INACTIVATION OF *ZYGOSACCHAROMYCES ROUXII* USING POWER ULTRASOUND AT DIFFERENT TEMPERATURES, PH AND WATER ACTIVITY CONDITIONS

S. KIRIMLI[1] and B. KUNDUHOGLU[2]*

[1]Institute of Science, University of Eskisehir Osmangazi, 26480 Eskisehir, Turkey
[2]Department of Biology, Science and Arts Faculty, University of Eskisehir Osmangazi, 26480 Eskisehir, Turkey
*Corresponding author: Tel. +90 222 2393750 ext. 2845, Fax +90 222 2393578, email: bkunduh@gmail.com

ABSTRACT

In this study, the effect of ultrasound treatments (20 kHz) combined with mild temperatures (thermo-sonication) on the inactivation of *Z. rouxii* was examined. Additionally, the effect of pH (4 and 7) and water activity (aw 0.99 and 0.94) of the sonication medium on yeast inactivation was determined. The $D_{(40-55)}$ values at a thermo-sonication amplitude of 80% were shorter than that obtained at 40%. Using thermo-sonication, particularly at a low aw, was associated with a significant synergistic effect for *Z. rouxii* inactivation ($p<0.05$). In most thermo-sonications at 50° and 55°C, the FDA requirement of a 5-log cycle reduction could be achieved (>5.7-log reductions in <0.2-0.2 min). Our findings show that sonication offers advantages in terms of reduced duration and temperature of pasteurization, without a reduction in structural and sensory quality particularly for fruit juices.

- Keywords: D value, inactivation, ultrasound, *Zygosaccharomyces rouxii* -

INTRODUCTION

The yeast *Zygosaccharomyces rouxii* represents a major cause of spoilage of foods and drinks that are packaged according to good manufacturing practices (GMP), including fruit juices, sauces, carbonated drinks, wine, salad dressings, and ketchups (JAMES and STRATFORD, 2003; PITT and HOCKING, 1985; LOUREIRO and MALFEITO-FERREIRA, 2003; FUGELSANG and EDWARDS, 2007; DEÁK, 2008). Typical physiological characteristics of *Z. rouxii* include tolerance to low-acidity preservatives, extreme osmotolerance, and the ability to adapt to high glucose concentrations, low water activity (aw) and thermal treatment (EMMERICH and RADLER, 1983; JAMES and STRATFORD, 2003; MARTORELL *et al.*, 2007). Thus, *Z. rouxii* is important to consider in examining spoilage during the processing of foods with low-acidity and high-sugar content.

The food industry most frequently uses traditional pasteurization methods such as low temperature long time (LTLT) and high temperature short time (HTST) to achieve shelf-life stability for fruit juices and drinks due to these methods' effectiveness and low cost. However, these procedures are associated with the loss of vitamins and volatile aromatic substances (KÖRMENDY, 2007; VASANTHA RUPASINGHE and LI JUAN YU, 2012). In addition to thermal pasteurization, other methods that are commonly utilized to prolong shelf-life include chemical preservatives such as potassium sorbate, sodium benzoate (VASANTHA RUPASINGHE and LI JUAN YU, 2012), citric acid and sulfur dioxide (WILEY, 1994; BATES *et al.*, 2001). Chemical preservatives used to prolong shelf-life may be associated with adverse health consequences in humans, depending on the characteristics of the consumer population and the frequency of consumption (ISMAN, 2000).

Although thermal treatment is the most common technique to inactivate microorganisms in food, there is an increased interest in the use of alternative food preservation methods as a response to consumer demand for food with conserved innate characteristics and no artificial preservatives (CORBO *et al.*, 2009; VASANTHA RUPASINGHE and LI JUAN YU, 2012; ALZAMORA *et al.*, 2003). Some of the non-thermal food preservation methods that may represent an alternative to thermal treatment include electric or magnetic fields, microwave radiation, ionizing radiation, high-intensity light pulses and high-hydrostatic pressure (CORBO *et al.*, 2009; DI BENEDETTO *et al.*, 2010). Additionally, power ultrasound (US) is a promising novel technology that minimizes the need for treatment, increases food quality, and conserves the characteristics and sensory qualities of the food. Power US is defined as the use of pressure waves between 20 and 100 kHz. The lethal effect of ultrasonic processing on microorganisms is achieved through the conversion of electrical energy to ultrasonic sound waves via the ultrasonic transducer and through the formation and collapse of vast numbers of small bubbles in each second during the propagation of ultrasonic waves within liquids. The quick formation and collapse of these bubbles (cavitation) creates very high local temperatures (5500°C) and pressures (50 MPa), which cause disruption of the cell wall and damage to the cell membrane and DNA (JIRANEK *et al.*, 2008; MANVELL, 1997; KNORR *et al.*, 2004; O'DONNELL *et al.*, 2010; CÁRCEL *et al.*, 2012; LEIGHTON, 1998; SORIA and VILLAMIEL, 2010). The duration and temperature of the procedure, the composition and volume of the liquid, and the form and dimensions of the microorganism are among the determinants of the antimicrobial efficiency of ultrasonic processes (BEVILACQUA *et al.*, 2013).

The possible areas of use for microbial inactivation by power US have been relatively well studied in the food industry. It has been reported that to achieve the FDA-required 5-log reduction in microorganisms, sonication should be used in combination with mild heat treatment and/or pressure (FDA, 2001; WALKLING-RIBEIRO *et al.*, 2009; BAUMANN *et al.*, 2005; D'AMICO *et al.*, 2006; UGARTE-ROMERO *et al.*, 2006; SALLEH-MACK and ROBERTS, 2007; TIWARI *et al.*, 2009). Many studies have reported the synergistic effect of the combination of non-thermal technologies and heat treatment on microbial inactivation (GUYOT *et al.*, 2007; LEE *et al.*, 2009; LEISTNER and GORRIS, 1995; RASO *et al.*, 1998; REDDY *et al.*, 2006; ROSS *et al.*, 2003). However, to our knowledge, there are no studies examining the combined effect of heat, pH and aw on *Z. rouxii* inactivation using US. Therefore, the aim of this research was to evaluate the effect of US with heat (thermo-sonication) on the inactivation of *Z. rouxii* at different pH and aw conditions. For this purpose, citrate buffer was chosen as the model medium, and the effect of thermo-sonication on *Z. rouxii* was tested under different pH and aw conditions. Thus, the optimum procedural parameters defined for *Z. rouxii* inactivation may be utilized as a model for the US-assisted pasteurization of real fruit juices and other drinks at mild temperature conditions.

MATERIALS AND METHODS

Maintenance of test strain

Zygosaccharomyces rouxii (NRRL Y-229) was obtained as a lyophilized culture from the ARS Culture Collection (Northern Regional Research Laboratory, United States Department of Agriculture, Midwest Area-National Center for Agricultural Utilization Research Microbial Genom-

ics & Bioprocessing Research Unit 1815 N University Street, Peoria, IL 61604). The culture tube was opened aseptically, the contents were transferred to a 2% Sabouraud Dextrose Broth (SDB, Merck, Germany), and the mixture was incubated for 48-72 h at 30°C. The stock cultures were then grown on Sabouraud Dextrose Agar (SDA, Merck, Germany) slants and stored at 4°C until use.

Preparation of yeast culture for inactivation studies

Z. rouxii subcultures were prepared by inoculating a test tube that contained 5 ml of sterile SDB with one single colony from a culture plate. The tubes were then incubated at 30°C for 48 h. Erlenmeyer flasks (250 mL) containing 50 mL of SDB were inoculated with this subculture. The flasks were incubated under agitation (130 rpm). The broth cultures were transferred to sterile centrifuge tubes, and pellets were obtained at 5500 rpm for 10 min. The pellets were then washed with saline water (0.85% NaCl) and resuspended in the same medium. *Z. rouxii* suspensions prepared in this way were used to inoculate sonication vessels at a final concentration of 10^8 CFU/mL.

Preparation of citrate buffer

All sonication and control group treatments in this study were applied in citrate buffer medium. Citrate buffer was prepared as two stock solutions (Stock Solution A: 0.1 M citric acid, $C_6H_8O_7.H_2O$ reagent, Carlo Erba, Italy; and Stock Solution B: 0.2 M di basic sodium phosphate, $Na_2HPO_4.2H_2O$, Merck, Germany). The final pH of the citrate buffer was measured using a pH meter (WTW InoLab 730, Germany).

Water activity (a_w) of the citrate buffer was adjusted to a_w 0.94 with glycerol (Merck, Germany). The aw values of the citrate buffer medium were measured at room temperature (23-25°C) with an AquaLab water activity meter (Decagon Devices, Inc., USA).

Combined treatments (Thermo-sonication treatments; TS-T)

Sonication was performed with a VC-750 Watt US generator and a Vibracell® WCX 750 (Sonics and Materials, CT, USA) model ultrasonic processor at a frequency of 20 kHz (maximum 124 µm amplitude). A solid sonication probe (13 mm in diameter) was used in all treatments. Levels of 40% (49.6 µm amplitude) and 80% ultrasonic power (99.2 µm amplitude) were applied in each case. Most of the sonication treatments were applied for 20 min. A 100 ml sterile water-jacketed vessel (Part No. 830-00010, Sonics and Materials, CT, USA) was used to hold the citrate buffer. The temperature of the citrate

buffer in the vessel was controlled by a refrigerated circulating water bath (Polyscience-9102, IL, USA). The temperature of the medium in the vessel was monitored during the sonication process using the digital thermometer (Sonics and Materials, CT, USA) of the ultrasonic processor. The vessels and probes were sterilized at 121°C for 15 min before and after each experiment. The preparation of the sonication vessels and the sonication process are described below. Additionally, the experimental design of the combined treatments (TS-T at different medium conditions) and thermal treatments alone (T-T: control group treatments, at the same medium conditions) are summarized in Table 1.

(1) A total of 99 mL of citrate buffer was placed in a water-jacketed vessel.

(2) A sonication probe was immersed in the center of the vessel.

(3) The sonication procedure produces heat in a liquid medium; thus, to fix the temperature of the citrate buffer in the vessel at the target treatment temperature (40, 45, 50 or 55°C) during the sonication process, the temperature of the circulating water bath was adjusted to 7-10°C less than the target temperature. Then sonication was started.

(4) Immediately after reaching the target temperature, 1 mL of yeast suspension was added to produce a final concentration of 10^8 CFU/ml in the citrate buffer in the sonication vessel.

(5) At the beginning and during the treatment, 1 ml samples of citrate buffer samples were collected from the vessel and serially diluted in sterile saline water (1:10). If necessary, the sampling intervals and treatment times were adjusted (e.g., in the case of high temperature

Table 1 - Summary of the experimental design with thermo-sonication (TS-T) and thermal treatments (T-T) at different pH and aw levels.

| Treatment Temperatures | Variables | | | Treatments | |
	Sonication Levels	pH	aw	TS-T	T-T
40°C, 45°C, 50°C and 55°C	-	4	0,99		+
			0,94		+
		7	0,99		+
			0,94		+
	40%	4	0,99	+	
			0,94	+	
		7	0,99	+	
			0,94	+	
	80%	4	0,99	+	
			0,94	+	
		7	0,99	+	
			0,94	+	

-: no sonication.

levels). Survival was determined using the drop-plate and spread-plate techniques. Aliquots of 0.02 ml (for drop-plate technique) or 0.1 mL (for spread-plate technique) were taken from the dilutions and plated on SDA. The plates were incubated at 30°C for 48 h, and counts of survivors in treated samples were conducted. All experiments were repeated at least two times.

Thermal Treatments (T-T) alone

The survival and growth of *Z. rouxii* was also determined in citrate buffer at different temperatures (40, 45, 50 or 55°C) and under different medium conditions (pH 4 and 7 and aw 0.99 and 0.94) without sonication. Treatments were performed in a shaking water bath (Memmert, Germany). The **T-T** process is described below and given in Table 1.

(i) A total of 99 ml of citrate buffer was placed in a flask.

(ii) To reach the target temperatures (40°, 45°, 50° and, 55°C), 99 mL of citrate buffer in flasks was pre-heated in a shaking water bath. The temperature of the citrate buffer in the flasks was monitored using a digital thermometer.

(iii) The citrate buffer reached the target temperature level.

(iv) One milliliter of yeast suspension was added to achieve a final concentration of 10^8 CFU/ml in the citrate buffer. This step corresponded to the beginning of the treatment time.

(v) During the treatment, 1 ml samples of the citrate buffer were collected from the flasks and serially diluted in saline water (1:10). The sampling intervals were 0, 1, 2, 4, 8, 12, 24 and 48 h. Viability counts were conducted as described above.

As shown in Table 1, 48 different (32 TS-T + 16 T-T) treatment conditions were studied to determine yeast inactivation, and each treatment was repeated in parallel at least two times.

Determination of D values

In this study, the inactivation of *Z. rouxii* was described using the first-order inactivation kinetic model. The D values were directly calculated from the k values (the slope of the inactivation curve) and the R^2 values.

First-Order Kinetic Model:

$$N/N_0 = e^{-kt} \text{ or } \ln N = \ln N_0 - kt$$

$$\log N = \log N_0 - k't$$

$$D = -\frac{1}{k'},$$

Where; No= initial cell number (CFU/mL), t = treatment time (min),

N = number of the surviving cells (CFU/mL) after t minutes of treatment,
k = slope of inactivation curve (min^{-1}),
k´ = log of slope of inactivation curve (min^{-1}), and
D = decimal reduction time, or the time required for a 1-log cycle reduction in the microbial population.

Data were fitted to this model with a linear regression using the Microsoft Excel program. Additionally, log reductions (log CFU/mL) for each process were calculated using data of the initial and final yeast numbers in the vessel.

Viability of yeast cells in treated samples during storage

In this step of the study, we determined the growth of the survivors during storage at different temperatures (4° and 25°C). Two samples (10 mL) were taken from each treatment, with 5-log cycle reductions achieved; they were aseptically transferred into 10 mL double-strength glass bottles containing SDB and stored at 4° and 25°C for 60 d in the dark. During storage, 1 ml aliquots were taken predetermined intervals from each bottle and were then transferred into SDB; the tubes were incubated at 30°C for 3-5 days, and yeast growth was checked. The sampling intervals were 1, 7, 15, 30, 45 and 60 d.

Statistical analysis

Variance analysis was used to determine the effect of inactivation factors on D values. The plate-count data were logarithmically transformed for statistical analysis. The results ($\log_{10}$ CFU/ml) were subjected to an analysis of variance (SPSS Ver. 11.5, Chicago, Il, USA). For all experiments, a *p* value ≤0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

In the present study, the inactivating effect of ultrasound waves (20 kHz) on *Z. rouxii* was investigated in a model medium (citrate buffer). A total of 48 different experiments were performed to determine the effect of heat (40, 45, 50 and 55°C), pH (4 and 7), and aw (0.99 and 0.94) on ultrasonic inactivation (40 and 80% amplitude) of *Z. rouxii*. During the sonication procedure, periodical sampling from the sonication chamber was conducted to determine the number of viable cells of *Z. rouxii* (CFU/ml). A first-degree kinetics reaction was used to establish inactivation plots for *Z. rouxii* that were subsequently utilized to estimate the "D values" based on slope and R^2. Additionally, yeast reduction was determined based on a comparison of the cell numbers before and after the procedure. The difference in D values, as defined by the TS-T and T-T processes, were assessed using variance analysis.

Furthermore, the growth pattern of sublethally injured yeasts following TS-T and T-T processes were evaluated under different storage conditions (at 4° and 25°C for 60 d).

Inactivation of *Z. rouxii* at 40°C

An overall assessment of the results of all combined procedures at 40°C showed a smaller D value at 80% amplitude (0.94 aw and 0.99 aw; pH = 4 and pH = 7) than at 40% amplitude (*p*<0.05) (Fig. 1a). A generally reduced microbiological resistance to heat occurs in an acidic environment. However, in our study, the D_{40} values in the combined and thermal procedures at pH= 4 were statistically significantly higher than those at pH=7 (*p*=0.012).

The aw of the medium also had an impact on *Z. rouxii* inactivation. The D_{40} values estimated at 0.94 aw in all combined and thermal procedures were higher than those estimated at 0.99 w (*p*<0.05). Thus, a low aw was considered to give *Z. rouxii* a higher resistance to heat and sonication. Similar to this study, ALVAREZ *et al.* (2003) observed a 30-fold increase in the thermal decimal reduction time for *Salmonella enteriditis* by decreasing aw from 1 to 0.96, whereas only a two-fold increase was observed with mano-sonication, and a synergistic lethal effect with the combined use of heat and ultrasound was observed.

In our combined treatment procedures at 40°C, the reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 0.4-1.6 log CFU/mL and 0.8-3.6 log CFU/ml, respectively (Fig. 1b). In a study by BEVILACQUA *et al.* (2013), ultrasound was used to determine the reduction in several spoiling yeasts, including *Z. rouxii*, in fruit juices; similar to our observations, there was a maximum reduction of 1.7 log CFU/mL *Z. rouxii* in orange juice after sonication (40°C, 20 kHz, amplitude 60%, time 4 min, pulse 2 s).

According to the Hurdle concept, if the effect obtained via the combined use of two different inactivation factors is greater than the sum of the separate use of these methods, then a synergistic interaction is said to occur (LEISTNER and GORRIS, 1995). In the present study, treatment with a pH= 4 or 7 at 0.94 aw, with the combined use of ultrasound (40% and 80%) and heat, resulted in a significant synergistic interaction, although the D_{40} value was higher than that observed with an aw of 0.99. In control treatments performed at the same temperature, sonication at 0.94 (pH 4 and 7) and 0.99 aw (pH 4 and 7), the reduction in D_{40} values was, respectively 1/8-1/16 and 1/32-1/128 (Fig. 1a).

Inactivation of *Z. rouxii* at 45°C

The D_{45} values estimated for combined treatments at 0.94 aw were greater than those observed with 0.99 aw; however, the D_{45} values were lower than those obtained at 40°C (Fig. 2a). Overall, our results suggest that increased treatment temperatures resulted in increased yeast inactivation. Additionally, all sonications at 80% amplitude (0.94 aw and 0.99 aw; pH 4 and 7) had D values smaller than those found at 40% (p<0.05). The reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 0.5-2.0 log CFU/mL and 1.1-3.9 log CFU/mL, respectively (Fig. 2b).

In treatments at 0.99 aw (pH 4 and 7), a synergistic interaction for *Z. rouxii* inactivation was observed with the combined use of heat and ultrasound (40% and 80% amplitude). While synergy was present at 0.94 aw and pH values of 4 and 7 (40% and 80%), the D_{45} value was greater than that observed at 0.99 aw. Compared with control treatments at the same temperature and pH, the reductions in D_{45} obtained with the combined treatments at 0.94 and 0.99 aw were from 1/8-1/32 and 1/64-1/128, respectively (Fig. 2a). LOPEZ-MALO *et al.* (2005) assessed the sonication inactivation (20 kHz, 90 μm) of *Z. bailii* in 2% Sabouraud Glucose Broth with a pH of 3.5 and at three different aw (0.99, 0.97 and 0.95) and temperatures (45, 50 and 55°C) levels. Consistent with our findings, the D value at 45°C obtained with thermal treatment (TT) was significantly greater than that obtained with thermoultrasonication (TUT) (*p*<0.05). These authors found that at 45°C and at 0.99, 0.97, and 0.95 aw, the D value was reduced from 15.4 to 7.4, 26.8 to 8.6 and 43.5 to 12.9, respectively, with TT and TUT. Additionally, along with the reduction in aw, an increase in the D values was observed. Furthermore, a lower aw was associated with a greater synergistic effect in TUT. In the present study, the average D_{45} values of *Z. rouxii* for 0.99 and 0.94 aw at 45°C T-T (pH=4) were 98.66 and 140.1 min, respectively. In contrast, treatment TS-T under the same conditions (80% amplitude: 99.2 μm) resulted in D_{45} values of 0.58 and 4.33 min at 0.99 and 0.94 aw, respectively.

Inactivation of *Z. rouxii* at 50°C

In our sonication treatments, the minimum possible sampling interval from the sonication vessel was 20 s. Therefore, in some combined treatments, especially those conducted with high temperatures and high aw values (i.e., aw=0.99; 50 and 55°C), samples were taken after the first 20 s, and there were typically no viable yeast cells (for this reason, some D values in Figs. 3a and 4a are shown as <0.2 min). Additionally, yeast reductions are shown as >5.7-log CFU/mL because the maximum yeast reduction was determined as 5.7-log CFU/mL in this study (Figs. 3b and 4b).

Similar to our results obtained at 45°C, the estimated D_{50} for *Z. rouxii* at 50°C and 0.94
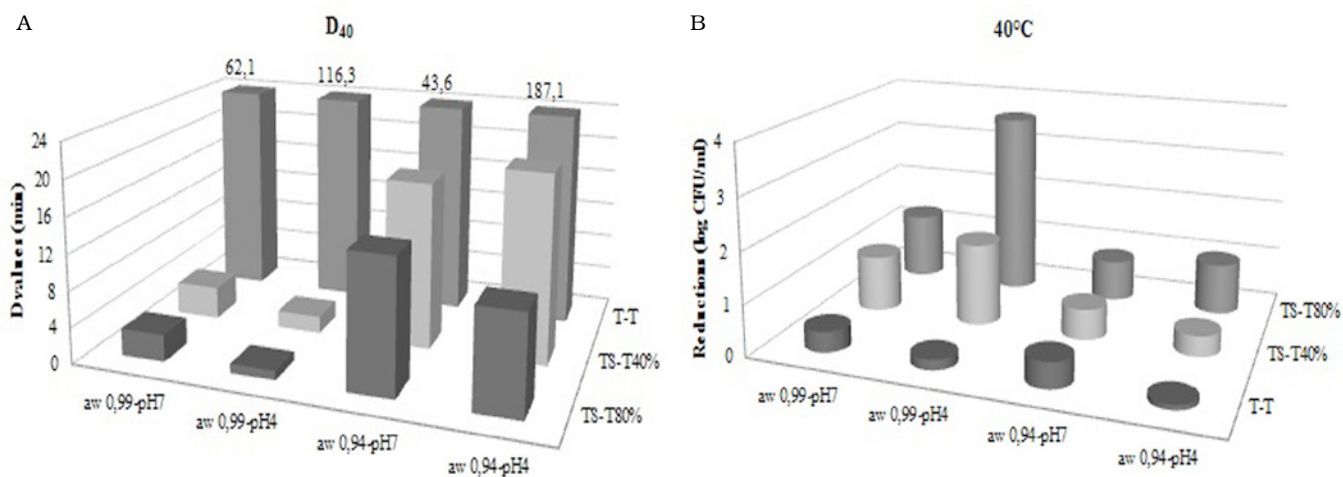
Fig. 1 - D_{40} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 40°C (B).
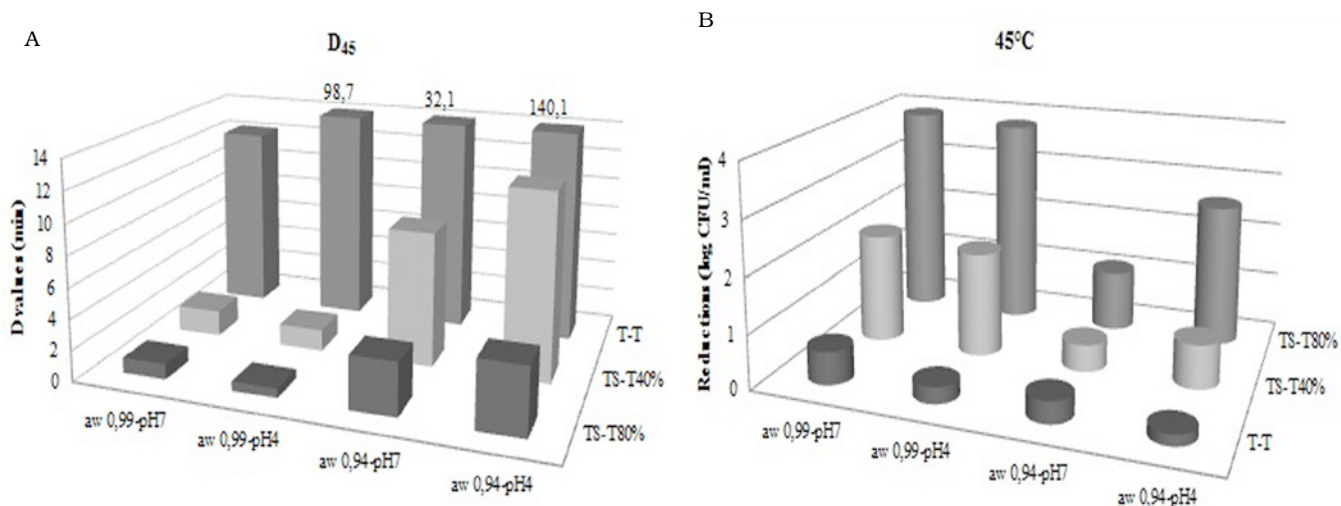

Fig. 2 - D_{45} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 45°C (B).
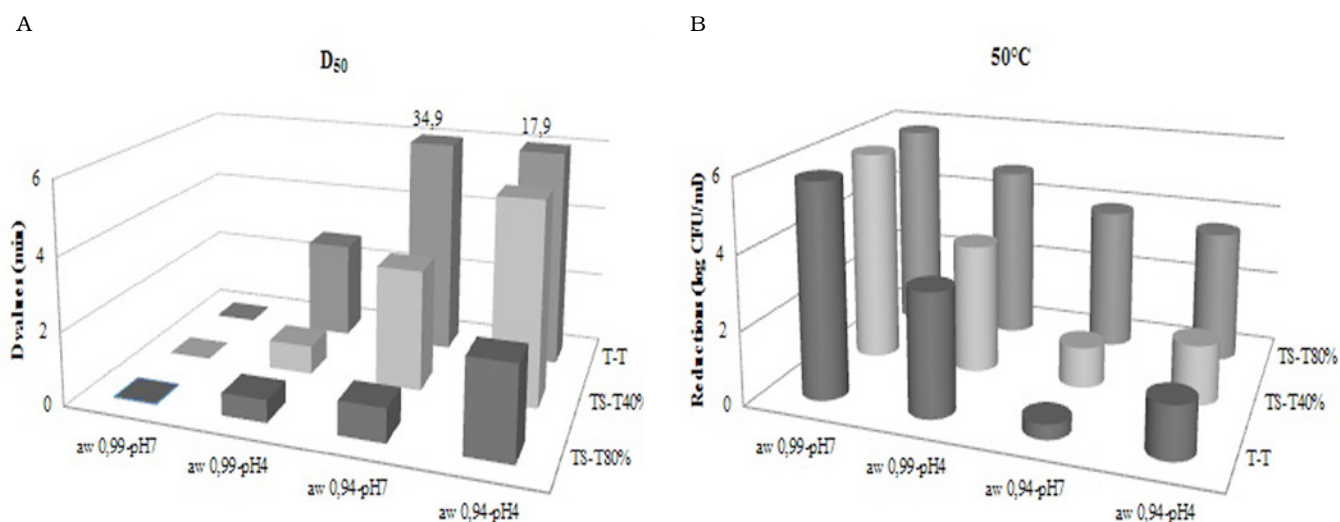

Fig. 3 - D_{50} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 50°C (B).
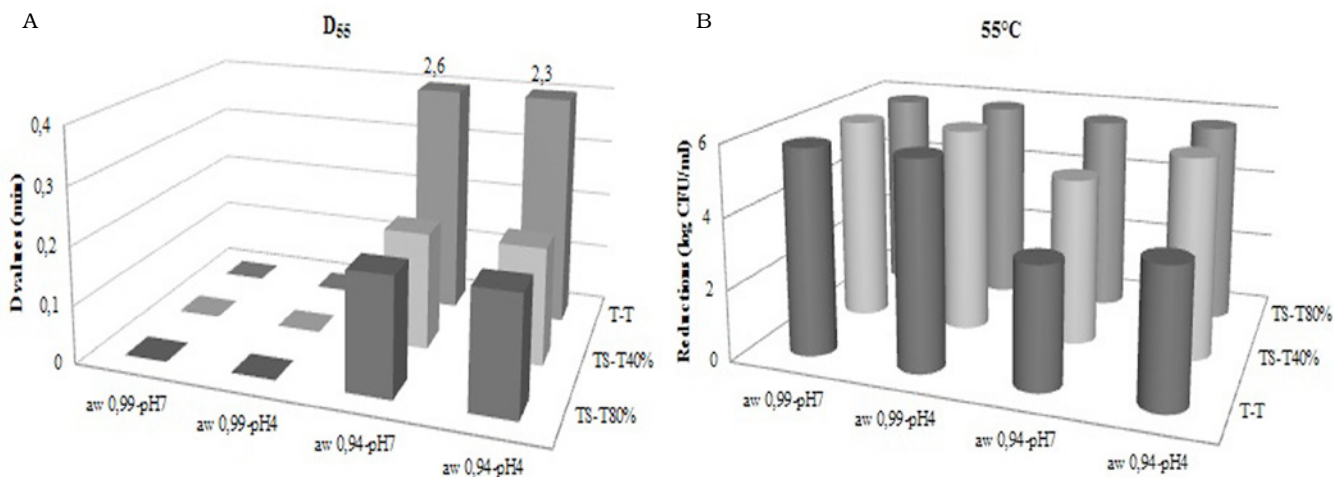
Fig. 4 - D_{55} values of *Z. rouxii* obtained from the TS-T and T-T (A) and reductions of *Z. rouxii* after TS-T and T-T at 55°C (B).

aw was greater than that observed at 0.99 aw (Fig. 3a). *Z. rouxii* showed a greater resistance to combined treatments at a pH of 4 than at a pH of 7, most likely because *Z. rouxii* is a yeast with good adaptation to lower pH values. The maximum D_{50} at 0.99 aw and pH 4 was 0.8 minutes, whereas the D_{50} value at pH 7 was <0.2 minutes. In treatments at 0.99 aw and pH 4, the combined use of ultrasound (40% and 80%) provided a significant synergistic interaction for *Z. rouxii* inactivation. However, no such synergy could be observed at 0.99 aw and pH 7 for the combined treatment. A synergistic effect could be observed at 0.94 aw, with pH values of 4 and 7, with sonication (40% and 80%), and with D_{50} values greater than that observed with 0.99 aw. Compared with controls under the same temperature conditions, the reduction in D_{50} in sonications of 0.94 aw (pH=4 and 7) and 0.99 aw (pH = 4 and 7) was 1/4-1/16 and 1/3-1/6, respectively. And the reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 1.1->5.7 log CFU/ml and 3.6->5.7 log CFU/ml, respectively (Fig. 3b).

Inactivation of *Z. rouxii* at 55°C

Compared with control treatments, the D_{55} values obtained with the combined treatments at 55°C and 0.99 aw suggested that the use of ultrasound did not result in a significant differences ($p>0.05$) in yeast inactivation and that heat was the primary determinant of inactivation.

In all combined treatments (at 0.94 aw), the D_{55} values for *Z. rouxii* were determined to be 0.2 minutes; at 0.99 aw, the D_{55} values were determined to be <0.2 minutes (Fig. 4a). Compared with controls at the same temperature levels, the reduction in D_{55} at sonications at 0.94 aw and pH levels of 4 and 7 was 1/8. In a similar study by GUERRERO *et al.* (2001), the inactivation of *S. cerevisiae* was examined at differ-

ent amplitude levels (20 kHz, 71.4 and 107.10 μm), pH values (3 or 5.6), and temperatures (35°, 45°, and 55°C) in Sabouraud broth. In line with our findings, the D value at 55°C was lower that obtained at other temperature levels (i.e., 35° and 45°C) ($p<0.05$), whereas sonication, amplitude, and medium pH were not associated with a change in that reduction. However, in our study, the combined treatment with 0.94 aw resulted in an increased yeast inactivation ($p<0.05$), regardless of the pH and amplitude, and was associated with a synergistic effect. The D_{55} values obtained for all sonication procedures at 80% were lower than those obtained at 40%, although the differences were not statistically significant ($p>0.05$).

The reduction in *Z. rouxii* for the 40% and 80% amplitude levels was 4.7->5.7 log CFU/mL and 5.6->5.7 log CFU/mL, respectively (Fig. 4b).

Viability of yeast cells in treated samples during storage

The growth during storage of sublethally injured yeast after combined treatments was tested. Samples were taken from treatments in which the 5-log cycle yeast reductions had been achieved (Figs. 3b and 4b) and stored for 60 d under different storage temperatures (4° and 25°C). As a result, none of the samples exhibited yeast growth during storage. This findings suggests that thermo-sonication is associated with irreversible cell damage. In a study by MARX *et al.* (2011) examining the effect of continuous and pulsed thermo-sonication (20 kHz frequency, at 60°C, 100% amplitude, for 30 min) on *S. cerevisiae* inactivation, the structural damage occurring in yeast cells after treatment was examined using scanning electron microscopy. They observed more broken cells using continuous rather than pulsed thermo-sonication treatments; however, they did not find any viable cells in their samples.

CONCLUSIONS

Compared with controls, all thermo-sonication procedures at 40, 45, 50 and 55°C resulted in a significant decrease in the D values (p=0.00) for *Z. rouxii* in our study. This finding shows a decreased resistance of *Z. rouxii* cells to heat together with the use of US.

The amplitude of the ultrasound waves was effective in the reduction of yeast cells, with lower D values obtained at the 80% amplitude than at 40%.

The use of US, particularly in medium with a low aw, resulted in significant synergistic effects for *Z. rouxii* inactivation. However, thermo-sonications performed at low aw (0.94) were associated with a more prolonged D value and a less marked reduction. Additionally, low aw was associated with the relative protection of yeast cells against thermo-sonication, particularly at lower temperatures.

Furthermore, as the sonication temperatures increased, the effects of amplitude, medium pH and aw on yeast reduction tended to weaken. Increased sonication temperatures (50° and 55°C) resulted in significant yeast inactivation (>5.7-log reductions). In most of the combined treatments at 50° and 55°C, the FDA requirement of a minimum of 5-log cycle reduction (within <0.2-0.2 min) could be met. However, although heat was the primary determinant of the yeast inactivation in combined treatments with high aw (55°C), the synergistic effect of US was more prominent than at 0.94 aw. The absence of yeast growth at 60 d that was observed in the samples obtained from the sonication chamber after combined treatments indicates that thermosonication was associated with irreversible yeast damage.

The findings of this study indicate that US combined with mild heat treatments (50° and 55°C) has the potential to inactivate *Z. rouxii* in fruit juices and beverages as an alternative to traditional pasteurization methods. Particularly for pasteurizing fruit juices to retain their structural and sensory qualities at higher temperatures, the use of US may offer certain advantages with respect to reducing the duration and temperature of the treatment.

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