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Monitoring the influence of high power ultrasound treatment and thermosonication on inactivation of *Brettanomyces bruxellensis* in red wine

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Abstract

Brettanomyces bruxellensis is one of the most important spoilage microorganisms in winemaking. It is harmful for wine industry because it produces volatile phenols, compounds primarily responsible for off-odours in wine. One of the possible solutions for preventing its growth is using new non-thermal processing technologies. The aim of this study was to investigate the application of one non-thermal processing technology, high power ultrasound and its combination with heating (thermosonication) on the inactivation of B. bruxellensis in red wine in batch systems. Various parameters, such as treatment duration (1, 2, 3, 6, 10 and 15 minutes), temperature (25, 35, 40 and 43 ºC) and probe diameter (12.7 mm and 19.1 mm), were examined. The combination of high power ultrasound and heating (thermosonication) proved to be a better method compared to solely using high power ultrasound. However, the production of volatile phenols by B. bruxellensis was also reduced after high power ultrasound treatment. The optimal treatment of 3 min at 43 ºC with high power sonicator at ultrasound frequency of 20 kHz with 12.7 mm diameter ultrasonic probe for complete inactivation of B. bruxellensis was determined.

Keywords*: red wine, spoilage yeast, B. bruxellensis, high power ultrasound treatment, thermosonication*

Sažetak

Brettanomyces bruxellensis jedan je od najvažnijih mikroorganizama koji uzrokuju kvarenje vina. Ovaj kvasac je štetan za industriju vina jer ima sposobnost stvaranja hlapivih fenola, spojeva koji su odgovorni za neugodan miris vina. Za sprečavanje njegova rasta mogu se primijeniti nove procesne tehnologije tzv. netermalne tehnologije. U ovom istraživanju ispitana je primjena ultrazvuka visoke snage kao i zajednička primjena ultrazvuka visoke snage i zagrijavanja (termosonifikacija) za šaržnu inaktivaciju B. bruxellensis u crnom vinu. Tijekom istraživanja ispitan je utjecaj različitih parametara kao što su trajanje tretiranja (1, 2, 3, 6, 10 i 15 minuta), temperatura (25, 35, 40 i 43 ºC) i promjer sonde (12,7 i 19,1 mm). Utvrđeno je kako je zajednička primjena ultrazvuka visoke snage i zagrijavanja (termosonifikacija) bolja metoda za inaktivaciju ovog kvasca u odnosu na samostalnu primjenu ultrazvuka visoke snage. Ipak primjenom ultrazvuka visoke snage postignuta je smanjena proizvodnja hlapivih fenola pomoću kvasca B. bruxellensis. Potpuna inaktivacija kvasca B. bruxellensis postignuta je pri 43 ºC primjenom ultrazvuka frekvencije 20 kHz i sonde promjera 12,7 mm kroz 3 minute.

Ključne riječi*: crno vino, kvasac uzročnik kvarenja, B. bruxellensis, ultrazvuk visoke snage, termosonifikacija*

Introduction

Brettanomyces bruxellensis is a non-*Saccharomyces* yeast that has been found in different stages of winemaking as well as in bottled wine (Jolly et al., 2014). This yeast is generally associated with spoilage, especially in red wines. It is able to convert hydroxycinnamic acids into volatile phenols (4-ethylphenol and 4-ethylguaiacol), providing off-odours described as 'horse sweat', 'animal leather' and 'medicinal' even at low concentrations at order of magnitude of 3 log CFU mL^{-1} (Kheir et al., 2013). This effect causes a decrease in wine quality, leading to economic losses. *B. bruxellensis* is a slow growth yeast, but it can grow in adverse conditions (low pH, in the presence of high levels of ethanol and in presence of sulphur dioxide). The growth of *B. bruxellensis* can be controlled by the use of chemical, physical and biological methods (Mehlomakulu et al., 2015). Sulphur dioxide is the most used chemical preservative in winemaking. It can be used to control the growth of spoilage microorganisms such as *B. bruxellensis,* but it can affect human health (e.g. allergic reaction, asthma, diarrhea, headache or nausea) (Guerrero and Cantos-Villar, 2015). In recent years, there is a growing interest to replace the use of sulphur dioxide by applying non-thermal process technologies. Until now, several different non-thermal technologies such as pulsed electric fields (PEF), low electric current treatment (LEC), high hydrostatic pressure (HHP) and high power ultrasound (HPU) have already been tested for inactivation of *B. bruxellensis* in wine (Puértolas et al., 2009; Lu-

strato et al., 2015; Delsart et al., 2016; González-Arenzana et al., 2016; van Wyk and Silva, 2017; Luo et al., 2012; Gracin et al., 2016; Bermúdez-Aguirre and Barbosa-Cánovas, 2012). Among them, HPU has physical (cavitation and micro-mechanical shocks) and chemical (formation of free radicals) effects on inactivation of microorganisms (Piyasena et al., 2003; O'Brien, 2007; Chandrapala et al., 2012). HPU combined with heat treatment (thermosonication), pressure treatment (manosonication) or both (manothermosonication) has been shown to increase the inactivation of microorganisms (Awad et al., 2012; Abdullah and Chin, 2014; Huang et al., 2016). Additionally, HPU treatment can be performed in batch and continuous flow systems (Bermúdez-Aguirre and Barbosa-Cánovas, 2012; Mohideen et al., 2015). The use of HPU can also reduce treatment duration, which prevents the loss of organoleptic qualities of wine such as flavour, colour or taste (Awad et al., 2012). HPU can be applied in winery to minimize the influence of undesirable microorganisms during processing of must, alcohol fermentation, malolactic fermentation and sanitation of barrels (Jiranek et al., 2008; Yap et al. 2008).

The aim of this work was to investigate the impact of high power ultrasound (HPU) and the combination of HPU and heating (thermosonication) on the inactivation of *B. bruxellensis* in red wine during treatment in batch systems.

Materials and methods

Inoculum preparation

Brettanomyces bruxellensis CBS 2499 (from Westerdijk Fungal Biodiversity Institute) was grown in YPD (10 gL^{-1} yeast extract, 20 gL-1 peptone, 20 gL-1 glucose) liquid culture medium at 28 ºC without shaking. The inoculum was prepared as described by Delsart et al (2016). When the population reached the stationary phase in this medium, another medium was prepared and supplemented with ethanol (4 % v/v) to receive the previous culture (inoculated at 10 % v/v). Following that, a new medium was prepared with ethanol (8 % v/v) and inoculated via the second medium at 10 % v/v. The last medium was prepared with ethanol (12 % v/v) and inoculated via the third medium at 10 % v/v. Growth was followed by measuring the absorbance at 600 nm. Also, samples were decimally diluted and cellular culturability was determined by surface plating 0.01 mL onto YPD medium (with 20 gL-1 agar), in duplicate and incubating at 24 ºC for up to 7 days.

Cells of *B. bruxellensis* were harvested by centrifugation at 4000 × *g* for 10 min and inoculated into red wine (Cabernet Sauvignon from Croatia, 2016) at approximately 6 log CFU-mL-1. The initial cell concentration in wine was then confirmed by plate counts. Inoculation was performed 24 h before HPU treatment. Bottles were incubated at 20 ºC ± 2 °C and shaken prior to sampling.

High power ultrasound (HPU) treatments

Red wine samples (200 mL) inoculated with *B. bruxellensis,* were placed in a round-bottom glass vessel (250 mL), which served as a treatment chamber. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, CT, USA), set at nominal power of 600 W and 20 kHz was used for HPU treatments. Diameters of probes were 12.7 mm and 19.1 mm. Each probe was immersed in red wine (2.5 cm) and placed at the center of treatment chamber. Ultrasonication was carried out at the amplitude of 100 % or 120 µm (for 12.7 mm probe) and 60 µm (for 19.1 mm probe). The samples were isothermally treated for 3, 6, 10 and 15 min on 25 °C. For the thermosonication experiments, before the ultrasonic treatment, the samples were heated in water bath (about 2 min) at temperatures of 35, 40 and 43 °C. The samples were then treated by ultrasound with a 12.7 mm diameter probe for 1, 2 and 3 min. Isothermal conditions during the ultrasound treatment were achieved by cold water cooling of the treatment chamber. After HPU treatments samples were analyzed, and placed in equal size sterile bottles (100 mL) and stored for 90 days at 20 °C ± 2 °C. Samples were analyzed right after the thermosonication treatment.

Wine parameters

The physico-chemical wine parameters (pH, total and volatile acidity, reducing sugars and alcoholic strength) were determined before inoculation with *B. bruxellensis* using Fourier transform infrared spectroscopy (FTIR; Bacchus II, Microdom) equipped with autosampler. Determination of sulphur dioxide in wine was done by titration with iodine / iodine solution where the iodine was reduced and sulfur dioxide oxidized by potentiometric determination of the titration point via the LED indicator (LDS Sulfilyser, Laboratoires Dujardin-Salleron, Noizay, France).

Analysis of *B. bruxellensis* cell number

Before and after the HPU treatment and thermosonication treatment and during storage after 30, 60 and 90 days wine samples were analysed for the colony forming unit (CFU) of *B. bruxellensis* on commercial Brettanomyces agar plates (with 100 mgL-1 chloramphenicol, 10 mgL-1 cycloheximide and 100 mgL-1 coumaric acid; Conda, Spain) incubated for 7-10 days at 24 °C. Serial dilutions were carried out using sterile saline solution. From each dilution, 0.01 mL was plated out in duplicate. The number of colony forming units (CFU) can be calculated according to the following formula:

GC/MS analysis of volatile compounds

Wine sample volatile compounds were analyzed by gas chromatography coupled with mass spectrometry (GC/MS) using an Agilent Gas Chromatography 6890 series equipped with an Agilent 5973 Inert mass selective detector (Agilent Technologies, Santa Clara, CA, USA). Prior to GC/MS analysis, volatile compounds were extracted from wine by headspace solid-phase microextraction (HS-SPME) using 100 µm PDMS fiber (Supelco, Bellefonte, USA). 10 mL of a wine sample, containing internal standards (20 mgL-1 n-amyl alcohol and 0.5 mgL-1 *p*-cresol), were placed into a 20 mL headspace vial containing NaCl p.a. (2 g) and capped with a crimp cap and silicone-PTFE septum. The fiber was exposed to the wine headspace for 30 minutes at 40 °C with constant stirring. This was followed by thermal desorption for 5 minutes in the injector (splitless mode) at 250 °C (Tomašević et al., 2016). The target analytes were separated by gas chromatography using a BP20 capillary column (SGE Analytical Science, Victoria, Australia), dimensions 50 m x 220 µm with 0.25 µm film thickness. The interface temperature of the detector was kept at 250 °C and the ion source working in EI mode at 70 eV was

held at 280 °C. Helium 5.0 was used as a vector gas (Messer Croatia Plin d.o.o., Zagreb, Croatia). The initial temperature was set to 40 °C and maintained for 5 minutes, then raised to 200 °C for 3 °Cmin-1 and finally raised to 240 °C for 30 °Cmin-1 (Tomašević et al., 2016). Volatile compounds were identified using the Enhanced Chemstation software (Agilent Technologies, Santa Clara, CA, USA) and by comparing the peak retention times against those of authentic standards and matching the mass spectra against Nist05 mass library (Wiley & Sons, Hoboken, NJ, USA). For quantification, calibration curves for each compound were prepared and analyzed using GC/MS and the same extraction and chromatographic methods as for wine samples. Identified volatile compounds included ethyl esters (ethyl hexanoate, ethyl octanoate and ethyl decanoate), acetate esters (*i*-amyl acetate) and volatile phenols (4 ethyl phenol and 4-ethyl guaiacol).

Data analysis

Significant differences among control and treated wine samples for each of the constituents was determined by one-way analysis of variance (ANOVA) using the Statistica V.10 software (StatSoft Inc., Tulsa, USA). Tukey's honestly significant difference (HSD) test ($p<0.05$) was used for comparison when samples differed significantly after ANOVA was performed.

Table 1. *Physico-chemical parameters of red wine*

Parameter	Red wine
pH	3.46 ± 0.00
Total acidity (gL^{-1} tartaric acid)	5.85 ± 0.78
Volatile acidity (gL^{-1} acetic acid)	0.60 ± 0.01
Reducing sugars (gL^{-1})	3.90 ± 0.28
Alcoholic strength (% vol)	13.00 ± 0.14
Free SO_2 (mgL^{-1})	11.5 ± 0.7
Total SO_2 (mgL^{-1})	24.0 ± 1.4

Data are presented as average value of two repetitions ± standard deviation (n=2).

Results and discussion

To investigate the possibility of using HPU for batch inactivation of *B. bruxellensis* in red wine, the experiments were performed at 25 °C and heated to 35, 40 and 43 °C. During the HPU treatment at 25 °C, two probes (12.7 and 19.1 mm diameter) and four treatment durations (3, 6, 10 and 15 minutes) were used (Figure 1 and Figure 2). A slightly better inactivation of *B. bruxellensis* was achieved using a 12.7 mm diameter probe in comparison to 19.1 mm diameter probe. After 15 minutes treatment the maximum inactivation of 0.5 log CFUmL-1 was achieved using the 12.7 mm diameter probe, while the maximum inactivation achieved using the 19.1 mm diameter probe was 0.18 log CFUmL-1.

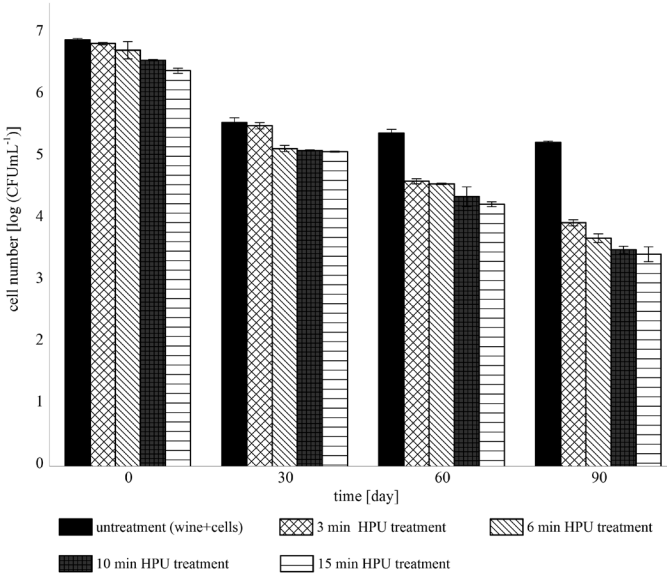

Figure 1. *B. bruxellensis CBS 2499 population in red wine before and after different HPU treatment duration with 12.7 mm diameter ultrasonic probe at* 25 °C *and during storage (30, 60 and 90 days). All parameters are expressed as average value of two repetitions ± standard deviation (n=2).*

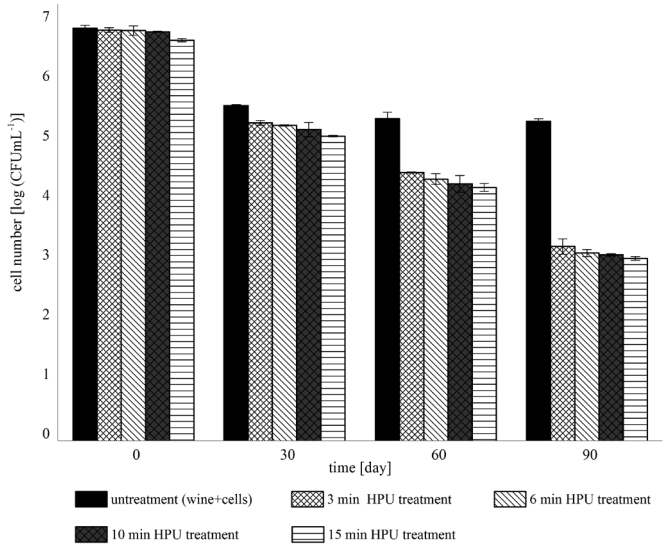

Figure 2. *B. bruxellensis CBS 2499 population in red wine before and after different HPU treatment duration with 19.1 mm diameter ultrasonic probe at* 25 °C *and during storage (30, 60 and 90 days). All parameters are expressed as average value of two repetitions ± standard deviation (n=2).*

Generally, for the HPU treatment ultrasonic intensity depends on ultrasonic power and surface area of the probe (Margulis and Margulis, 2003; Piyasena et al. 2003; Gao et al., 2014; Kentish and Feng, 2014; Harvey et al., 2014; Režek Jambrak et al., 2017;). Therefore, a different diameter probe is used depending on the volume of the treated medium. For the volume of 200 mL (used in our experiments), both 12.7 mm and 19.1 mm probes could be used. Since slightly better inactivation of *B. bruxellensis* was achieved using 12.7 mm diameter probe, this probe was chosen for further research. Furthermore,

obtained data demonstrate that HPU treatment at 25 °C for 15 minutes is not effective enough to notably inactivate *B. bruxellensis* population in red wine (Figure 1 and Figure 2). Contrary to our research, Luo et al. (2012) reported significant inactivation in *B. bruxellensis* in wine (about 2.15 log CFUmL-1) after 20 min of HPU treatment at 23 - 25 °C. However, they used a higher frequency (24 Hz) in their work.

During the 90 days monitoring period of *B. bruxellensis* population, a constant trend of reduction in all samples treated with both probes was observed (Figure 1 and Figure 2). However, the population in the untreated sample decreased for about 1 log CFUmL-1 after 30 days, while no further inactivation was observed during the following 60 days. After 90 days, the population in all treated samples decreased for about 3 log CFUmL-1, while the population in untreated samples decreased for about 1 log CFUmL-1. The physico-chemical parameters of wine used in this work (Table 1) are common in winemaking and are not expected to have a significant effect on the survival of *B. bruxellensis*. The observed difference in *B. bruxellensis* population between treated and untreated samples was probably due to HPU treatment that may have caused damage to cellular components and interfered with cellular functions. This is supported by the previous research where it was showed that ultrasonic treatment singly could not break down a yeast cell, but could damage cell wall and cytoplasmic membrane (Chemat and Khan, 2011; Chandrapala et al., 2012; Huang et al., 2014; Režek Jambrak et al., 2017; Wu et al., 2015; Ferrario and Guerrero, 2017). However, further study is required to confirm this hypothesis.

Also, the presence of volatile compounds (4-ethylphenol, 4-ethyl guaiacol, ethyl hexanoate, ethyl octanoate, ethyl decanoate, isoamyl acetate) in treated and untreated samples after 90 days was determined (Table 2). The concentration of 4-ethylphenol in the untreated sample was 1742 µgL-1, while in treated samples was determined in the range of 919 to 1595 µgL-1. The concentration of 4-ethyl guaiacol was 668 µgL-1 in the untreated sample and in the range of 186 to 398 µgL-1 in treated samples. The concentration of the analyzed esters (ethyl decanoate, ethyl hexanoate, isoamyl acetate and ethyl octanoate) were higher in the untreated sample (88 to 646 µgL-1) compared to the treated samples (34 to 456 µgL-1). The concentration of volatile phenols produced in wine depends on many parameters such as hydroxycinnamic acid composition in wine, developed active *Brettanomyces* population and ability of different *B. bruxellensis* strains to produce and accumulate volatile compounds (Kheir et al., 2013). In addition to volatile phenols, *B. bruxellenasis* could produce acetic acid and volatile esters (Steensels et al., 2015). In our research, lower concentration of volatile phenols in treated wines compared to untreated wine was the effect of reduced active *Brettanomyces* population as well as possible reduced ability of *B. bruxellensis* to produced volatile compounds due to cell damage (Table 2). Cells of *B. bruxellensis* with a damaged cell wall or membrane are more sensitive to their environment, which could affect enzyme activity (Chemat and Khan, 2011; Chandrapala et al., 2012; Huang et al., 2016). This effect observed during our work is supported by observations conducted by other authors (Lustrato et al., 2015), that reported lower production of volatile phenols by non active *B. bruxellensis* population treated with non-thermal technology, low electric current (LEC). An interesting topic for further research would be to more precisely determine how sonification affects the cell wall or membrane, and consequently how it affects the volatile phenols production.

Table 2. *B. bruxellensis production of volatile compounds in red wine after 90 days under different experimental conditions*

Treatment	Concentration (μgL^{-1})					
	4-ethyl phenol	4-ethyl guaiacol	ethyl hexanoate	ethyl octanoate	ethyl decanoate	isoamyl acetate
untreatment (wine+cells)	1742±8^{c}	668±1^{d}	269±3^{c}	646±40^{b}	88±3^{b}	543±57^{d}
12.7 diameter probe						
3 min	919±5^{a}	183±11^{a}	174±22^{a,b}	280±3^{a}	34±3^{a}	456±50^{c,d}
6 min	1474±41^{b,c}	398±26^{b,c}	165±28^{a,b}	357±48^{a}	42±2^{a}	410±29^{b,c}
10 min	1066±105^{a,b}	313±16^{b}	143±11^{a}	451±73^{a}	47±9^{a}	124±4^{a}
15 min	1191±52^{a,b,c}	321±38^{b}	147±8^{a,b}	344±24^{a}	45±6^{a}	303±10^{b}
19.1 diameter probe						
3 min	1595±142^{c}	511±49^{c}	206±24^{b}	396±84^{a}	52±20^{a}	337±27^{b,c}
6 min	1137±34^{a,b,c}	313±24^{b}	159±12^{a,b}	306±31^{a}	40±6^{a}	345±17^{b,c}
10 min	1236±47^{a,b,c}	339±18^{b}	185±3^{a,b}	366±23^{a}	56±1^{a,b}	380±4^{b,c}
15 min	927±74^{a,b}	187±7^{a}	169±1^{a,b}	367±35^{a}	58±7^{a,b}	384±28^{b,c}

All parameters are expressed as average value of two repetitions ± standard deviation (n=2). ANOVA to compare data; different letters indicate statistical differences between wines of all treatments at the same time (Turkey's test,<0.05).

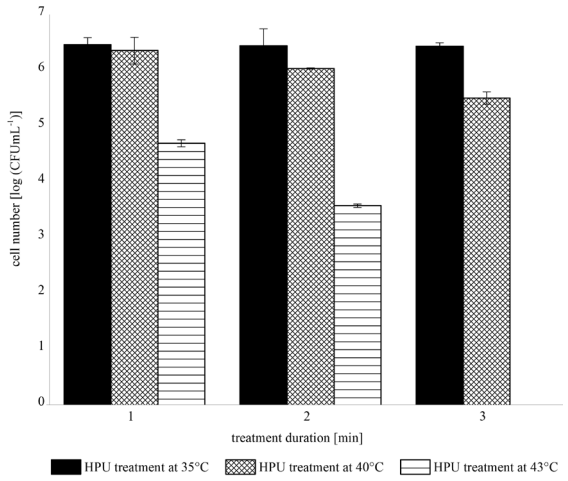

Figure 3. *Influence of thermosonication parameters (treatment temperature, treatment duration and 12.7 mm diameter ultrasonic probe) on B. bruxellensis CBS 2499 population in red wine immediately after thermosonication treatment. All parameters are expressed as average value of two repetitions ± standard deviation (n=2).*

In order to increase inactivation of *B. bruxellensis* yeast, HPU treatments with a 12.7 mm diameter probe were performed at 35, 40 and 43 °C for 1, 2 and 3 minutes (Figure 3). The number of *B. bruxellensis* cells was determined immediately after thermosonication treatment. The *B. bruxellensis* population changed only slightly over all treatment durations at 35 °C. At higher temperatures (40 and 43 °C), an increased inactivation of *B. bruxellensis* population was observed. After 3 -minute treatment, the population decreased for 1 log CFUmL-1 at 40 °C and for 6 log CFUmL-1 at 43 °C. Since the initial growth was 6 log CFUmL-1, a complete inactivation of *B. bruxellensis* was achieved at 43 °C after 3 minutes. An increase in *B. bruxellensis* inactivation compared to solely HPU, could be explained by the fact that HPU causes the cell wall weakening which could facilitate thermal coagulation of the intracellular proteins (Yap et al. 2008; Chandrapala et al., 2012; Cruz-Cansino et al., 2016; Wu et al., 2015). According to previous research data for *S. cerevisiae* (Cacciola et al., 2013), maximal inactivation of this yeast in wine occurred after 3 minutes and at similar temperature (47.6 °C) with similar diameter ultrasonic probe (13 mm). The ultrasound affects combined physical and chemical mechanisms occurring during cavitation and increases yeast sensitivity to heat (Piyasena et al., 2003; Wu et al., 2015).

The influence of thermosonication treatment on inactivation of bacteria, yeasts and molds depends on many conditions such as ultrasonic power and amplitude, temperature, treatment duration as well as composition of the treated medium (Patil et al., 2009;Wong et al., 2010; Bermúdez-Aguirre and Barbosa-Cánovas, 2012; Gao et al., 2014; Ortuño et al., 2014; Mohideen et al., 2015). Combination of HPU and heating (thermosonication) can have a greater effect on *B. bruxellensis* inactivation because it damages cells at the subcellular level (Abdullah and Chin, 2014; Huang et al., 2016; Cruz-Cansino et al., 2016; Režek Jambrak et al., 2017). Proportion between sonification and heat influence on *Brettanomyces* cells is hard to estimate. Our experiments show that sonification without heating is not very effective for inactivation of *Brettanomyces* cells in wine environment. However, it is possible that sonification accelerates yeast inactivation by heating.

Conclusions

In the present work the use of high power ultrasound treatment (HPU) and combination of HPU and heating (thermosonication) to control the spoilage yeast *B. bruxellensis* in red wine during the treatment in batch systems has been tested. The results clearly indicate improved inactivation by combined treatment (thermosonication) compared to solely using HPU. Parameters that appear to have the greatest effect are temperature of the treatment and treatment duration. Complete inactivation of *B. bruxellensis* was achieved by treatment with 12.7 mm diameter ultrasonic probe at temperature of 43 °C during 3 min. After HPU treatment, *B. bruxellensis* population has also shown lower ability to produce volatile phenols.

Acknowledgments

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