22} + \gamma_{22} x_4)x_2^2$$
$$+ (\beta_{23} + \gamma_{23} x_4)x_2 x_3 + (\beta_{33} + \gamma_{33} x_4)x_3^2 \quad (8)$$

With the hybrid model proposed it is possible to perform a statistical evaluation of the effect of HPU through the significance of the γ parameters (Neter & Wasserman, 1978). The same model was fit separately to the two sets of experimental results: the inactivation data for the sonication vessel outlet (x_1 defined in Eq. 3a) and for the separation vessel outlet (x_1 defined in Eq. 3b).

3. Results and Discussion

The continuous flow SC-CO_2-HPU equipment was used for the evaluation of the effect of pressure, temperature and residence time on *S. cerevisiae* inactivation, for the experimental design and under the conditions described in sections 2.1-2.5. The effect of the process variables on inactivation was quantified through Eq. 8 parameters. The value of the parameters obtained for the two sets of experimental results (samples extracted in the sonication vessel and in the separator) and their statistical significance are listed in Table 2. The fitted model had a determination coefficient (r^2) of 0.92 for the sonication vessel and 0.88 for the separator and an estimated variance (s^2) of

0.418 and 0.364 for the sonication and separator vessels, respectively. The general behavior of the model with respect to residence time, pressure and operation mode (with and without US) is plotted in Figs. 2 and 3, and the individual effects of the process variables on the microbial inactivation are plotted in Figs. 4-6.

3.1. Effect of residence time and HPU

In Table 2, the significance probability of parameters β_1, β_{12}, β_{13}, β_{11}, γ_1, γ_{12}, γ_{13} and γ_{11} indicates that residence time and operation mode (with and without US) are highly statistically significant in *S. cerevisiae* inactivation both in the sonication vessel and in the separator. The significance of second order interactions (β_{12}, γ_1,...) and third order interactions (γ_{11}, γ_{22} ...), complicates the interpretation of the effects. Therefore, the graphical behavior of the model was plotted in Figs. 2 and 3. The model relates four process variables with one response, so, in order to be plotted in a 3D graph, it is necessary to fix two variables and plot the response as a function of the remaining two process variables. Fig. 2 plots the model behavior in the sonication vessel at a fixed temperature of 31 °C ($x_2 = -1$) and for the two possibilities of the boolean variable: without ultrasound ($x_4 = 0$) and with ultrasound ($x_4 = 1$). Fig. 3 shows the same information for the separator. Figs. 4, 5 and 6 show Eq. 8 behavior in 2D projections and compare the fitted behavior with the experimental results under several process conditions. Fig. 4 plots Eq. 8 at a constant pressure and temperature (300 bar and 31 °C), whereas Eq. 8 at a constant pressure and flow is plotted in Figs. 5 (100 bar and 15 mL/min) and 6 (200 bar and 15 mL/min).

From Figs. 2 and 3 and the γ_1, γ_{12}, γ_{13}, γ_{11} values and their significance, it can be concluded that there is a significant effect ($p<0.01$) of HPU on microbial inactivation. In particular, compared to treatments without US, HPU increases the

293 inactivation of *S. cerevisiae* in the sonication and separation vessels by an average of
294 1.5 log cycles and 2 log-cycles, respectively. The effect of HPU may be attributed to
295 the enhancement of CO_2 mass transfer into the juice that accelerates the pH decrease
296 in the liquid phase and the extraction of components, such as phospholipids and
297 hydrophobic compounds, from S. cerevisiae cells. Another possible mechanism is the
298 cavitation produced by HPU in the liquid phase (Gogate et al., 2011). Cavitation refers
299 to the formation, growth, and implosion of tiny bubbles of CO_2 or water vapor in a liquid
300 when ultrasounds travels through it. Cavitation has been proven to cause cracked or
301 damaged cell walls, which enhances the penetration of SC-CO_2 inside the cells,
302 changing the cellular equilibrium and facilitating the extraction of intracellular
303 compounds, thus accelerating the death of the microbial cells. Ortuño et al. (2014)
304 observed that after the SC-CO_2 + HPU treatment, the cell wall and cell membrane were
305 totally disrupted, thus easing the disintegration of the cytoplasm and the inactivation of
306 cells. The damage caused by the treatment was serious enough to prevent a possible
307 regrowth of cells. Another effect of HPU is the increase in the internal cell component
308 mass transfer and interfacial turbulence, accelerating the inactivation effect of CO_2
309 (Gao *et al*., 2009).

310 Table 2 β_0 value parameters and their significance indicate that there exists a
311 maximum inactivation difference of 4 log-cycles in the separator with respect to the
312 reactor. This difference shows that, although some inactivation is obtained in the
313 sonication vessel, the holding tube as analog of a PFR complements the inactivation,
314 providing enough contact time between the SC-CO_2 and the microbial cells for the
315 microbial inactivation to be completed. In this regard, the SC-CO_2 dissolved into the
316 juice in the holding tube penetrates the damaged cells and completes the inactivation
317 mechanisms, leading to the maximum microbial death.

318 Figs. 4 a and b show that, regardless of the use of HPU, inactivation increases
319 at the longest residence times. The effect of ultrasound is dependent on the total

residence time. At the shortest residence times, the HPU intensification can be clearly observed (Figs. 4a and b) and greater inactivation levels are obtained compared with treatments which only use SC-CO_2. For example, at 9.2 min an inactivation of around 8 log-cycles of *S. cerevisiae* was obtained regardless of whether HPU was used or not; however, at 3.06 min, 4.2 log-cycles were obtained without HPU and 5 log-cycles with HPU (Fig. 4b). The results relating the microbial inactivation to the residence time in the sonication vessel (Fig. 3a) are consistent with Ortuño et al. (2013). These authors reported an average inactivation of 5 log-cycles of *S. cerevisiae* (in YPD Broth) in 1.3 min and 7 log-cycles in 2 min, for a SC-CO_2-HPU batch treatment at different pressures (100, 225, 290 and 350 bar) and temperatures (31, 36 and 41 °C). However, using a batch SC-CO_2 (without HPU) system, Ortuño et al. (2013) reported an inactivation of *S. cerevisiae* in YPD Broth of less than 1 log-cycle after 50 min of contact time at 225 bar and 31-41 °C. Therefore, considering the inactivation obtained in the present study (8 log-cycles at 9.2 min total residence time) without HPU, the continuous SC-CO_2 treatment system allows a better mixing of CO_2 in the juice and hence a greater dissolution and penetration into the microbial cells due to the fluid flow compared with batch treatments (Gunes et al., 2005; Shimoda et al., 1998).

3.2. Effect of pressure

The significance probability of Table 2 β_3, β_{13}, β_{23}, β_{33} and γ_{13} parameters indicates that pressure and its interaction with the operation mode are statistically significant as regards *S. cerevisiae* inactivation both in the sonication vessel and the separator. The generalized behavior of the pressure effect can be appreciated in Figs. 2 and 3. It can be observed that the pressure effect is dependent on the other process variables. For example, the pressure effect is almost negligible at 1.33 min residence time ($x_1 = -1$) without ultrasound in the sonication vessel (Fig 2b); and reaches a

maximum, with a difference of 4.3 log-cycles of inactivation between 300 and 100 bars, at 4 min ($x_1 = 1$) without US in the sonication vessel (Fig 2b).

Several authors have previously studied *S. cerevisiae* inactivation. Spilimbergo and Mantoan (2005) and Erkmen (2003) concluded that the pressure increase had a positive effect on the inactivation treatment. CO_2 can diffuse into the cellular membrane and accumulate within the cells, since the plasmic membrane of a microbial cell consists of a lipid bilayer structure. At higher pressures, the amount of dissolved CO_2 increases and, therefore, a large number of CO_2 molecules can cross through the cell membrane and lower the internal pH enough to exceed the buffering capacity of the cytoplasmic content. The lowering of pH inside the cells might cause the inhibition and/or inactivation of key enzymes essential for metabolic and regulating processes, such as glycolysis, amino acids and peptide transport, the active transport of ions and proton translocation (Spilimbergo and Bertucco, 2003).

3.3. Effect of temperature

The significance of Table 2 β_{12} and β_{23} parameters for the sonication vessel indicates that temperature has a significant effect on *S. cerevisiae* inactivation. However, no interaction was found between temperature and the use of HPU in the sonication vessel, which can be observed in Figs.5a and 6a, where, although the application of HPU increases the inactivation by 3.7 log-cycles (quantified in γ_0 parameter), the slopes of both model lines are almost the same. On the contrary, in the separator, the significance of β_2, β_{12} β_{23}, β_{22}, γ_{12} and γ_{23} parameters indicates that both temperature and its interaction with the use of HPU are statistically significant as regards *S. cerevisiae* inactivation, as can be observed in Fig. 5b. For example, at 100 bar and 4.6 min of total residence time with ultrasound, in the separator vessel, a maximum inactivation difference of 1 log cycle can be obtained at the highest

372 temperature used (41 ºC) with respect to the lowest one (31 °C), which indicates that
373 the effect of temperature, although significant, is moderate.
374 Ortuño et al. (2013) studied the influence of HPU on *S. cerevisiae* inactivation
375 kinetics using a batch SC-CO_2 system. These authors found a similar, moderate effect
376 of temperatures between 31 and 41 °C on the microbial inactivation. The temperature
377 effect is explained by the decrease in the medium's viscosity at higher temperatures,
378 which causes an increase in the SC-CO_2 diffusivity, facilitating the penetration of SC-
379 CO_2 into the cells and causing the extraction of essential substances from cells or
380 membranes, cytoplasmic membranes and disorders in the organelles, and therefore,
381 the disruption of the biological system in the cell (Shimoda et al., 1998).
382
383 *3.4. Process optimization*
384 As can be observed in the significance of the second and third order interaction
385 parameters (Table 2), it is evident that the effect of process variables has strong
386 interactions. However, the fitted descriptive model (Eq. 8) can be used to find the
387 maximum potential of the proposed process. In order to increase the process
388 productivity, the maximum potential of the process would be given by the minimum
389 residence time that could be handled (maximum product flow), ensuring the desired
390 number of log-cycles reduction. For the sonication vessel, Eq. 8 was considered for the
391 optimization in order to show the actual potential of HPU however, the inactivation level
392 reached in the separator was also calculated. Accordingly, a multi-objective
393 optimization problem of two competitive variables (residence time vs microbial
394 reduction) was formulated. The problem, solved as detailed in Carrillo-Ahumada et al.
395 (2011), was formulated as follows:
396
397 Problem 1,
398 $$\text{Min } \tau_{SoV}$$

399 Subject to $y_{SoV} \geq \phi$

400 and $1.333 < \tau_{SoV} < 4,\ 31 < T < 41,\ 100 < P < 300,\ 0 < x_4 < 1$

401

402 Where ϕ is the required number of log-cycles reduction. The optimization problem was
403 solved, applying the Box-Ruiz-Rodríguez-García constraint optimization algorithm
404 (Ruiz-López et al., 2006), at different values of ϕ in order to obtain the maximum flows
405 that could be achieved for microbial reductions ranging from 5 to 7 log-cycles. The
406 optimum results are listed in Table 3 with superscript 1, 2 and 3. The results indicate
407 that when optimization problem 1 was solved for $\phi = 5$, the competitive behavior
408 between residence time vs microbial reduction was lost. This can be observed by the
409 fact that the optimum was found in $y = 5.5$. Therefore, the maximum inactivation that
410 can be obtained at 1.35 minutes or less of sonication vessel residence time, was
411 sought by the following optimization problem,

412

413 Problem 2,

414 Max $f(\tau_{SoV}, T, P, x_4)$

415 Subject to $1.33 < \tau_{SoV} < 1.35,\ 31 < T < 41,\ 100 < P < 300,\ 0 < x_4 < 1$

416

417 The result of optimization problem 2 is listed in Table 3 with superscript 4. The
418 microbial reduction obtained in the sonication vessel at optimum τ_{SoV}, T and P for
419 problems 1 and 2, but with $x_4 = 0$ (without HPU), is also listed in Table 3. The effect of
420 HPU in the sonication vessel is clearly evident from the inactivation differences of 3.8,
421 4.1, and 4.2 log cycles when HPU is used and when it is not (Table 3). The different
422 inactivation parameters predicted by the optimization problems in the sonication vessel
423 were used to calculate the inactivation in the separator (Table 3). As expected, an
424 increase of inactivation (avg. 1 log-cycles with HPU and 3.3 without HPU) with respect

to the sonication vessel is obtained due to the longer residence time provided by the holding tube. The HPU effect in the separator vessel can be appreciated by the fact that, for the problem 2 optimum (subscript 4), there is a difference of 2.5 log cycles reduction when HPU is used and when not. The model developed was used to find the optimum working conditions by maximizing the flow; however, it could also be used for minimizing the process temperatures to increase the product quality.

4. Conclusions

The designed and built continuous regime HPU-SC-CO_2 plant demonstrated a high capacity to inactivate (>7 log cycles) *S. cerevisiae* in apple juice. The results allowed the quantification of the effect of the process variables through the development of a hybrid real-boolean model that described the effect of real variables (residence time, temperature and pressure) and the discrete variable (application or not of HPU). Multi-objective optimal problems were developed in order to calculate the minimum residence time that can be handled to reach different minimum inactivation levels. The optimization results showed that the system can achieve an inactivation of 6.8 log-cycles in 3.1 min of total residence time when HPU was applied, instead of 4.3 log cycles (under the same conditions) without HPU, which shows how important the influence of HPU is on SC-CO_2 inactivation.

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Highlights

- An ultrasound assisted continuous supercritical CO_2 pasteurization system was built
- Continuous SC-CO_2 juice flow treatment greatly enhanced microbial inactivation
- Ultrasound considerably intensified inactivation using the continuous system
- 7.8 log-cycles inactivation obtained after 9.2 min residence time (300 bars; 31^{o}C)
- Optimal process conditions were established through multivariate optimization

Figure Captions

Fig. 1. Supercritical CO_2 continuous treatment system. 1. CO_2 tank; 2. N_2 tank; 3. Chiller reservoir; 4. CO_2 Pump; 5. Liquid reservoir; 6. Liquid Pump; 7. Mixing point; 8.Sonication vessel; 9. Sonotrode; 10. Insulation joint; 11. Ceramics; 12. Power generation unit; 13. Thermostatic bath; 14. Continuous contact tube; 15. Separation vessel; 16. Treated sample; 17. CO_2 Recirculation; 18. Sonication vessel output, 19. Separation vessel output, V. valve; VS. non-return valve; VM. micrometric valve; P. Manometer; T. temperature sensor.

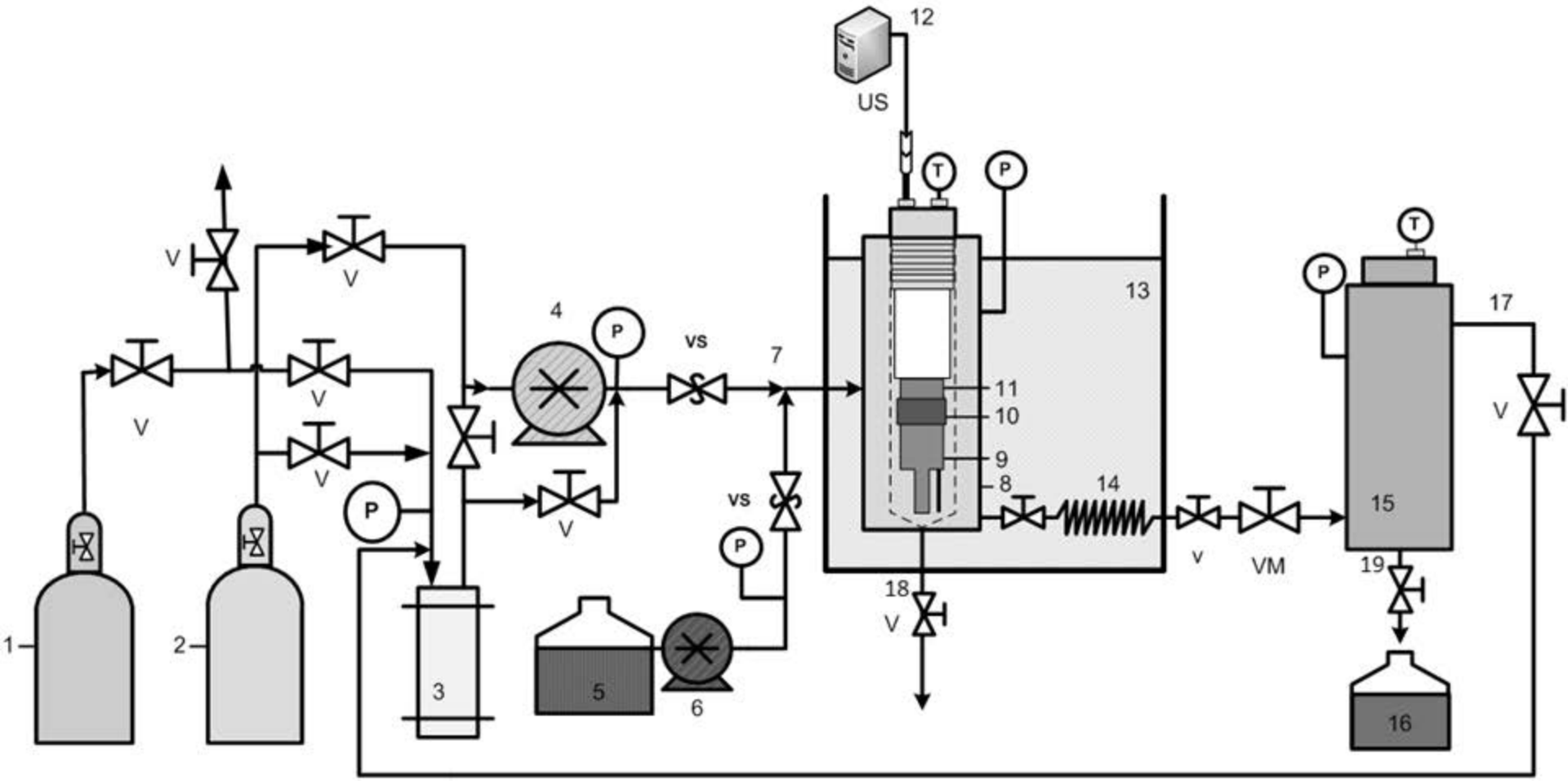
Fig. 2. Modeled (Eq. 8) *S. cerevisiae* inactivation (*y*) using a HPU assisted supercritical CO_2 continuous treatment system at 31 °C in sonication vessel. Effect of juice flow (x_1) and pressure (x_3). a) with ultrasound; b) without ultrasound.

Fig. 3. Modeled (Eq. 8) *S. cerevisiae* inactivation (*y*) using a HPU assisted supercritical CO_2 continuous treatment system at 31 °C in separator. Effect of juice flow (x_1) and pressure (x_3). a) with ultrasound; b) without ultrasound.

Fig. 4. Effect of juice residence time on *S. cerevisiae* inactivation (*y*) at 300 bar and 31 °C. Experimental data: SC-CO_2 (○) and SC-CO_2-HPU (x). Modeled (Eq. 8; continuous line). a) Sonication vessel; b) separator.

Fig. 5. Effect of temperature on *S. cerevisiae* inactivation (*y*) at 100 bar and 15 mL/min (4.6 min residence time). Experimental data: SC-CO_2 (○) and SC-CO_2-HPU (x). Modeled (Eq. 8; continuous line). a) Sonication vessel; b) separator.

Fig. 6. Effect of temperature on *S. cerevisiae* inactivation (*y*) at 200 bar and 15 mL/min (4.6 min residence time). Experimental data: SC-CO_2 (○) and SC-CO_2-HPU (x). Modeled (Eq. 8; continuous line). a) Sonication vessel; b) separator.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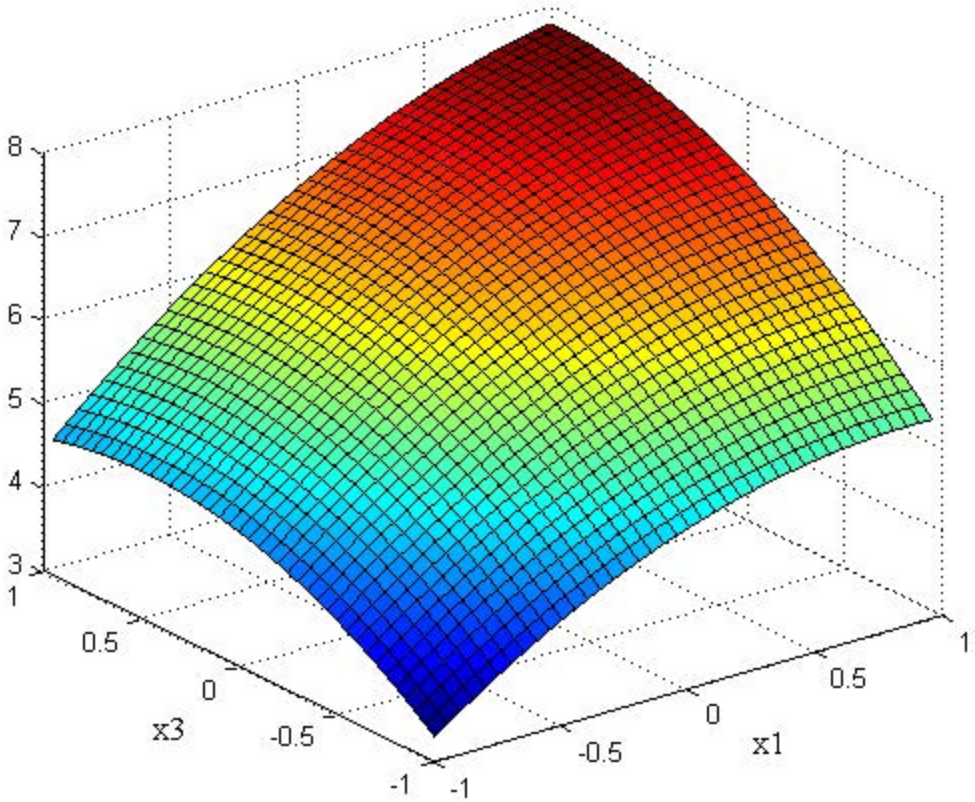
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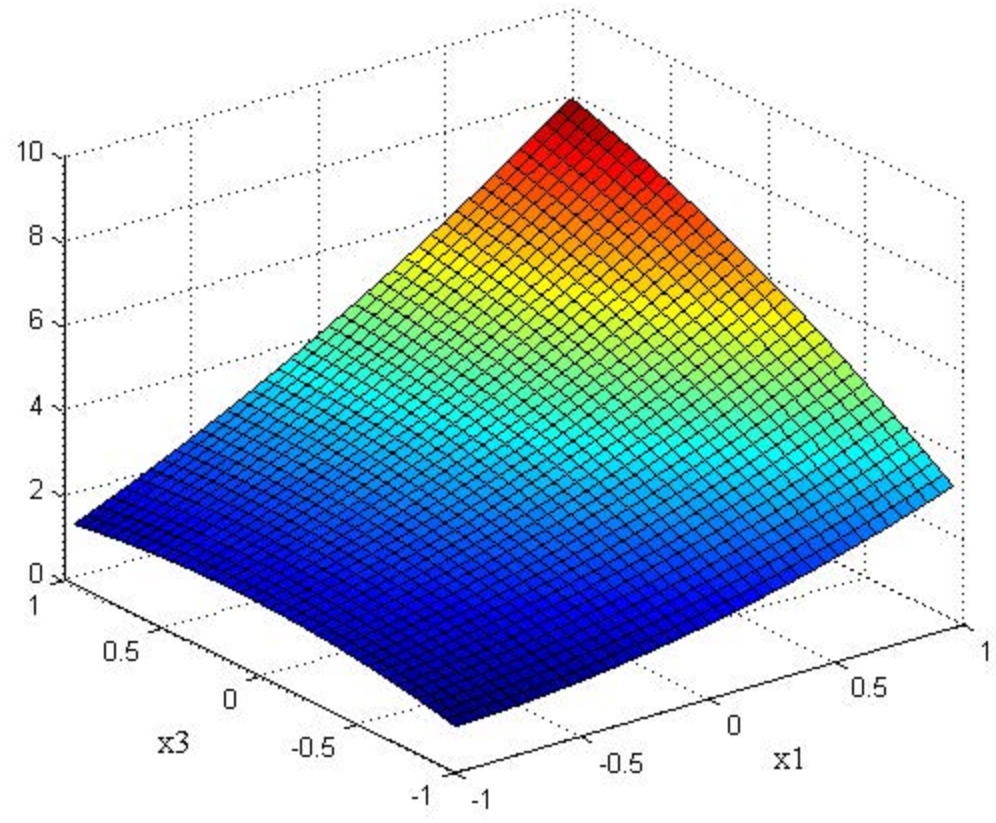
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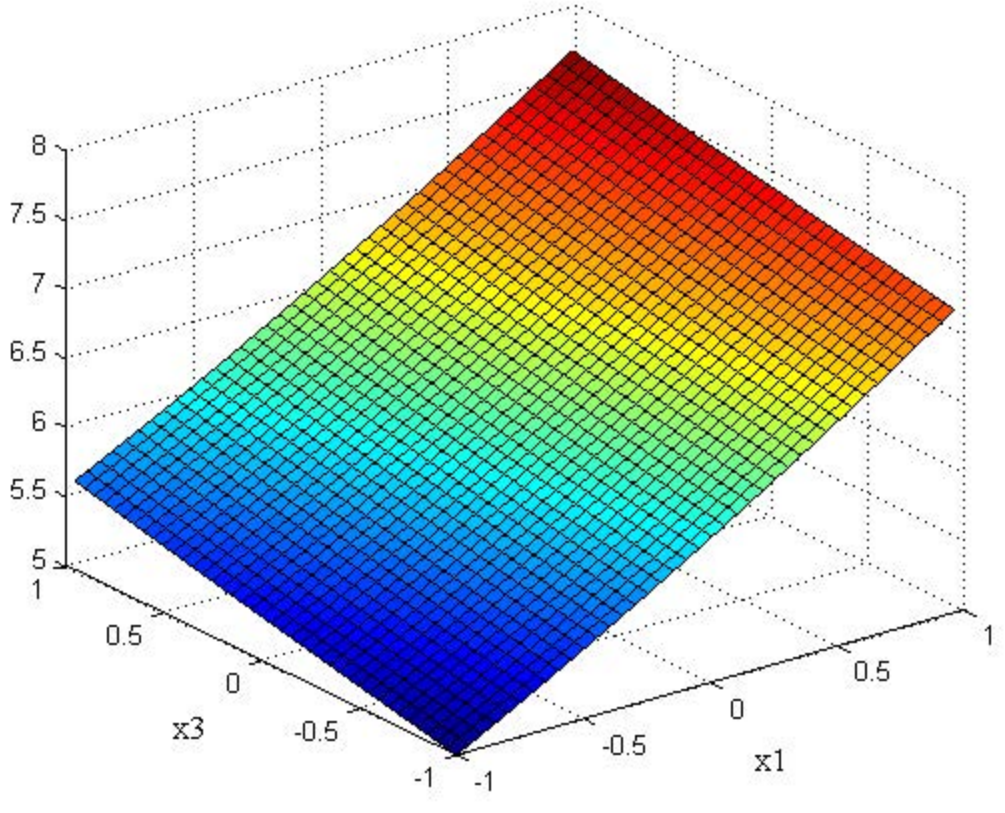
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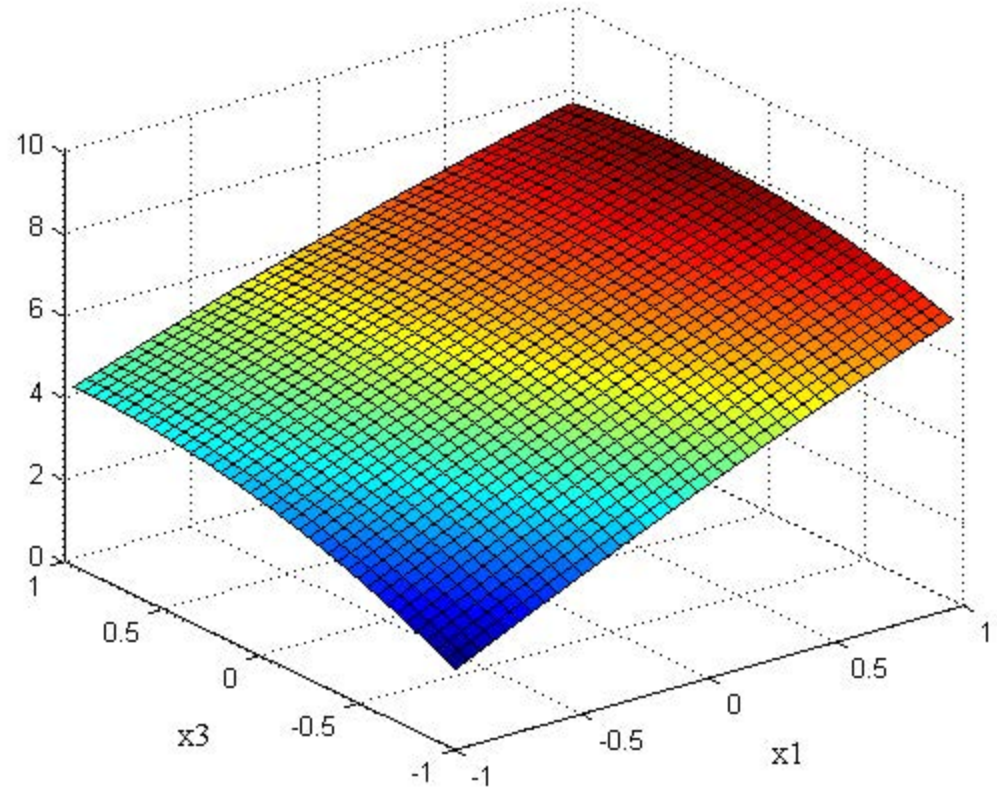
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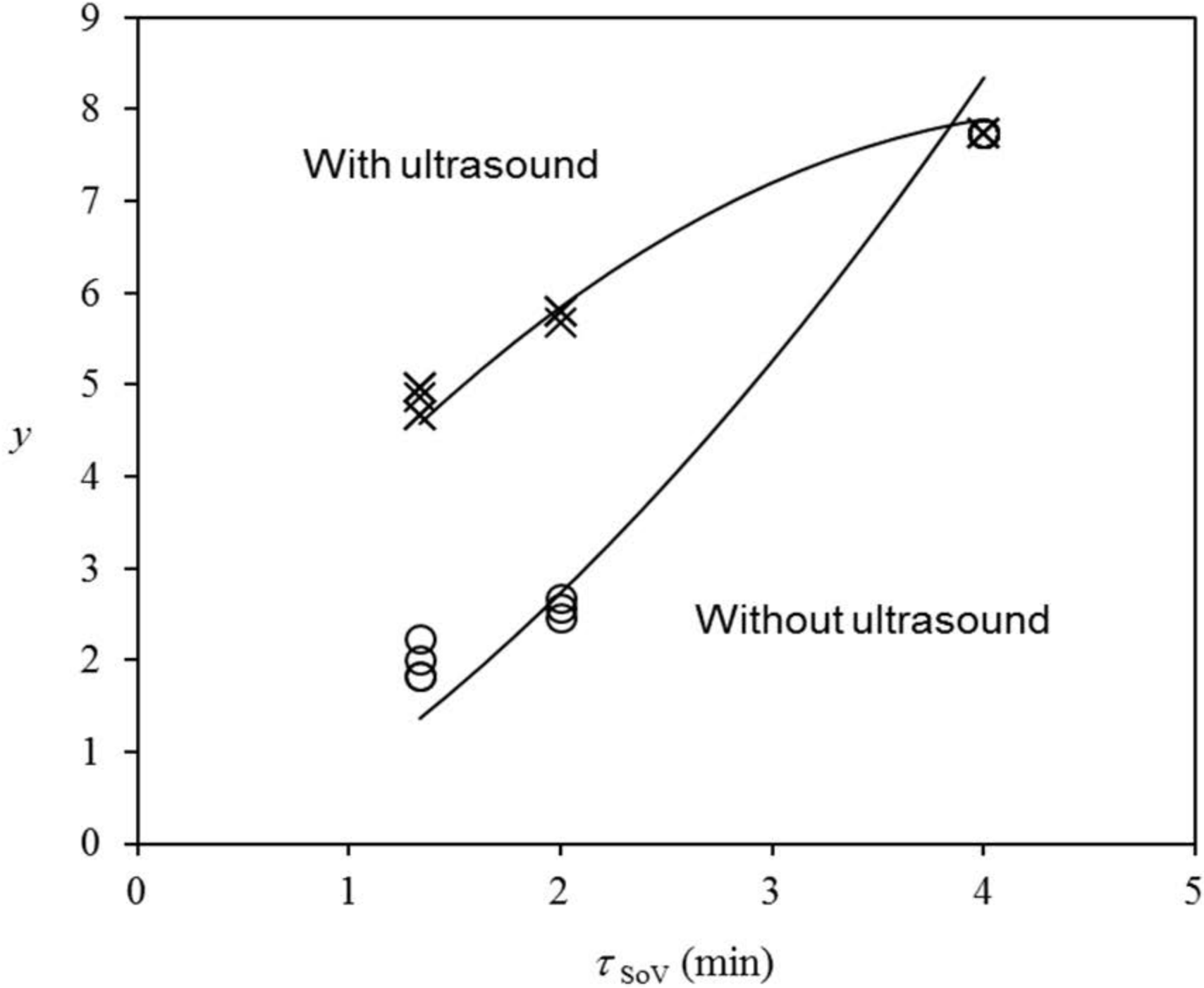
With ultrasound
Without ultrasound
y
τ_{SoV} (min)
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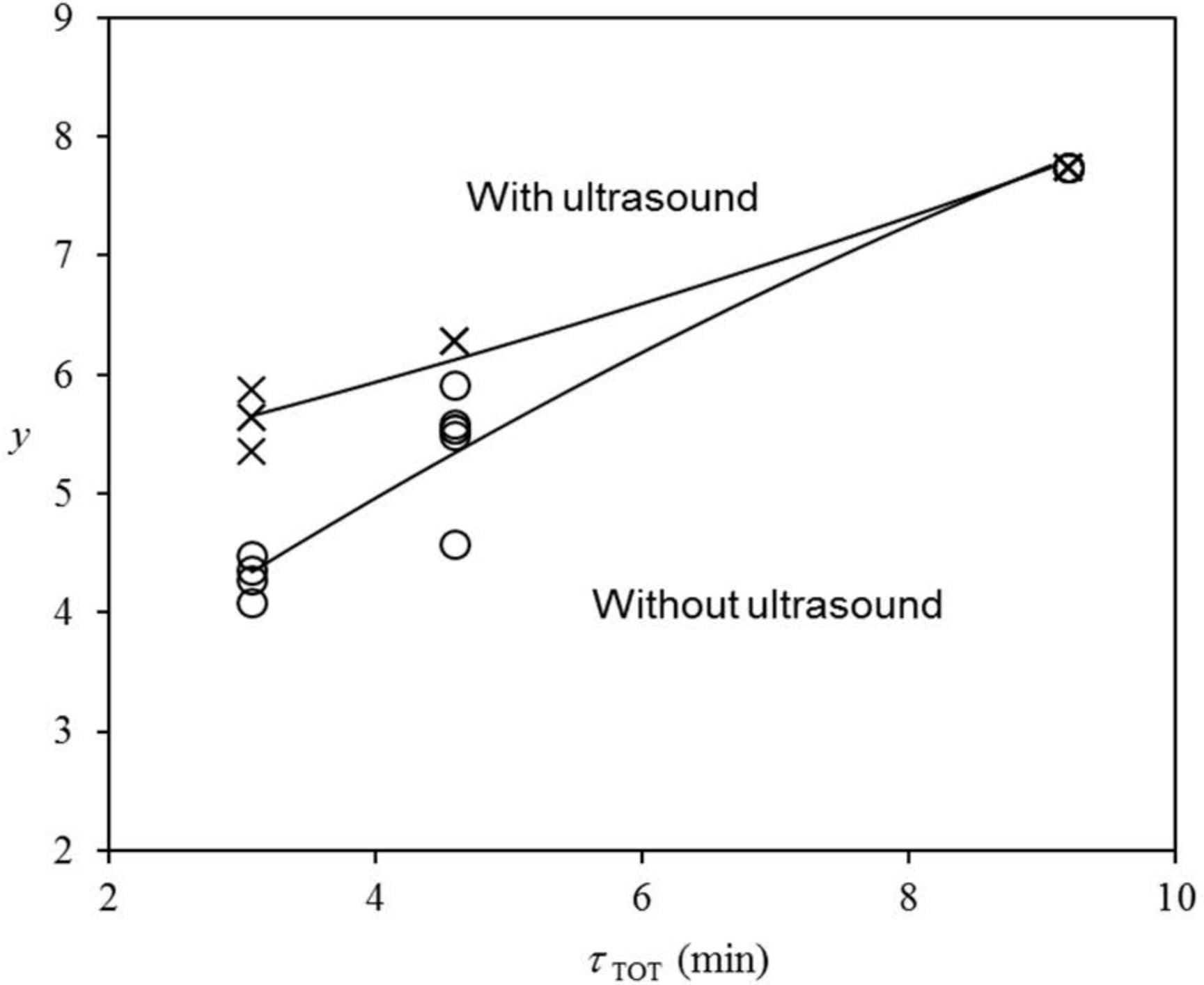
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With ultrasound
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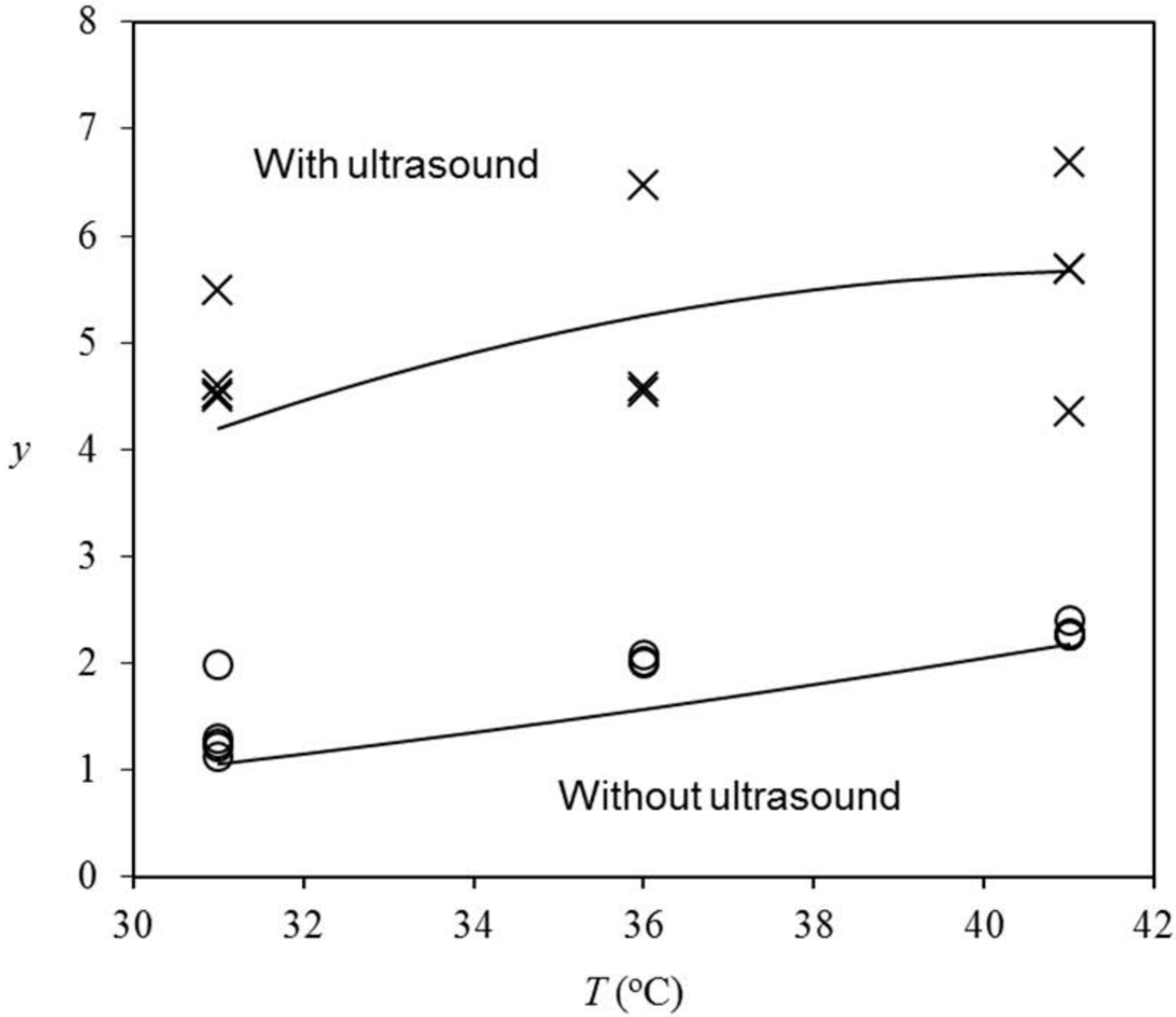
τ TOT (min)


With ultrasound
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T (°C)
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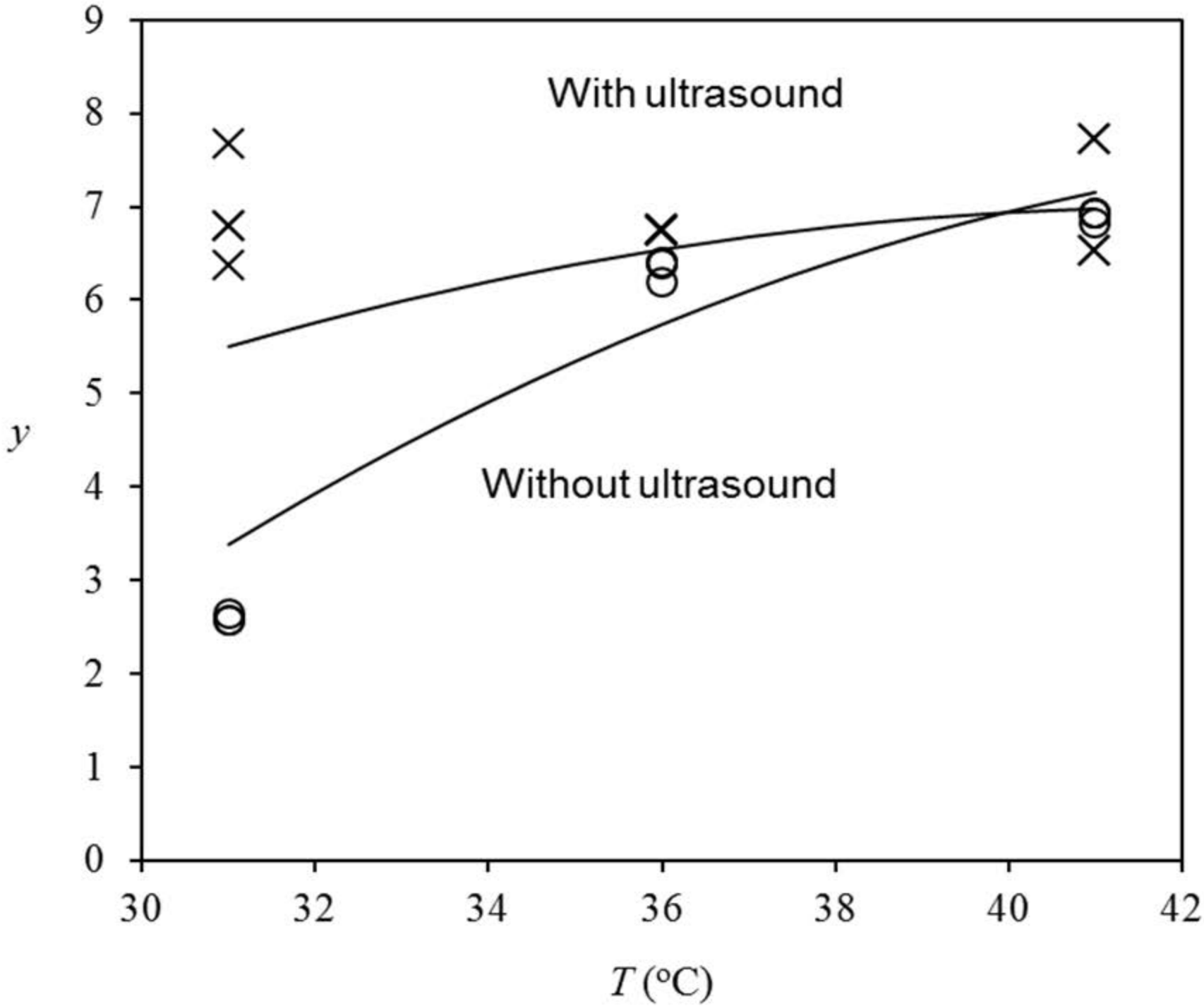
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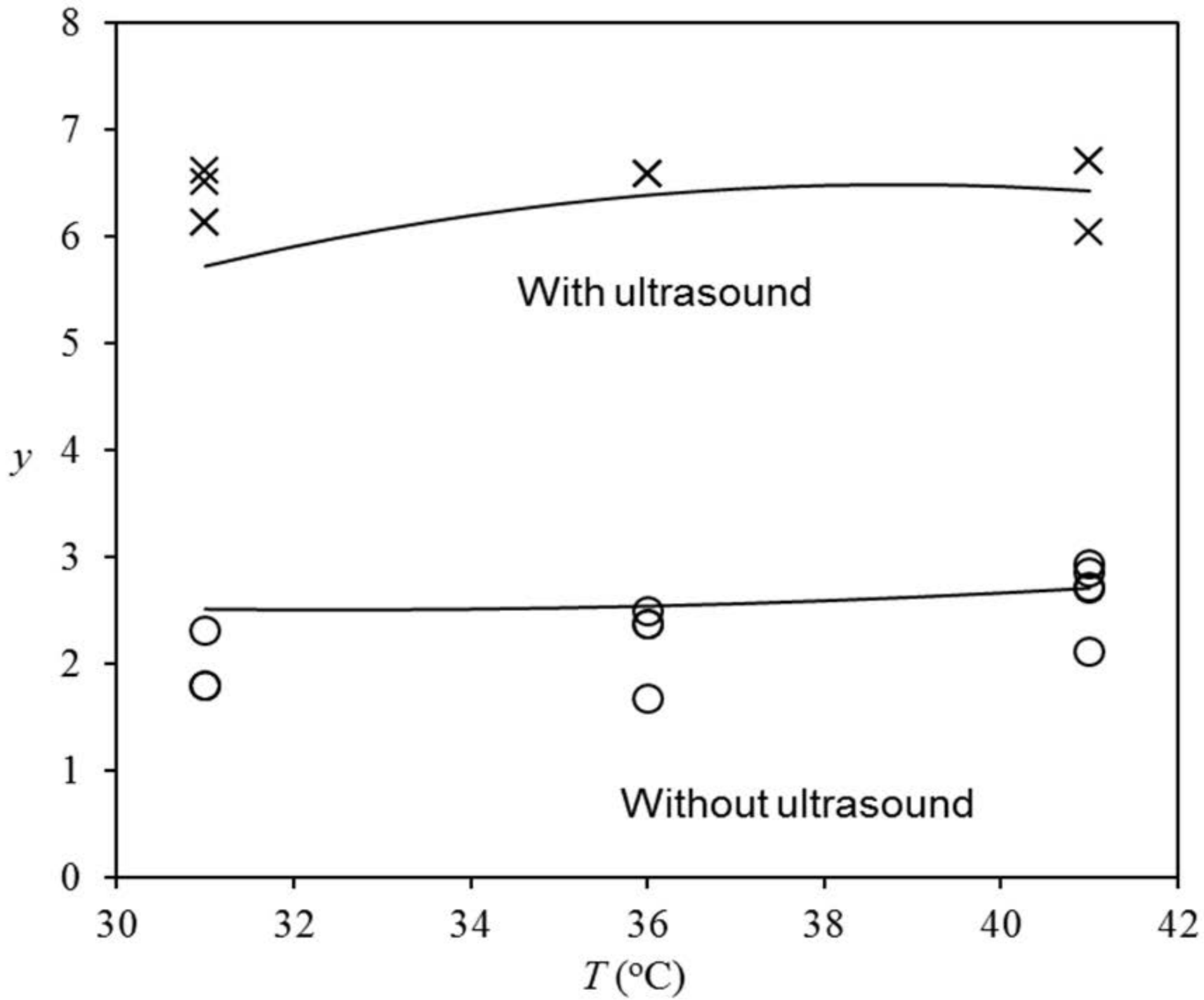
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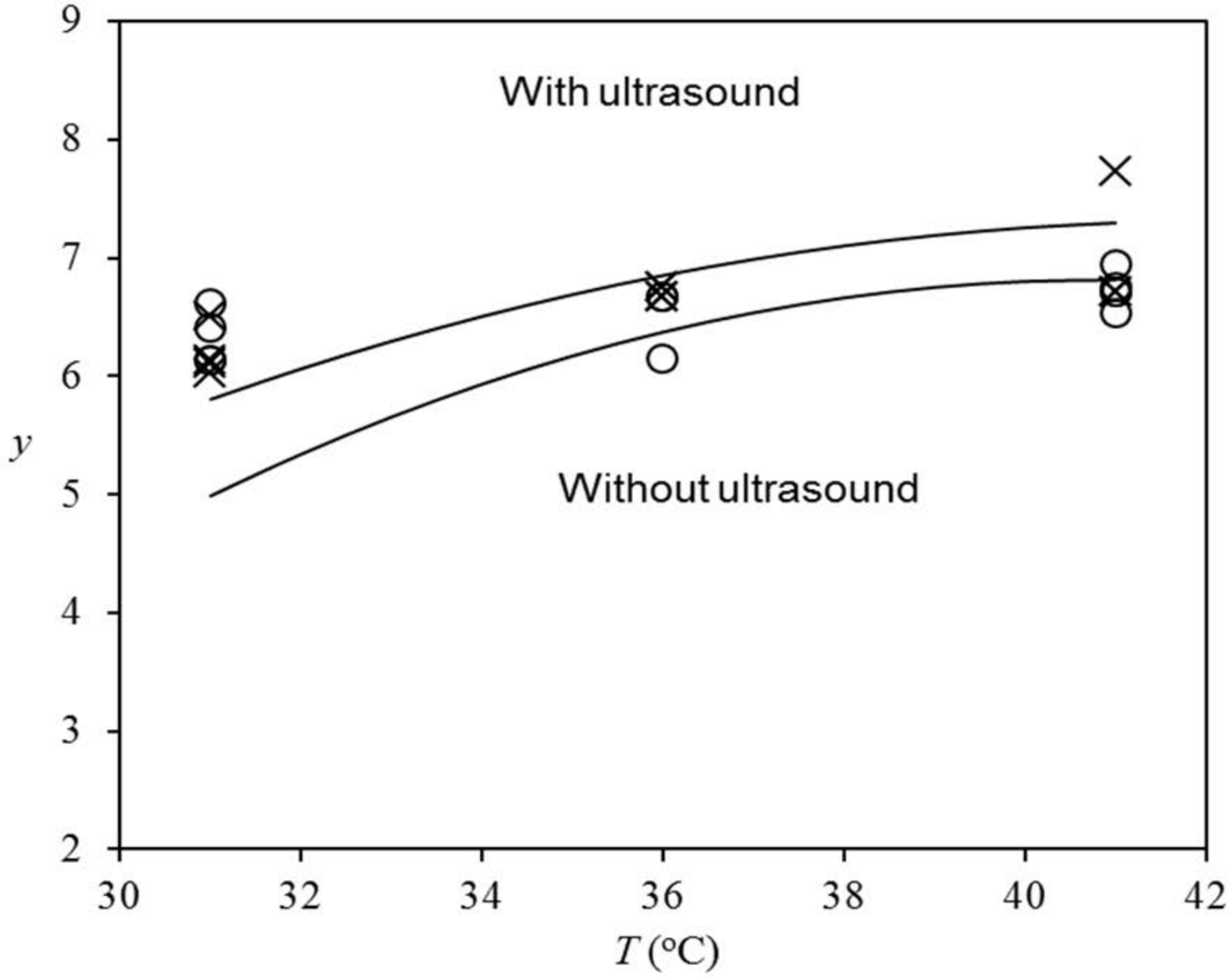
T (°C)


With ultrasound
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T (°C)
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Table 1. Experimental design performed to study the effect of pressure, temperature, use of ultrasound and residence time in the sonication vessel (τ_{SoV}) and total residence time (τ_{ToT}) on *S. cerevisiae* inactivation.

P (bar)	T (°C)	Juice flow (mL/min)	τ_{SoV} (min	τ_{TOT} (min)
100	31	5	4	9.2
200	31	5	4	9.2
300	31	5	4	9.2
100	31	15	2	4.6
200	31	15	2	4.6
300	31	15	2	4.6
100	31	25	1.333	3.0667
200	31	25	1.333	3.0667
300	31	25	1.333	3.0667
100	36	15	2	4.6
200	36	15	2	4.6
100	36	15	2	4.6
200	36	15	2	4.6
100	41	25	1.333	3.0667
200	41	25	1.333	3.0667
100	41	25	1.333	3.0667
200	41	25	1.333	3.0667

All of the treatments were carried out with and without ultrasound and were run in triplicate.

Table 2. Parameters of the model (Equation 8) used to describe the effect of pressure, temperature, residence time and use of ultrasound on the inactivation of *S. cerevisiae* using SC-CO_2-HPU.

Parameter	Sonication vessel	p	Separator vessel	p
β_0	3.891	<0.01	8.29	<0.01
β_1	2.956	<0.01	3.744	<0.01
β_2	0.409	0.12	1.703	<0.01
β_3	0.916	<0.01	-0.2011	0.24
β_{11}	0.499	<0.01	-0.183	<0.01
β_{12}	0.622	0.05	1.582	<0.01
β_{13}	1.155	<0.01	-0.423	<0.01
β_{22}	0.069	0.67	-0.471	<0.01
β_{23}	-0.373	0.02	-0.972	<0.01
β_{33}	-0.484	<0.01	-0.621	<0.01
γ_0	3.24	<0.01	-0.964	<0.01
γ_1	-1.757	<0.01	-2.755	<0.01
γ_2	-0.131	0.74	-1.012	0.01
γ_3	-0.326	0.19	0.508	<0.01
γ_{11}	-1.09	<0.01	0.263	0.37
γ_{12}	-0.77	<0.01	-1.69	<0.01
γ_{13}	-0.844	<0.01	0.395	0.02
γ_{22}	-0.3825	<0.01	0.170	0.46
γ_{23}	0.0119	0.95	0.980	<0.01
γ_{33}	-0.1818	0.42	0.633	<0.01

p: probability that the parameter could be zero and therefore non-significant in the model

Table 3. Optimization results in the sonication (y_{SoV}) and separation vessels (y_{SeV}), calculated to minimize the residence time (problem 1) and to maximize the microbial inactivation (problem 2).

τ_{SoV} (min)	q (mL/min)	τ_{TOT} (min)	T (°C)	P (bar)	x_4	y_{SoV}	y_{SeV}
2.40	11.7	5.52	37.6	231.1	1	7.0[1]	7.4
2.40	11.7	5.52	37.6	231.1	0	3.5	7.4
1.66	19.1	3.82	37.4	219.3	1	6.0[2]	6.9
1.66	19.1	3.82	37.4	219.3	0	1.9	5.4
1.35	24.6	3.10	39.0	216.7	1	5.5[3]	6.9
1.35	24.6	3.10	39.0	216.7	0	1.3	4.2
1.35	24.6	3.10	39.4	203.4	1	5.5[4]	6.8
1.35	24.6	3.10	39.4	203.4	0	1.3	4.3

Superscript 1-3: Optimization results obtained after minimizing the residence time, ensuring a given microbial inactivation (ϕ), 1: $\phi \geq 7$; 2: $\phi \geq 6$; 3: $\phi \geq 5$. Problem1.

Superscript 4: Optimization results obtained after maximizing the microbial inactivation, constrained by $1.33 < \tau_{SoV} < 1.35$

For all the operating conditions that optimize the criteria (Superscript 1-4), in the row bellow, the corresponding inactivation without HPU (x_4=0) is calculated.