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1 **Ultrasound-assisted fermentation for cider production from**
2 **Lebanese apples**

3

4 Marina AL DACCACHE ^{1,2}, Mohamed KOUBAA ^{3*}, Dominique SALAMEH ², Richard G.
5 MAROUN ², Nicolas LOUKA ², Eugène VOROBIEV ¹

6

7 ¹ Sorbonne University, Université de technologie de Compiègne, ESCOM, EA 4297 TIMR,
8 Centre de recherche Royallieu, CS 60319, 60203 Compiègne cedex, France.

9 ² Faculté des Sciences, Centre d'Analyses et de Recherche, UR TVA, Laboratoire CTA,
10 Université Saint-Joseph, Beyrouth, Lebanon.

11 ³ ESCOM, UTC, EA 4297 TIMR, 1 allée du réseau Jean-Marie Buckmaster, 60200
12 Compiègne, France.

13

14 Corresponding author: Mohamed KOUBAA

15 Email: m.koubaa@escom.fr

16 **Abstract**

17 The present work studies the impact of low-intensity ultrasound (US) on *Hanseniaspora*
18 sp. yeast fermentations. The effect of pulse duration and growth phase on US application was
19 first evaluated using a synthetic medium. The optimal conditions were then applied to apple
20 juice US-assisted fermentation. An US treatment chamber was first designed to allow the
21 recycling of the culture medium. The optimal US pulse duration on the yeast growth rate was
22 of 0.5 s followed by 6 s rest period, and during 6 h of both Lag and Log phases. These US
23 parameters led to a faster consumption of glucose in the medium during the fermentation,
24 compared to the untreated culture. The impact of US was also depending on the growth
25 phase, showing higher sensitivity of the yeast to US during the Lag phase rather than the Log
26 phase. US-assisted fermentation of apple juice showed a significant increase in biomass
27 growth and glucose consumption, along with a significant decrease in the ethanol yield. The
28 fastest growth kinetic (by 52%), and the highest ethanol reduction (by 0.55% (v, v)) were
29 obtained for the treatment during the first 12 h of fermentation, thereby, the stationary phase
30 was reached faster, and the maximum biomass growth rate was 10 folds higher compared to
31 the untreated culture. The results obtained in this study demonstrated the promising
32 efficiency of US-assisted fermentation in stimulating the biomass growth and reducing the
33 ethanol content in alcoholic beverages.

34

35 **Keywords:** *Hanseniaspora* sp.; ultrasound-assisted fermentation; stimulation; cider; low
36 alcohol content; Lebanese apples

37 **1. Introduction**

38 Ultrasound (US) is defined as acoustic waves whose frequency is equal or greater than
39 20 kHz. It has been widely applied in medical diagnosis, sonochemical treatment, ultrasonic
40 cleaning of surfaces, and in submarines [1,2]. Most applications for microbial inactivation in
41 food processing use low frequency US, with a frequency ranging from 20 to 100 kHz [1,3].
42 This is mainly related to the several benefits of US, which is considered as “green”
43 technology, compared to conventional processes. The benefits of US include shortening the
44 processing time (seconds to minutes) with high reproducibility and accuracy, reducing the
45 processing cost, generating highly pure product, eliminating some of the downstream
46 purification steps, as well as reducing the post-treatment of wastewater [4,5].

47 While high-intensity US ($10\text{-}1000\text{ W.cm}^{-2}$) is usually effective in disrupting microbial
48 cells, low-intensity US ($<10\text{ W.cm}^{-2}$) can result in an intensification of several
49 biotechnological processes without damaging the cells [1,6,7]. The mechanisms underlying
50 productivity improvements have not been so far identified, although factors such as improved
51 gas-liquid mass transfer (liquid phase gas absorption) and solid-liquid mass transfer
52 (dissolution of a solid in the liquid phase) play an important role [8]. Furthermore, US
53 induces cavitation that generates microbubbles at different sites in the liquid. The formed
54 microbubbles grow during the rarefaction phase and then implode and collapse during the
55 compression phase, releasing thereby a wave shock in the medium [4]. Besides, pressure
56 fluctuation during US treatments can induce stress on the cells, possibly promoting cell
57 growth and proliferation, as well as changes in the metabolic processes [8–10].

58 Several applications of low-intensity US have been described in food processing, and
59 particularly in food fermentation to improve the performance of microorganisms, and the
60 quality of the end product [11–13]. It has been reported that the application of US in the dairy

61 fermentation can induce some modifications in macromolecules such as enzymes [14,15],
62 reduce the yogurt fermentation time [16,17], and improve the yogurt's rheological properties
63 [18]. Besides, US was used for the proliferation of probiotics (e.g. *Bifidobacterium* sp.),
64 which caused an increase in the lactose hydrolysis, and transgalactosylation in milk [19,20].

65 One of the promising fields described in the literature for the application of US-assisted
66 fermentation is the production of alcoholic beverages. For instance, Matsuura and co-workers
67 reported a significant reduction of wine and beer fermentation time (by 50%) when applying
68 US intensity of 30 mW.cm^{-2} with a frequency of 43 kHz. An acceleration of ethanol
69 production and a decrease in the dissolved CO_2 were also noted [21]. A similar observation
70 was reported by Lanchun and co-workers by applying US with a frequency of 24 kHz, a
71 power of 2 W, and a stimulation time of 1 s after intermitting for 15 s during 30 min of the
72 exponential phase [22]. Results showed growth acceleration by 33% of the *Saccharomyces*
73 *cerevisiae* cells. The impact of US depends as well on the period of growth phase (e.g.
74 adaptation, exponential, etc). For example, the effect of the energy applied during the Lag
75 phase of *S. cerevisiae* yeast culture was studied [23]. Applying US energy between 330 and
76 360 W.s.m^{-3} decreased the Lag phase duration by 1 h compared to the untreated suspension,
77 whereas applying higher energy than 850 W.s.m^{-3} led to a longer Lag phase and a growth
78 reduction. There is a great variability in the sensitivity of different microorganisms to US,
79 which strongly depends on the properties of the medium and the parameters of the treatment
80 (intermittent or continuous application of US). For example, it has been shown that the
81 intermittent sonication (25 kHz, 300 W.m^{-3}) of *S. cerevisiae* yeasts throughout the
82 fermentation more than doubled the ethanol yield, whereas the same effect did not occur for
83 the continuously-treated cultures [24]. A 4-fold increase in ethanol productivity was observed
84 when applying US (35 kHz, 1.48 W.cm^{-2}) to a 10% cycle (equivalent to sonication for 1 min
85 followed by a rest period of 9 min) on *S. cerevisiae* MTCC 170 [25]. By contrast, a recent

86 study has reported that the application of either direct US (at different intensities ranging
87 from 23 to 32 W.L⁻¹) or indirect US (1.4 W.L⁻¹ intensity) had negative effects on yeast
88 performances [26]. Both reductions in the rates of glucose consumption and ethanol
89 production were also observed.

90 Thus, high variability was observed for the impact of US on fermentation, which
91 depends on the type of microorganism, the US parameters, the growth phase, etc. This makes
92 understanding the mechanisms underlying the process more difficult, and therefore more
93 studies are required. In this line, the current work is devoted to study the impact of US-
94 assisted fermentation on *Hanseniaspora* sp. growth and glucose uptake during cider
95 production. The genus *Hanseniaspora* was the major yeast developed during spontaneous
96 apple juice fermentation [27,28]. Besides, Capozzi and co-workers demonstrated the positive
97 impact of *Hanseniaspora* sp. in alcoholic fermentation and in cider fermentation [29], which
98 can positively contribute to the aroma profile of the fermented apple juice [30].

99 **2. Materials and methods**

100 *2.1. Chemicals, yeast, and apples*

101 Peptone and ethanol (C₂H₆O) were bought from Fisher Scientific (Illkirch, France).
102 Yeast extract was supplied by Sigma-Aldrich (Saint-Quentin-Fallavier, France). Glucose and
103 fructose were provided by Merck Millipore (Guyancourt, France).

104 *Hanseniaspora* sp. strain used in this work belongs to the authors' laboratory collection.
105 The strain was isolated previously from spontaneous fermentation of a Lebanese apple juice
106 obtained from the "Ace spur" variety [31].

107 The apples ("Ace spur" variety) used to conduct fermentation experiments were supplied
108 from Bqaatouta village (Keserwan region, Lebanon).

109 2.2. *Fermentation processes*

110 2.2.1. *Hanseniaspora* sp. *pre-culture*

111 The pre-culture of the *Hanseniaspora* sp. strain was performed by taking one colony
112 from YPD agar (1 % yeast extract, 2 % peptone, 2 % dextrose, and 2 % agar) plate into 250
113 mL of YPD (1 % yeast extract, 2 % peptone, and 2 % dextrose) medium under sterile
114 conditions (20 min at 121 °C in an autoclave HMC HV-110L (HMC Europe GmbH,
115 Germany)). The pre-culture was incubated during 45 h at 30 °C temperature and 250 rpm
116 agitation speed in a shaker (Thermo Scientific, France).

117 2.2.2. *Fermentations in synthetic medium*

118 2.2.2.1. *Control fermentations*

119 A volume of 1.5 L of YPD medium (1 % yeast extract, 2 % peptone, and 2 % dextrose)
120 was introduced into a 2-L fermenter (LSL Biolafitte S.A.). After sterilization as above-
121 mentioned and cooling to 30 °C, the medium was inoculated by a pre-culture to get an initial
122 concentration of 4.5×10^6 cells.mL⁻¹. The control fermentation in batch mode without medium
123 circulation (Figure 1.A) was performed at 250 rpm agitation speed, and the temperature was
124 maintained at 30 °C. After recording the kinetics of growth and glucose consumption in batch
125 mode without medium circulation, a treatment chamber was designed to allow the circulation
126 of the medium and the yeast treatment by US during the fermentation (Figure 1.B). For this
127 purpose, two parallel stainless-steel plates (3 mm width, 6 cm diameter) were fixed between
128 two Teflon rings creating a channel for the flowing liquid. The volume of the treatment
129 chamber was around 40 mL (corresponding to a ratio of 1:50 between the volume of the
130 fermenter and the volume of the chamber). Two pipe fitting connectors were adjoined to
131 make it run as a continuous chamber. A transducer (100 W, 40 kHz) fixed to the chamber and
132 connected to the power supply (220 V) converted the available electrical voltage line into

133 high-frequency waves. A peristaltic pump allowed the medium circulation from the reactor to
134 the treatment chamber and back into the reactor. Various circulation flow rates were tested
135 (25, 50, and 75 mL.min⁻¹) without US application to get the same growth and glucose
136 consumption kinetics obtained in batch mode without circulation (Figure 1.A). The total
137 length of the tubes used was equal to 90 cm. The treatment chamber and the tubes were
138 sterilized as above-mentioned.

139 2.2.2.2. *US-assisted fermentations*

140 The designed treatment chamber (Figure 1.B) was used for the stimulation of
141 *Hanseniaspora* sp. yeast. The stimulation conditions were first optimized using the synthetic
142 medium. The US treatment was done in a cyclic mode with variable periods of US pulses of
143 $\Delta t_p = 0.5, 1, \text{ and } 2$ s, followed by pauses of $\Delta t_w = 6$ s (Figure 2). The variation of US
144 application period allowed the optimization of the treatment. US-assisted fermentation was
145 initially performed during the first 6 h of fermentation (associating both the Lag and the Log
146 phases). US-assisted fermentation was then performed during either Lag phase (3 h) or Log
147 phase (3 h) using each time optimal US treatment.

148 2.2.3. *Fermentations in apple juice*

149 2.2.3.1. *Control fermentations*

150 The apples were first washed and then used to extract the juice using a fruit juice
151 centrifuge separator (Moulinex, France). A volume of 1.5 L of apple juice was introduced
152 into a 2-L sterile fermenter and inoculated by the pre-culture to get an initial concentration of
153 3.0×10^6 cells.mL⁻¹. Cider production was conducted for 100 h in batch mode, with medium
154 circulation at 30 °C temperature and 250 rpm agitation speed. The flow rate used for the
155 circulation was fixed to allow obtaining the same growth kinetics compared to the control
156 without medium circulation. The kinetics of growth, substrates consumption, and ethanol

157 production were recorded during the fermentation. The content of soluble compounds was
158 followed by measuring the °Brix using a digital refractometer (Anggur DR401, France).

159 2.2.3.2. US-assisted fermentations

160 The kinetics of growth, substrate consumption, and ethanol production of US-assisted
161 fermentation were compared to that of control fermentation (section 2.2.3.1). US-assisted
162 fermentation of apple juice was recorded during the first 12 h of the fermentation (including
163 the Lag phase). The application of US was also performed on the pre-culture for 6 h
164 (including the Lag and Log phases), followed by apple juice fermentation without US
165 treatment inoculated with the treated pre-culture.

166 US treatment was done in a pulsed mode with sequential application of n US pulses. The
167 total time of US cycles was 6 h (21600 s) and can be defined as follows: $t_t = n(\Delta t_p + \Delta t_w)$, where
168 Δt_p corresponds to the US pulse duration ($\Delta t_p = 0.5, 1, \text{ and } 2 \text{ s}$), and Δt_w is the pause duration
169 ($\Delta t_w = 6 \text{ s}$). The number of pulses was $n = 21600 / (\Delta t_p + \Delta t_w) = 2700, 3086 \text{ and } 3324$, while the
170 effective time of US treatment was $t_{US} = n \cdot \Delta t_p$ corresponding to 1662 s, 3086 s, and 5400 s.

171 The US generator allowed recording the energy E (Wh) during the treatment. The
172 specific energy consumption W ($\text{J} \cdot \text{mL}^{-1}$) was estimated according to equation (1):

$$173 \quad W (\text{J} \cdot \text{mL}^{-1}) = \frac{E \cdot 3600}{V} \quad \text{Equation (1)}$$

174 where E is the energy (Wh) provided by the generator, and V is the volume of the culture
175 medium in the bioreactor (mL).

176 2.2.4. Kinetics of growth, substrates consumption, and ethanol production

177 The kinetics of growth, substrates consumption, and ethanol production were recorded
178 for all the fermentation processes (control and assisted by US).

179 *2.2.4.1. Determination of the growth kinetics*

180 The growth kinetics were followed by both measuring the optical density (absorbance) at
181 600 nm, and counting the cell number (cells.mL⁻¹). Samples were taken periodically from the
182 bioreactor and their absorbance values were determined using a Thermo Spectronic BioMate
183 3 UV-visible spectrophotometer (Thermo Scientific, France). The samples were diluted
184 several times in water, and the cells were counted using a Thoma cell counting chamber
185 (Preciss, France). To detect the viable cells, the samples were mixed with a 0.01 % v/v
186 solution of methylene blue. The optical density was followed only for the fermentation in the
187 synthetic medium, while for the apple juice fermentations the biomass concentration was just
188 recorded by cell counting. The biomass concentration X (cells.mL⁻¹) was calculated according
189 to equation (2):

190
$$X \text{ (cells.mL}^{-1}\text{)} = \frac{a \cdot 250000 \cdot d}{b} \qquad \text{Equation (2)}$$

191 where a is the number of cells counted, d is the dilution factor, b is the number of squares
192 counted in the chamber, and 250000 corresponds to a constant included in the formula of the
193 Thoma cell.

194 The maximal specific growth rate μ_{max} (h⁻¹) was determined by plotting Ln X (cells.mL⁻¹)
195 versus time (h). The μ_{max} (h⁻¹) values corresponded to the slopes of the linear curves obtained
196 during the exponential phase.

197 *2.2.4.2. Carbon substrates quantification*

198 The carbon substrates were quantified enzymatically using a glucose and fructose assay
199 kit (LTA, Milano, Italy).

200 *2.2.4.3. Ethanol quantification*

201 The samples taken at different times of the fermentation processes were centrifuged at
 202 6000 rpm for 10 min to eliminate the yeast suspensions and apple particles. The ethanol
 203 content was determined by density measurements using a 5-mL glass pycnometer (Thermo
 204 Fisher, France). Calibration curve for ethanol quantification was performed using
 205 reconstituted solutions of apple juice/ethanol (1-10%, v/v).

206 The ethanol production rate γ ($\text{g}_{\text{ethanol}} \cdot \text{g}_{\text{biomass}}^{-1} \cdot \text{h}^{-1}$), the biomass yield $Y_{X/S}$ ($\text{g}_{\text{biomass}} \cdot \text{g}_{\text{substrate}}^{-1}$),
 207 and the product yield ($Y_{P/S}$ ($\text{g}_{\text{ethanol}} \cdot \text{g}_{\text{substrate}}^{-1}$) were calculated according to equations (3),
 208 (4), and (5), respectively.

$$209 \quad Y_{X/S} (\text{g}_{\text{biomass}} \cdot \text{g}_{\text{substrate}}^{-1}) = \frac{X_f - X_0}{S_0 - S_f} \quad \text{Equation (3)}$$

$$210 \quad Y_{P/S} (\text{g}_{\text{ethanol}} \cdot \text{g}_{\text{substrate}}^{-1}) = \frac{P_f - P_0}{S_0 - S_f} \quad \text{Equation (4)}$$

$$211 \quad \gamma (\text{g}_{\text{ethanol}} \cdot \text{g}_{\text{biomass}}^{-1} \cdot \text{h}^{-1}) = \frac{P_f - P_0}{X(t_f - t_0)} \quad \text{Equation (5)}$$

212 where X_0 and X_f are respectively the initial and the final biomass concentrations, P_0 and P_f are
 213 respectively the initial and the final ethanol concentrations, and S_0 and S_f are respectively the
 214 initial and the final substrate concentrations.

215 2.2.5. Statistical analyses

216 Each experiment was repeated three times. One-way ANOVA was used for the statistical
 217 analysis of the data with the help of Statgraphics Plus software (version 5.1, Statpoint
 218 Technologies Inc., Warrenton, VA, USA). A significance level of 5% was taken for each
 219 analysis. The error bars presented in the figures correspond to the standard deviations.

220 3. Results and discussion

221 3.1. Optimization of the experimental parameters

222 3.1.1. Treatment during the 6 first hours of fermentation

223 A medium circulation flow rate of 75 mL.min⁻¹ allowed obtaining the similar growth
224 kinetics than that of the control fermentation without circulation. It was thereby used for US-
225 assisted fermentations. The US parameters were optimized on the synthetic medium by
226 applying US, first during both Lag (adaptation) and Log (exponential) growth phases (6 h),
227 and then separately during either the Lag phase (3 h) or the Log phase (3 h). Results in figure
228 3 show an increase of the biomass (yeast) concentration with US treatment during both Lag
229 and Log phases (6 h). The biomass concentration increased more rapidly under the US
230 treatment than for the control samples, especially for the pulses duration $\Delta t_p = 0.5$ and 1 s,
231 which corresponded to respectively 1662 and 3086 s of effective US treatment. The
232 stimulation effect was more pronounced when applying US pulses for 0.5 s compared to that
233 for pulses of 1 s. Furthermore, increasing the pulse time up to 2 s (effective US treatment of
234 5400 s) seemed to decrease the yeast growth (Figure 3). The results of the US treatment of
235 *Hanseniaspora* sp. cells concur with those reported in the literature showing that cell
236 stimulation is energy-dependent, and the application of high energy can lead to a cell growth
237 reduction and yeast inactivation [23].

238 Treating the cell suspension for 6 h (pulses of 0.5 s followed each by a pause of 6 s)
239 allowed obtaining a significant increase in the final biomass concentration (after 8 h of
240 fermentation) by 63 %, which corresponded to an increase in the number of cells from
241 $9.0 \cdot 10^7$ (for the control (untreated) medium) to $1.5 \cdot 10^8$ cells.mL⁻¹ (for the US-treated
242 medium). These results concur with the measurements of the optical densities (absorbance
243 values at 600 nm), which showed a significant increase after 8 h of fermentation (from 2.43
244 for the control experiment to 2.97 for the US-assisted fermentation) (Figure 3.B). Moreover,
245 the highest specific growth rates μ_{max} of 0.54 and 0.56 h⁻¹ were observed for the US-assisted
246 fermentations with pulses of 0.5 s and 1 s, respectively, with no significant differences

247 between both growth rates (Table 1). These results concur with those of Barukčić and co-
248 workers, who reported an intensification of the cell growth by ≈ 1 log cycle following US
249 treatment, which led to a decrease in the fermentation time [32]. Besides, stimulation of
250 probiotics in milk was reported for the cultures exposed to low-frequency US [19,33]. Ewe
251 and co-workers noted as well an increase up to 9% of the US-treated cells compared to that of
252 the control during the fermentation of biotin-soymilk [34].

253 Figures 3.C and 3.D present respectively the evolution of glucose concentrations and
254 °Brix values for the US-treated and untreated fermentations. The results show that the values
255 of the °Brix concur with that of glucose concentrations. Both significantly depleted faster for
256 the 6 h US-treated cultures, compared to the untreated ones, with the fastest glucose depletion
257 achieved with the US pulse duration of $\Delta t_p = 0.5$ s. Likewise, after 8 h of fermentation, the
258 °Brix decreased from 5.9% (g.100 mL⁻¹) for the untreated culture to 4.8% (g.100 mL⁻¹) for
259 the US-assisted fermentation (with $\Delta t_p = 0.5$ s). Accordingly, the biomass yields per gram of
260 glucose consumed were significantly higher for the US-treated cultures (pulses of 0.5 and 1
261 s), compared to that of the control fermentation (Table 1). It has been reported in the
262 literature that US may increase the permeability of the cell membrane through the micro-
263 bubbles activity, creating temporary pores on the cell membrane [35]. Furthermore, the
264 formation of reversible pores could improve the transmembrane flow and thereby decreasing
265 the membrane resistance [36] and accelerating the transfer of substrates, which leads to
266 stimulating the cell growth [14,22].

267 *3.1.2. Treatment during either Lag or Log phase*

268 For the suspensions treated during the first 3 h (Lag phase), an increase in the yeast
269 biomass concentration by 43% was observed after 8 h of fermentation (Figure 4.A).
270 However, only an increase of 17% of yeast stimulation was recorded for the cells treated

271 during the Log phase (from 3 to 6 h of fermentation). Additionally, faster sugar depletion was
272 noticed during the Lag phase, compared to the Log phase (Figure 4.C). It was also confirmed
273 by a faster reduction of the °Brix for the treatment during the Lag phase (Figure 4.D). This
274 observation was previously reported by Lanchun and co-workers, who mentioned that the
275 effects of US on the growth of *S. cerevisiae* were significant during the Lag and the Log
276 phases, but not during the stationary phase [22]. They also noted that the yeast cells were less
277 resistant to environmental stress during the Lag phase, preparing for cell growth and
278 reproduction [22].

279 3.1.3. Energy consumption

280 Regarding the energy consumption, the US treatment performed during 6 h with pulse
281 duration of 2 s consumed the highest energy (269 J.mL⁻¹), and led to a growth kinetics
282 decrease compared to the US treatments with pulses of 0.5 s and 1 s (Table 1), which
283 consumed less amount of energy (respectively 155 and 197 J.mL⁻¹). The same amount of
284 energy (77 J.mL⁻¹) was consumed for the US treatments during one growth phase (either Lag
285 or Log); however, different impacts on the growth kinetics were observed.

286 Thus, the most effective parameters for *Hanseniaspora* sp. cells stimulation, among
287 those tested during this study, could be the application of 0.5 s US pulses followed by 6 s rest
288 period during either both Lag and Log phases or the Lag phase alone.

289 3.2. US-assisted fermentation of apple juice

290 The impact of US on ethanol accumulation during cider production was evaluated using
291 *Hanseniaspora* sp. yeast. An US treatment with pulse duration of 0.5 s followed by a pause
292 of 6 s was applied to either the pre-culture (YPD medium) or the apple juice yeast medium.
293 The growth phase durations of *Hanseniaspora* sp. on apple juice were determined during the

294 conventional fermentation of the apple juice (without US treatment, with or without medium
295 circulation).

296 3.2.1. Biomass growth kinetics

297 Results in figure 5.A show an increase in the biomass concentration by $\approx 52\%$ when the
298 apple juice yeast suspension was treated by US during the first 12 h of fermentation (without
299 treating the pre-culture). On the other hand, the biomass concentration increased by 42%
300 when the pre-culture was treated by US, followed by apple juice fermentation without any
301 treatment (Figure 5.A). The raise in the biomass concentration could be assigned to the
302 stimulation of the yeast cells' division during the US treatment. Different impacts of US have
303 been reported at the cellular and molecular level. US could promote microbial growth by
304 separating the cell groups, increasing thereby the membrane permeability and impacting the
305 cellular functions and components [14]. The values of the maximal growth rate, μ_{max} (Table
306 2) showed accelerated growth kinetics by 10 folds for the US-assisted fermentation. As a
307 result, the stationary phase was reached faster. These results concur with that of the literature,
308 where higher μ_{max} values were reported for *Lactobacillus sakei* cells treated with US [37].

309 Moreover, the treatment of the pre-culture showed a significant effect on the
310 fermentation kinetics. A higher μ_{max} value was obtained compared to the control, which
311 indicates that the stress of the yeasts during the pre-culture may influence later the
312 fermentation kinetics of apple juice. These results concur with the results of Matsuura and co-
313 workers, who observed a reduction of the fermentation time by 50% when using US (30
314 $\text{mW}\cdot\text{cm}^{-2}$, 43 kHz) [21]. Besides, an increase in the biomass production was reported for
315 *Kluyveromyces marxianus* fermentation treated by 20 kHz sonication at an intensity of 11.8
316 $\text{W}\cdot\text{cm}^{-2}$ for a duty cycle less than 20% (a 10% duty cycle: 1 s of sonication followed by a
317 pause of 10 s) [38]. Furthermore, when the fermentation was treated during the Lag phase, a

318 1 h reduction of the Lag phase was observed by Jomdecha & Prateepasen [23]. Finally, an
319 ultrasonic treatment of 1 s after intermitting for 15 s during 30 min can stimulate the growth
320 of *S. cerevisiae* by around 33.3%, and then the stationary phase was reached 4 h earlier
321 compared to that of the control [22,39]. Besides, Yang et al. have reported that US treatment
322 enhanced the growth of *Brevibacterium* sp. [40].

323 3.2.2. Ethanol production and sugar consumption kinetics

324 In terms of ethanol production, results in figure 5.B show a decrease in the ethanol
325 concentration by 0.41 and 0.55%, respectively for the treated pre-culture for 6 h, and the
326 treated juice during the first 12 h, compared to the control. Besides, a decrease in the specific
327 ethanol production rate γ_{\max} was observed in comparison with the control (Table 2). Figures
328 5.C and 5.D show an increase in glucose consumption while the same quantity of fructose
329 was used for the control and US-assisted fermentations. This result is also reflected by the
330 yield values, showing that the ethanol yield production per gram of fructose was constant for
331 all the fermentation processes, whereas the ethanol yield per gram of glucose decreased for
332 the US-assisted fermentations (Table 2).

333 These results could be explained by the fact that the fastest biomass kinetic (juice treated
334 by US during the first 12 h) could be associated with the lowest ethanol content (reduction of
335 0.55% (v,v) and ethanol production rate, and with the highest glucose consumption. The
336 yeast cells seem to consume more glucose to produce more biomass and CO₂ rather than
337 producing ethanol. In fact, it was reported that one of the probable impacts of the US is
338 improving the substrate consumption and oxygen uptake leading to a higher cell biomass
339 concentration [14,41,42]. Some studies have also reported that US may enhance the enzyme
340 activity [20,33,41,43]. In fact, applying US causes a strong micro-convection in the medium,
341 which may increase the mass transfer of substrates through the cell membrane, and alters the

342 enzyme conformation and the enzyme-substrate affinity [7]. Since higher concentration of
343 substrate is accessible by cells, associated enzyme activities may be promoted, leading to
344 higher glucose consumption and thereby higher biomass production. A thorough study of
345 enzymes' kinetics could be of great interest for a better understanding of microbial
346 stimulation by ultrasound.

347 It is important to note that different impacts of US on fermentation have been reported in
348 the literature depending on the intensity, the duration of the treatment, and the type of
349 microorganism being treated [44,45]. A recent study demonstrated a decrease in ethanol
350 production for the fermentations assisted by US [26]. A direct US treatment at 23 and 32
351 W.L⁻¹, or an indirect US (1.4 W.L⁻¹) treatment had undesirable effects on *S. cerevisiae*
352 activity and viability and induced a reduction in glucose consumption rates and ethanol
353 production [26]. Ojha and co-workers studied the impact of US on *L. sakei*, and revealed that
354 several metabolic pathways were affected by US [37]. Results also showed that the US-
355 treated *L. sakei* had significant variations in nutrient consumption. The ultrasonicated cells
356 preferred to consume other carbon sources in comparison with the untreated cells. These
357 results may explain the enhancement in the glucose uptake for the US-treated cells in the
358 current study (Figure 5.C). The metabolic pathway of *Hanseniaspora* sp. treated cells may be
359 redirected to produce more biomass than to produce ethanol. Thus, the results obtained in the
360 current study may highlight that US treatment of *Hanseniaspora* sp. cells could be a
361 promising way to reduce the ethanol content in alcoholic beverages and to control the
362 sugar/ethanol conversion rate. Particularly, US is classified as a technology that preserves the
363 qualitative characteristics of the treated products, such as flavor, odor, and visual appearance.
364 However, different disadvantages were reported in some studies affecting the final product's
365 texture [46]. In the current study, organoleptic analyses are required in order to evaluate the
366 volatile organic compounds and the aromatic profile of the untreated and US-treated ciders.

367 4. Conclusions

368 In this work, the effects of US treatment on *Hanseniaspora* sp. yeast cells were first
369 studied and optimized using synthetic medium. For an effective treatment time of 1662 s,
370 applied during both the Lag and Log phases (6 h) for pulse duration of 0.5 s followed by 6 s
371 pause, the yeast concentration and the biomass yield increased significantly. The depletion of
372 glucose was also significantly faster. For this short period of US exposure, the cells were
373 more sensitive to the treatment during the Lag phase rather than during the Log phase. The
374 yeast concentration and the biomass yield increased significantly during the Lag phase.

375 Afterward, the impact of US treatment on *Hanseniaspora* sp. cells was studied during the
376 apple juice fermentation. The yeast concentration and the biomass yield increased
377 significantly when the cells were treated during the first 12 h of the fermentation. Besides, the
378 ethanol content was reduced by 0.55% (v/v) and the biomass production rate was 10 folds
379 higher than the control. Furthermore, the yeast concentration and the biomass yield increased
380 significantly for the US-treated cells.

381 The obtained results demonstrate the potential for the practical implementation of the US
382 fermentation technology to reduce the ethanol content during the fermentation processes and
383 especially using *Hanseniaspora* sp. yeast cells. Nevertheless, further investigations are
384 required to understand the mechanisms behind microbial stimulation and ethanol reduction.

385

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389

390 **Conflict of interest**

391 The authors declare no conflict of interest

392

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520 **Figure captions**

521 **Figure 1.** **A.** Control batch mode fermentation without medium circulation, **B.** Control batch
522 mode fermentation with medium circulation (without US treatment). Insert represents a
523 schematic diagram of the designed treatment chamber.

524

525 **Figure 2.** Experimental set-up for the optimization of US-assisted fermentation of
526 *Hanseniaspora* sp. on YPD medium.

527

528 **Figure 3.** **A.** Growth kinetics, **B.** Optical densities at 600 nm, **C.** Glucose consumption
529 kinetics, and **D.** Soluble compounds (°Brix) depletion kinetics for the control and the US-
530 assisted fermentations with US treatment during the first 6 h (both Lag and Log phases). 0.5 s
531 pulse / 6 s pause denotes applying US pulses of 0.5 s followed by a pause period of 6 s.

532

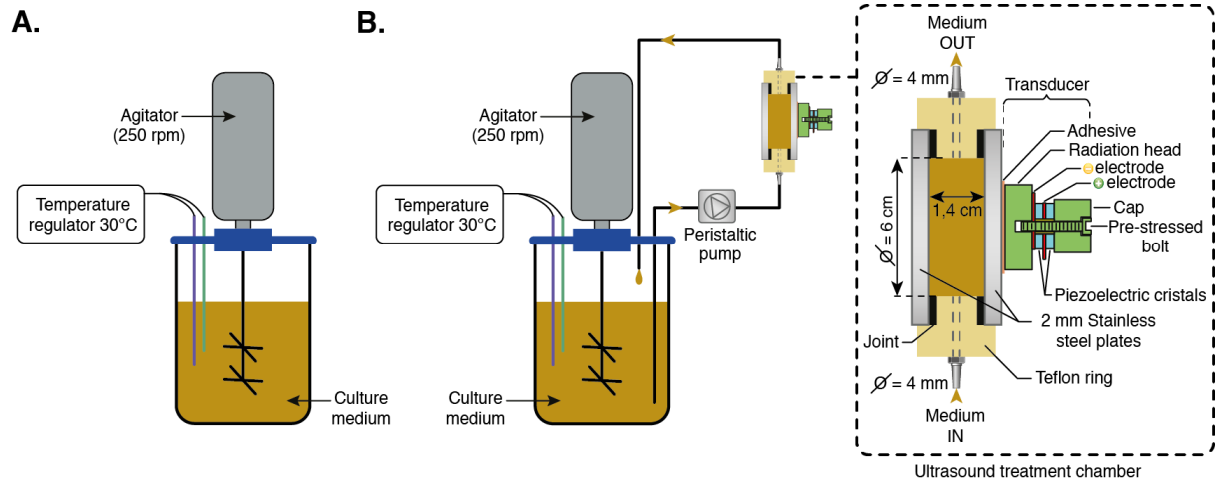
533 **Figure 4.** **A.** Growth kinetics, **B.** Optical density measurements at 600 nm, **C.** Glucose
534 consumption, and **D.** Soluble compounds depletion kinetics, for the control and the US-
535 assisted fermentations, during the Lag (0-3 h of fermentation) and the Log (3-6 h of
536 fermentation) phases separately, and during both phases (0-6 h of fermentation) treated with
537 pulse duration of 0.5 s, followed by pause of 6 s.

538

539 **Figure 5.** **A.** Growth kinetics, **B.** Ethanol production kinetics, **C.** Glucose consumption
540 kinetics, and **D.** Fructose consumption kinetics for the control and the US-assisted
541 fermentations (pulses of 0.5 s followed each by a pause of 6 s).

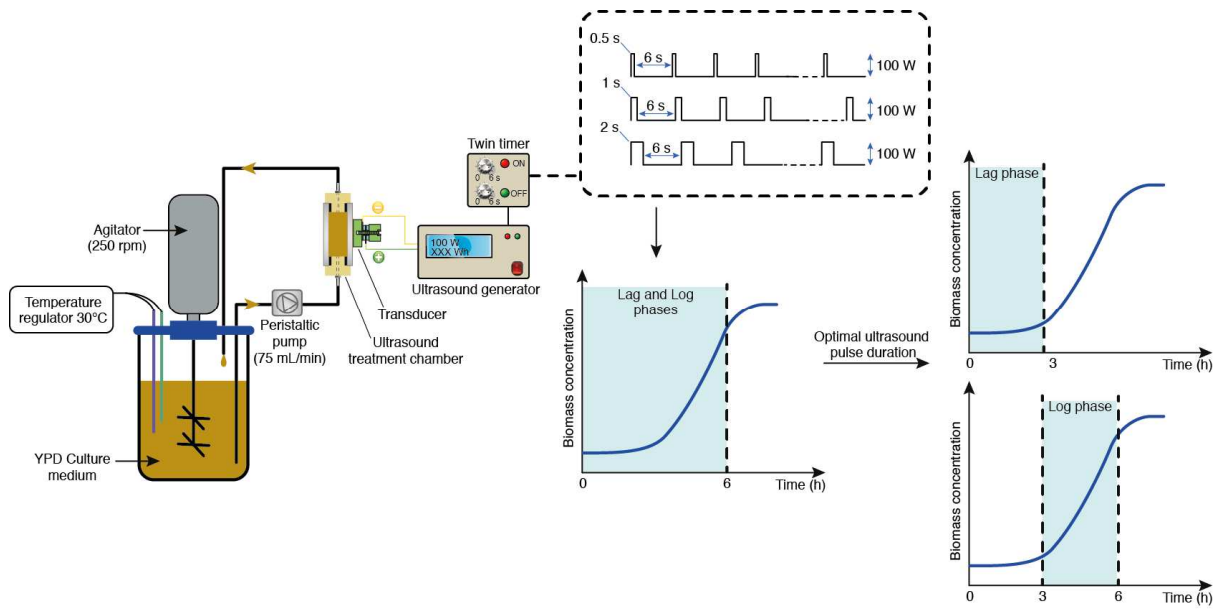
542 **Figures**

543 **Figure 1.**



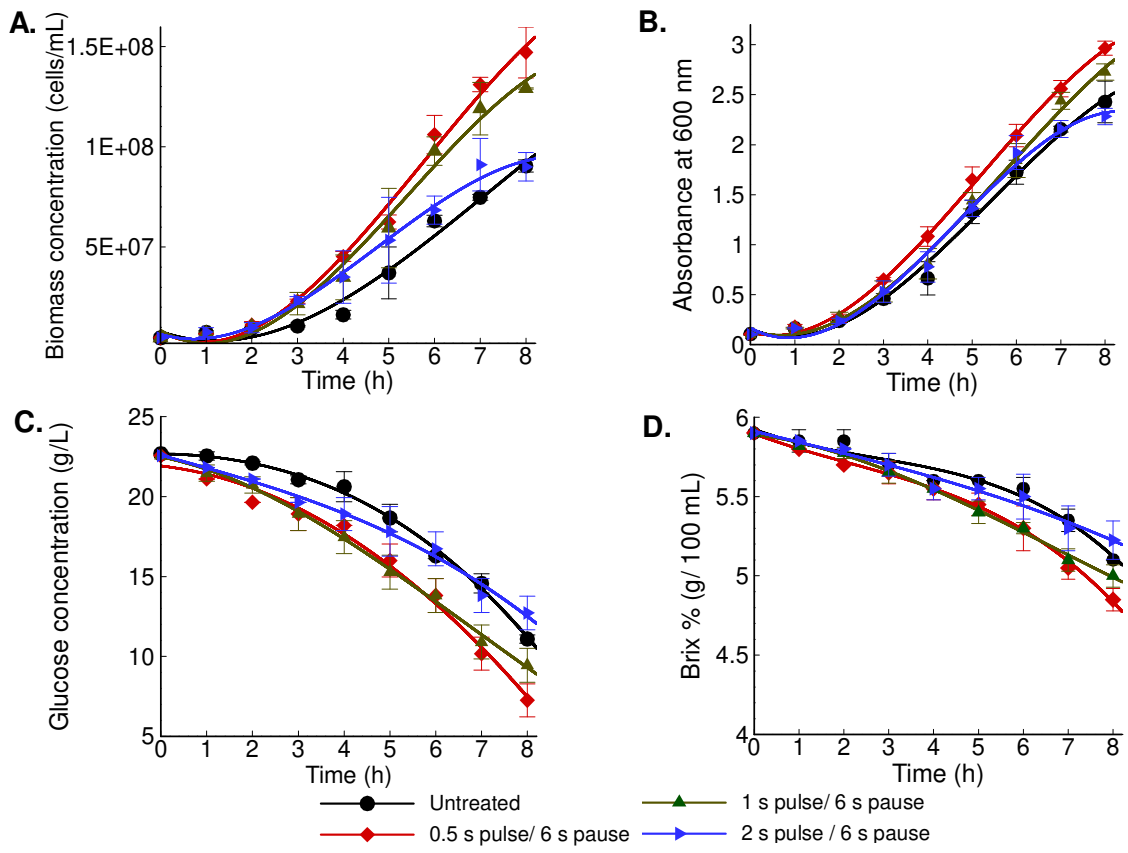
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545 **Figure 2.**

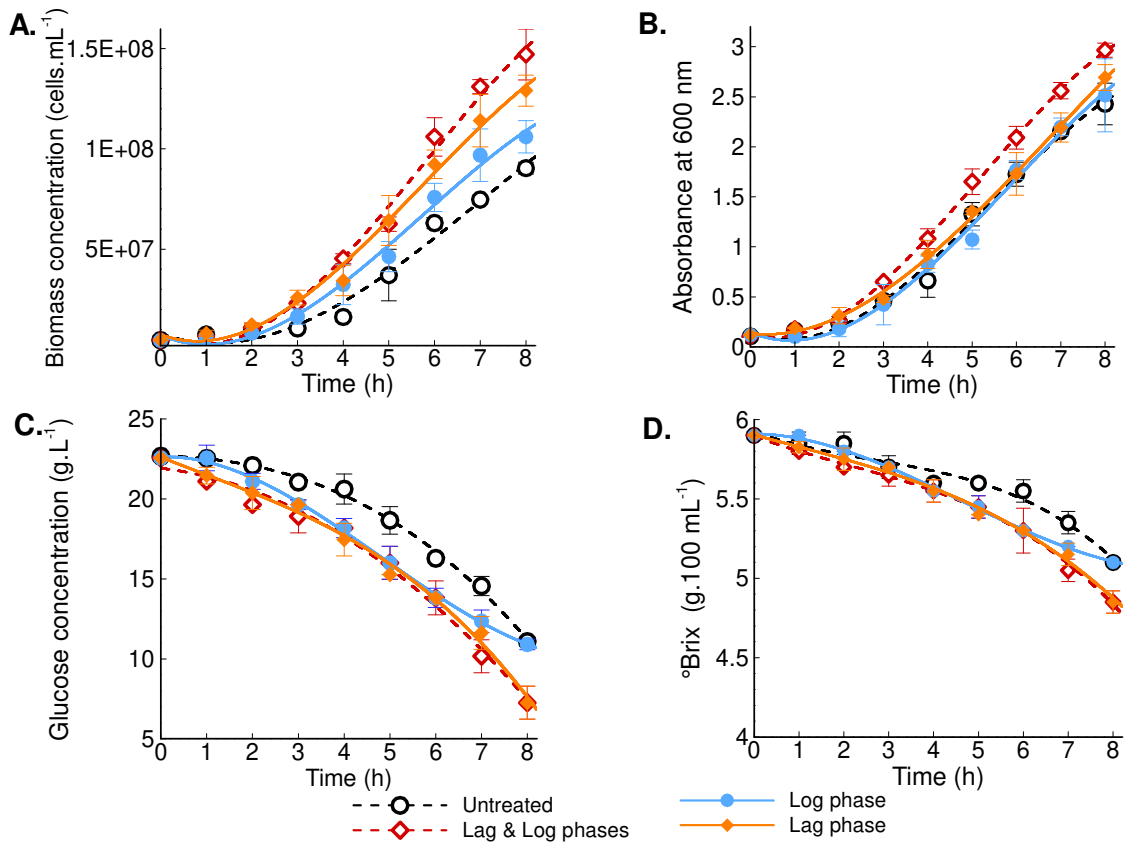


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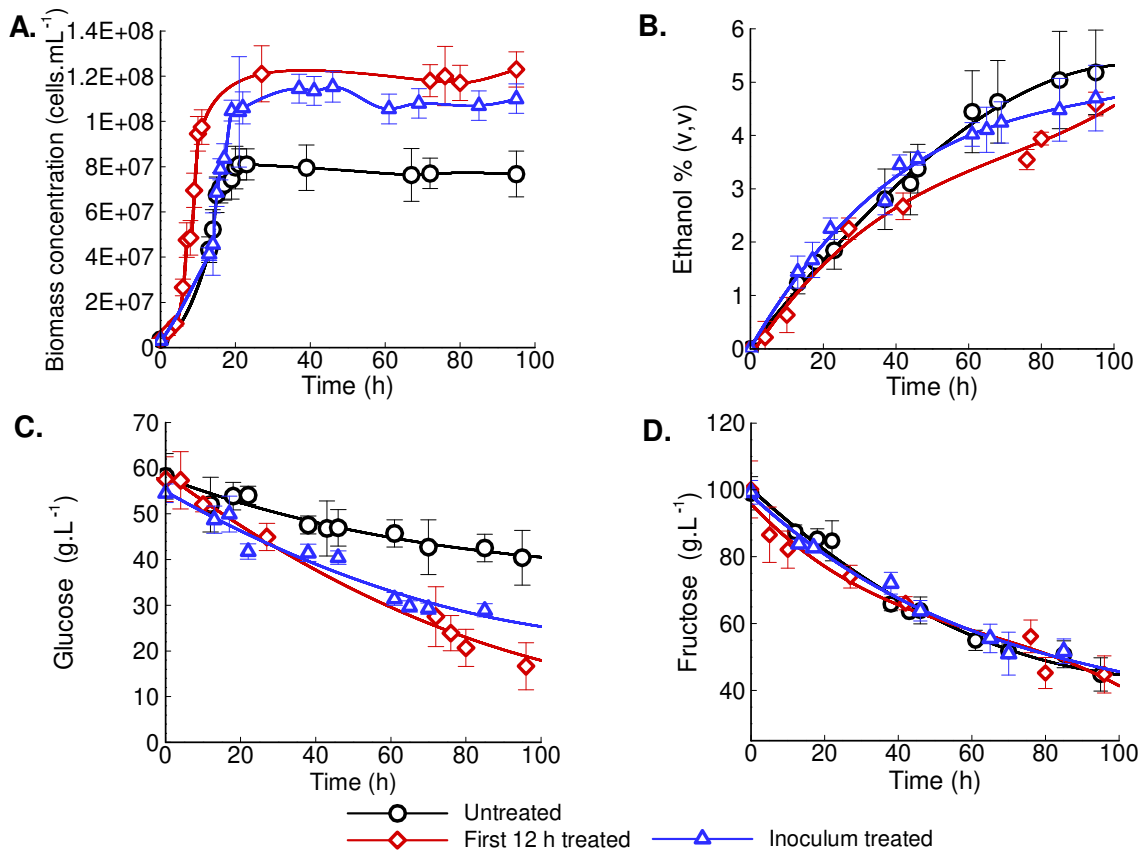
547 **Figure 3.**



548



551 **Figure 5.**



552

553 **Tables**

554 **Table 1.** Energy consumptions, and fermentation process performances (Y (g biomass . g glucose⁻¹),
 555 μ_{max}) during US treatment. 0.5 s pulse / 6 s pause denotes applying US pulses of 0.5 s
 556 followed by a pause period of 6 s.

	W (J.mL⁻¹)	μ_{max} (h⁻¹)	Y (g biomass . g glucose⁻¹)
Untreated	-	0.39 ± 0.004	0.08 ± 0.003
US treatment during both Lag and Log phases (6 h)			
0.5 s pulse / 6 s pause	155 ± 3	0.54 ± 0.01	0.15 ± 0.002
1 s pulse / 6 s pause	197 ± 5	0.56 ± 0.003	0.14 ± 0.001
2 s pulse / 6 s pause	269 ± 7	0.43 ± 0.002	0.12 ± 0.003
US treatment (0.5 pulse/6 s pause) during either Lag or Log phases (3 h)			
Lag phase treated	79 ± 1.5	0.50 ± 0.005	0.12 ± 0.002
Log phase treated	77 ± 1.4	0.48 ± 0.01	0.12 ± 0.004

557

558 **Table 2.** Fermentation process performances, and energy consumptions for the control and
 559 US-assisted fermentations.

	W (J.mL ⁻¹)	μ_{max} (h ⁻¹)	Y (g biomass . g glucose ⁻¹)	Y (g biomass . g fructose ⁻¹)	Y (g ethanol . g glucose ⁻¹)	Y (g ethanol . g fructose ⁻¹)	\mathcal{Y}_{max} (g ethanol . g biomass ⁻¹ .h ⁻¹)
Untreated	-	0.03 ±0.002	0.04 ± 0.001	0.01 ± 0.002	0.27 ± 0.007	0.07 ± 0.001	0.30 ± 0.01
First 12 h treated	311.4 ± 4.56	0.32 ± 0.05	0.05 ± 0.002	0.03 ± 0.001	0.07± 0.002	0.06 ± 0.002	0.15 ± 0.003
Inoculum treated	158.2± 3.2	0.05 ±0.003	0.05 ± 0.001	0.02 ± 0.001	0.13± 0.002	0.07 ± 0.002	0.28 ± 0.004

560