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The Effect of High Power Ultrasound and Cold Gas-Phase Plasma Treatments on Selected Yeast in Pure Culture

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Abstract High power ultrasound (US) and cold gas-phase plasma (CP) are non-thermal processing technologies that maybe used in food processing industry. The main objective of this research was to study the effect of both treatments on selected yeasts (*Rhodotorula* spp. 74 and *Candida* spp. 86) in pure culture. Samples were treated by ultrasound with 57.50-, 86.25- or 115-μm amplitude, for 3, 6 or 9 min at 20 °C, and 40 or 60 °C in the case of thermosonication. For cold gas-phase plasma treatments, samples were treated at a gas flow of 0.75, 1 or 1.25 L min^{-1}, treatment time of 3, 4 or 5 min, and sample volume of 2, 3 or 4 mL. Each technology has its own advantages and is able to give the best effect on the desired target product. The experiment was designed using central composite design (CCD), and results were analysed and presented using response surface methodology (RSM). The greatest reduction of yeasts was observed after ultrasound treatments at 60 °C (thermosonication) and after plasma treatments, after the longest treatment time (5 min) and the lowest sample volume (2 mL). For high power ultrasound treatment, reduction in the number of yeast cells (*N*) can be attributed to elevated temperature (60 °C), cavitation and free radical formation. For plasma treatment, the inactivation can be attributed to UV radiation and plasma reactive oxygen species (ROS).

Keywords High power ultrasound · Cold gas-phase plasma · Yeast · Response surface methodology · Radicals

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Introduction

Ultrasound range can be divided into three different frequency ranges: diagnostic ultrasound (1–10 MHz), high-frequency ultrasound (100 kHz-1 MHz) with low sound intensity (0.1–1 W cm^{-2}) and low-frequency power ultrasound (20–100 kHz) with high sound intensity (10–1000 W cm^{-2}) (Mason 1998, 2003; Ashokkumar and Kentish 2011). Power ultrasound can provide the mechanical effect of cavitation in liquid systems which can alter the physical and chemical properties of food depending on the type of material involved. The collapsing bubbles will create energy accumulated hot spots which can generate high temperature (5000 K) and pressure (1000 atm) resulting in high shear energy zones and turbulence in the cavitation zone of the liquid (Chemat et al. 2011). Ultrasonication is a non-thermal method of food processing that has the advantage of preserving food without causing the common side effects associated with conventional heat treatments (Salleh-Mack and Roberts 2007; Jambrak et al. 2008; Chemat et al. 2011; Herceg et al. 2012, 2013). The effect of high-intensity ultrasound on the inactivation of food spoilage bacteria was investigated by Herceg et al. (2013). The results indicate that inactivation of microorganisms increased under longer period of treatments, particularly in combination with higher temperature (thermosonication) or with pressure (manothermosonication) and/or higher/lower amplitude. Also, inactivation of *Staphylococcus aureus* and *Escherichia coli* in milk was carried out using a 20-kHz power ultrasound (Herceg et al. 2012). It was found that Gram-negative bacteria are more susceptible to the ultrasonic treatment than Gram-positive bacteria.

Most microorganisms show greater sensitivity to ultrasound at increased temperatures over 50 °C (Herceg et al. 2012). However, some authors claim that it is possible to inactivate microorganisms even at a temperature of 40 °C (Herceg et al. 2012, 2013). Elevated temperature weakens

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the bacterial membrane, which enhances the effect of cavitation due to the ultrasound. In particular, the use of high power ultrasound has shown several advantages compared to heat pasteurization such as minimization of flavour loss in juices, greater homogeneity and significant energy savings (Herceg et al. 2012). In combination with heat (thermosonication) and pressure (manothermosonication), ultrasound can accelerate the rate of heat treatment of foods, thereby lessening the duration, and intensity of thermal treatment and the resultant damage (Piyasena et al. 2003). Many researches have attempted to understand the mechanism played by ultrasound on the inactivation of microorganisms (Bermudez-Aguirre et al. 2011; Raso et al. 1998; Herceg et al. 2012, 2013). It can be explained by the phenomenon of acoustic cavitation and its physical, mechanical and chemical effects that inactivate bacteria and deagglomerate bacterial clusters or flocs (Mason 2003). The high temperatures produced during cavitation may also be responsible; changes occur momentarily, because only the liquid in the immediate surroundings is heated and therefore only a small number of cells are affected. Ultrasonic waves in water have been shown to form radicals due to homolytic cleavage ($H_2O \rightarrow H\cdot + OH$).

Plasma, the fourth state of matter, is ionized gas and can be generated using a range of gases or gas mixtures, typically argon, helium, nitrogen, air or oxygen. Plasma generated in air consists of reactive forms of atoms, excited molecules, charged particles, reactive oxygen species (ROS), reactive nitrogen species (RNS) and UV photons, all of which may contribute to its antibacterial properties. The plasma glow consists of UV radiation, excited atoms, molecules and free radical components that are perceived to be responsible for germicidal effect (Laroussi 2005). Until recent advances in the development and applications of atmospheric pressure plasma systems, cold plasma processes were carried out under vacuum and thus incompatible with food processing. Cold gas-phase plasma treatments are known as non-thermal because it has electrons at a hotter temperature than the heavy particles that are at room temperature. It has been shown recently that plasmas generated outside thermal equilibrium at atmospheric pressure produce an antimicrobial effect (Laroussi 2005; Roth et al. 2007; Peña-Eguiluz et al. 2010). This effect results from the interaction of the organic media with a wide variety of active oxidizing species, excited atoms and molecules, as well as ultraviolet radiation that are produced during plasma interaction with air. Bacteria, yeasts and fungi have different susceptibility, and, in many cases, bacteria are more vulnerable to plasma (Xiong et al. 2010; Lee et al. 2006; Muranyi et al. 2007; Tang et al. 2008; Kamgang-Youbi et al. 2009). In the study of Mitra et al. (2014), the seeds of *Cicer arietinum* were exposed to cold atmospheric plasma. A significant reduction of the natural microbiota attached to the seed surface was observed for 2- and 5-min plasma treatment to achieve a 1 and 2 log reductions.

Cold gas-phase plasma treatments offer distinct advantages for decontamination of foods. The term 'plasma' refers to an overall electrically neutral gas composed of molecules, atoms, ions and free electrons. The electron temperature is much higher than the ion and neutral temperatures which are typically equalled and close to room temperature ('cold' or non-thermal). The gas is at atmospheric pressure, e.g. ambient.

Mathematical modelling is important for analysis of interaction between investigated parameters that cannot be considered using simple statistical analysis, also reducing energy and chemical consumption by a lower number of experiments. Response surface methodology (RSM) may be employed to optimize critical processing parameters by estimating interactive and quadratic effects. A further benefit of using RSM is the reduction in the number of experiments needed as compared to a full experimental design (Myers and Montgomery 2002; Lu et al. 2008).

The aim of this study was to evaluate the effect of high power ultrasound and cold gas-phase plasma treatments on the number of viable yeast cells (*Rhodotorula* spp. 74 and *Candida* spp. 86) in pure culture.

Materials and Methods

Preparation of Yeast Culture

Yeast strains *Rhodotorula* spp. 74 and *Candida* spp. 86 were obtained from the Collection of Microorganisms of the Laboratory of General Microbiology and Food Microbiology, Faculty of Food Technology and Biotechnology, University of Zagreb (Zagreb, Croatia). The cultures were stored on nutrient agar (Biolife, Milan, Italy) at 4 °C. To prepare the pure culture for plasma and ultrasound treatments, the investigated yeasts were incubated on nutrient broth (Biolife) for 24/48 h at 30 °C (yeast cultures were incubated for 24 h, and then, if their growth would not be satisfactory, incubation would be prolonged up to 48 h) and then harvested young cells were suspended in sterile water solution. The initial number (given in Tables 1, 2 and 3 for each strain) and total viable cell count (N) after the high power ultrasound and plasma treatments were performed by standard dilution method on nutrient agar after incubation at 30 °C for 48 h. The yeast colonies were counted and reported as log colony-forming units per millilitre (log CFU mL^{-1}). All samples were analysed in triplicate, and the given score is the mean value of three determinations.

High Power Ultrasound Treatments

Samples of 50 mL with each strain have been separately treated with high power ultrasound. Samples were placed in a sterile glass (in a 100-mL flask), which were used as the

Table 1 Amplitude (*A*), treatment time (*B*), temperature (*C*), power (*P*), acoustic intensity (AI) and viable yeast cell count (*N*) of *Candida* spp. before and after ultrasound treatments

Sample	Amplitude (μm)	Time (min)	Temperature (°C)	Power (W)	AI (W cm^{-2})	*N* (log CFU mL^{-1})
C	–	–	20	–	–	5.60±0.01
CU1	115	9	20	42–44	109.19–110.51	3.23±0.04
CU2	86.25	6	20	29–30	75.39–76.21	4.20±0.02
CU3	57.50	9	20	18–20	46.80–47.61	4.38±0.03
CU4	57.50	6	40	16–17	41.60–42.32	3.04±0.01
CU5	86.25	9	40	27–30	70.19–71.24	2.00±0.04
CU6	115	6	40	39–41	101.39–102.14	2.15±0.02
CU7	86.25	3	40	26–27	67.59–68.10	3.63±0.02
CU8	115	3	60	36–37	93.59–94.34	3.11±0.02
CU9	115	9	60	38–40	98.79–99.26	0.00
CU10	86.25	6	40	26–27	67.59–68.22	3.00±0.03
CU11	115	3	20	43–45	111.79–112.34	4.38±0.05
CU12	86.25	3	60	25–27	59.00–60.21	3.18±0.02
CU13	57.50	6	40	19–22	47.59–48.25	3.00±0.05
CU14	57.50	3	20	18–20	46.80–47.32	4.38±0.01
CU15	86.25	6	60	26–28	67.59–68.25	0.00
CU16	57.50	9	60	15–16	49.00–50.01	0.00

All values are expressed as mean of three repetitions±standard deviation

treatment vessel. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, CT, USA), set at 600 W, 20 kHz (12–260 μm range of amplitude for the device) with a 7-mm diameter probe, was introduced into the vessel. The same part of the probe was immersed in the sample (about 1.5 cm) and placed at the 'centre' of the sample. Ultrasonication was carried out with 57.50-, 86.25- and 115-μm amplitude. Samples were treated by ultrasounds for 3, 6 and 9 min at 20 °C. In the case of thermosonication before ultrasonic treatments, the samples were heated at 40 and 60 °C

Table 2 Amplitude (*A*), treatment time (*B*), temperature (*C*), power (*P*), acoustic intensity (AI) and viable yeast cell count (*N*) of *Rhodotorula* spp. before and after ultrasound treatments

Sample	Amplitude (μm)	Time (min)	Temperature (°C)	Power (W)	AI (W cm^{-2})	*N* (log CFU mL^{-1})
R	–	–	20	–	–	4.00±0.01
RU1	115	9	20	47–48	122.19±123.56	3.32±0.02
RU2	86.25	6	20	30–32	77.99–78.62	3.60±0.03
RU3	57.50	9	20	18–19	46.80–47.62	3.43±0.01
RU4	57.50	6	40	17–18	44.20–45.21	2.91±0.01
RU5	86.25	9	40	28–30	72.79–73.26	2.30±0.02
RU6	115	6	40	41–42	106.59–107.23	2.48±0.05
RU7	86.25	3	40	28–29	72.79–73.25	3.49±0.01
RU8	115	3	60	39–40	101.39–102.68	2.95±0.01
RU9	115	9	60	38–40	98.79–99.24	0.00
RU10	86.25	6	40	27–28	70.19–71.24	2.78±0.04
RU11	115	3	20	45–47	116.99–117.65	3.81±0.04
RU12	86.25	3	60	26–28	71.60–72.57	3.30±0.02
RU13	57.50	6	40	17–18	40.19–41.23	2.78±0.01
RU14	57.50	3	20	18–20	46.80–47.25	3.91±0.02
RU15	86.25	6	60	26–27	67.59–68.24	0.00
RU16	57.50	9	60	16–18	41.60–42.30	0.00

All values are expressed as mean of three repetitions±standard deviation

Table 3 Gas flow (X_1), treatment time (X_2), sample volume (X_3) and viable yeast cell count (N) of *Candida* spp. and *Rhodotorula* spp. before and after non-thermal gas-phase plasma treatments (CP)

Samples	Gas flow (L min^{-1})	Time (min)	Sample volume (mL)	*N Candida* spp. (log CFU mL^{-1})	*N Rhodotorula* spp. (log CFU mL^{-1})
CP; RP	–	–		7.01±0.03	6.81±0.02
CP1; RP1	0.75	3	2	4.64±0.01	5.12±0.01
CP2; RP2	1.25	3	2	5.07±0.03	4.23±0.01
CP3; RP3	1	3	3	4.87±0.04	5.40±0.01
CP4; RP4	0.75	3	4	6.07±0.05	5.19±0.05
CP5; RP5	1.25	3	4	6.25±0.04	5.32±0.03
CP6; RP6	1	4	2	4.12±0.01	4.38±0.03
CP7; RP7	0.75	4	3	5.51±0.03	4.81±0.02
CP8; RP8	1	4	3	4.30±0.05	5.59±0.02
CP9; RP9	1	4	3	5.27±0.01	5.66±0.05
CP10; RP10	1.25	4	3	5.70±0.02	5.33±0.02
CP11; RP11	1	4	4	6.96±0.02	6.17±0.01
CP12; RP12	0.75	5	2	2.19±0.01	2.76±0.01
CP13; RP13	1.25	5	2	2.02±0.02	2.58±0.04
CP14; RP14	1	5	3	4.44±0.03	4.84±0.01
CP15; RP15	0.75	5	4	4.76±0.05	4.55±0.02
CP16; RP16	1.25	5	4	4.04±0.04	5.10±0.02

All values are expressed as mean of three repetitions±standard deviation

(Tables 1 and 2). During ultrasound treatments, overheating of the samples was prevented by ice-water cooling of the treatment chamber (vessel). The final temperature of microorganism suspensions after sonication at 40 or 60 °C was 40±1 °C or 60±1 °C. After the treatments of samples, by means of a high power ultrasound, the final number of viable yeast cells has been determined (log CFU mL^{-1}) (Herceg et al. 2013).

Determination of Acoustic Power

The most widely accepted method for determining the power of an acoustic horn in an aqueous solution is the calorimetric technique described by Jambrak et al. (2009). This method involves taking a known volume of water and applying ultrasound (for ~3 min) while monitoring the change in temperature with time at various ultrasonic amplitudes. The ultrasonic power (P) and the ultrasonic intensity (AI) can be readily determined from the following equations:

$$P = m \times C_p \times \left(\frac{\partial T}{\partial t}\right)_{t=0} \quad (1)$$

$$\mathrm{AI} = P/A \quad (2)$$

where P is the ultrasonic power (W), where m is the mass of the sonicated liquid (g), C_p is its specific heat at a constant pressure (J/g K), and dT/dt is the slope at the origin of the curve (T is temperature, t is time), AI is the ultrasonic intensity (W cm^{-2}), and A is the surface area of probe (cm^2).

A common problem in the sonochemical literature is that the power delivered to the system (as quoted by the manufacturer) is mentioned, but the actual power dissipated (P) in the treated system is rarely reported. One of the most common methods of measuring P is to use Eq. (2). This equation is based on the use of calorimetry and assumes that all of the power entering the system is dissipated as heat. This simple equation has been widely used throughout the sonochemistry literature.

Cold Gas-Phase Plasma Treatments

The cold atmospheric plasma jet was generated in argon (purity 99.99 %; Messer, Sulzbach, Germany) by applying a 25-kHz electric field through the electrode. The plasma source used was a single-electrode atmospheric jet (End-field Jet type), designed at the Institute of Physics (Zagreb, Croatia) (Fig. 1). It consist of Teflon body to which a glass capillary tube of 7.5 cm in length and 0.15/0.1 cm in outer/inner diameter is attached. Cu wire of 1×10^{-4} m in diameter is placed inside the capillary tube. The tube is connected to the high-voltage source through the vacuum tight connector. The high-voltage source of nominal 6-W power provides 2.5 kV of voltage at 25 kHz. The actual current through the electrode was measured to be typically 3 mA. The actual power of plasma was 4 W, as determined from the voltage-current waveforms (Zaplotnik et al. 2014). Such plasma source produces a plasma jet extending out of the capillary tube to the length of about 2.2 cm at argon gas flow of 1.5 L min^{-1}. A further increase of gas flow causes a decrease of the plasma jet length. Optical emission spectroscopy (OES) of Ar plasma jet in the region from 200 to 1000 nm was performed by means of

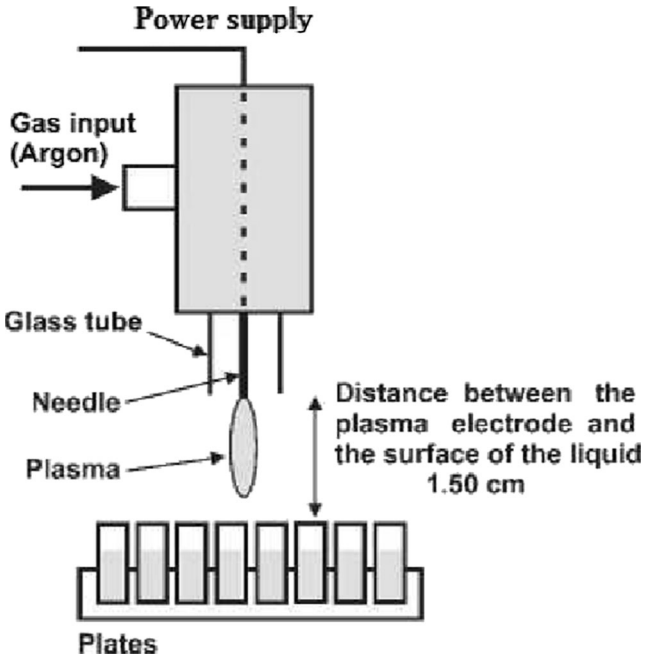

Fig. 1 Schematic description of the used plasma source

a miniature fibre spectrometer (Avantes 3600, Leatherhead, Surrey, UK) of a 0.8-nm spectral resolution. The light was collected from the region near the capillary tube exit by means of a quartz lens and a solar-resistant optical fibre (Kregar et al. 2011).

For the sample treatments, plasma was running at a constant power of 4 W, varying the gas flow, treatment time and volume of treated sample according the experimental design (Table 3). The three level values for cold gas-phase plasma (CP) treatments for gas flow were 0.75, 1 and 1.25 L min^{-1}, treatment time were 3, 4 and 5 min, and sample volumes were 2, 3 and 4 mL. The initial number of strain is given in Table 3. The distance of the plasma nozzle tip from the samples was fixed at 1.5 cm. Samples were placed in a tissue culture test plate that consisted of 16 sample positions (TPP Techno Plastic Products AG, Trasadingen, Switzerland), and each treatment was performed in triplicate. After the treatments of samples, by means of cold gas-phase plasma treatments, the final number of viable yeast cells has been determined (log CFU mL^{-1}).

Experimental Design and Statistical Analysis

The experimental design and statistical analysis were done using Statgraphics Centurion software (StatPoint Technologies, Inc., VA, USA). A separate setup of experiment has been designed for cold gas-phase plasma treatments and high power ultrasound treatments. A general factorial design comprising 16 experimental trials with one replication of the central point was chosen to evaluate the combined effect of three independent variables. For plasma treatments, the variables were as follows: gas flow (X_1, L min^{-1}), treatment time (X_2, min) and sample volume (X_3, mL) (Tables 1, 2 and 3). For high power ultrasound treatments, the experiment consists of 16 experimental trials (Tables 1, 2 and 3). The independent variables were amplitude A (μm), treatment time B (min) and temperature C (°C).

In order to determine the influence of each factor on the number of viable yeast cells, a central composite design (CCD) model was chosen. Experiments were performed in triplicate, in randomized order according to the trial number as arranged by the software (Šimunek et al. 2013). The responses obtained from the experimental design were log CFU mL^{-1} of each yeast strain. The design matrix for the experiment and the regression model for each response were calculated as follows (Khuri and Cornell 1996):

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} {X_i}^2 + \sum \beta_{ij} X_i X_j \quad (3)$$

where Y is predicted response, β_0 is the fixed response at central point, and β_i, β_{ii} and β_{ij} are the linear, quadratic and interaction coefficients, respectively.

The validity of the quadratic empirical model was tested using the analysis of variance (ANOVA). The confidence level used was 95 %. Furthermore, three dimensional response surface plots were generated by keeping one response variable constant and plotting it against the two other independent variables.

Results and Discussion

From the results shown in Tables 1, 2 and 3, the effect of ultrasound and plasma could be observed as a reduction in the number of yeasts, *Rhodotorula* spp. 74 and *Candida* spp. 86. The effect of ultrasound (US) treatments on microorganisms is attributed to cavitation phenomenon and free radical formation. Power values (W) after ultrasonication/thermosonication treatments for *Candida* spp. 86 ranges from 15 to 44 W and for *Rhodotorula* spp. 74 ranges from 16 to 48 W, depending of the amplitude applied. Acoustic intensity for *Candida* spp. 86 ranges from 49.00 to 112.34 W cm^{-2} and for *Rhodotorula* spp. 74 ranges 40.19–123.56 W cm^{-2} depending on amplitude applied. Cavitation is the formation of bubbles or cavities in liquids, and the collapse of these bubbles releases intense shock waves that can cause considerable damage to surrounding material (Lee et al. 2009). The greatest reduction (total removal of viable cells) in log CFU mL^{-1} of *Candida* spp. 86 (Table 1) and *Rhodotorula* spp. 74 (Table 2) were after ultrasound treatments at 60 °C (thermosonication) for 6 and 9 min (CU9, CU15, CU16, RU9, RU15 and RU16). The least reduction of yeasts was observed after ultrasound treatments at

20 °C (CU2, CU3, CU11, CU14, RU2, RU3, RU11 and RU14). Compared to thermal treatment alone, in both cases, for *Candida* spp. 86 (Table 1) and *Rhodotorula* spp. 74 (Table 2), there was a greater reduction in viable yeast cells (*N*) for thermosonication treatments (ultrasound treatments combined with elevated temperatures, 40 and 60 °C). Applying higher amplitude, there was also higher power and intensity delivered to the treated system.

The mechanism by which ultrasound acts in inactivation path could be explained by two competing theories. These theories explain chemical effects due to cavitation: the hot-spot theory and the electrical theory. The hot-spot theory postulates that when the bubbles cavitate, localized hot spots are formed which reach temperatures and pressures in excess of 5000 K and 1000 atm. Under extreme conditions, chemical compounds can be degraded through three distinct pathways: oxidation by hydroxyl radicals, pyrolytic decomposition and supercritical water oxidation which can be linked with micro-organism's inactivation (Hoffmann et al. 1996). The electrical theory postulates that an electrical charge is created on the surface of a cavitation bubble, forming enormous electrical field gradients across the bubbles which are capable of bond breakage upon collapse and also interfering with surface of microorganisms.

Plasma treatments lead to significant reductions in yeast loads (Table 3). The greatest reduction in the number of yeast cells (*N*) was observed for the longest treatment time (5 min) and the lowest sample volume (2 mL) (CP12, CP13, RP12 and RP13), whereas the least reduction in the number of yeast cells (*N*), *Rhodotorula* spp. 74 and *Candida* spp. 86, was shown for the highest sample volume (4 mL) (CP4, CP5, CP11, CP15, CP16, RP4, RP5, RP11, RP15 and RP16). For plasma treatment, it is generally believed that the inactivation is caused by UV radiation which penetrates deeply into the cell and causes DNA strand breaks. In contrast to conventional UV/C preservation, where shadowing of the UV radiation by multi-layered stacks of spores or by biofilms can largely reduce the sterilization efficiency, the combined effect of incident UV photons, ions and chemical active species makes plasma extremely efficient for decontamination purposes. Possible inactivation mechanisms by CP are likely to be associated to plasma-immanent reactive species such as atomic oxygen and OH^- radicals, since UV photons get easily absorbed in atmospheric air and charged particles cannot access the sample in its downstream position (Vleugels et al. 2005). In this paper, optical emission spectroscopy (OES) measurements were also performed at different wavelengths. The existence of excited NO, OH, O radicals within the plasma jet, as well as excited N_2 and Ar were observed as shown in Fig. 2. When there are more electrons per time unit (current) flowing through the electrode and the plasma, there are more inelastic collisions of electrons with the plasma particles. In these collisions, electrons ionize, dissociate and excite molecules and atoms. Generally, we could say that more electrons per time unit lead to more excited particles and increase the intensity of emission lines (Zaplotnik et al. 2014).

Sun et al. (2012) investigated the antifungal effect of cold plasma, as well as its combination with common antifungal drugs, against *Candida* biofilms. A direct current atmospheric pressure He/O_2 (2 %) plasma microjet (PMJ) was used to treat *Candida* biofilms. The *Candida* biofilms were completely inactivated after 1 min of PMJ treatment, where severely deformed fungal elements were observed in SEM images.

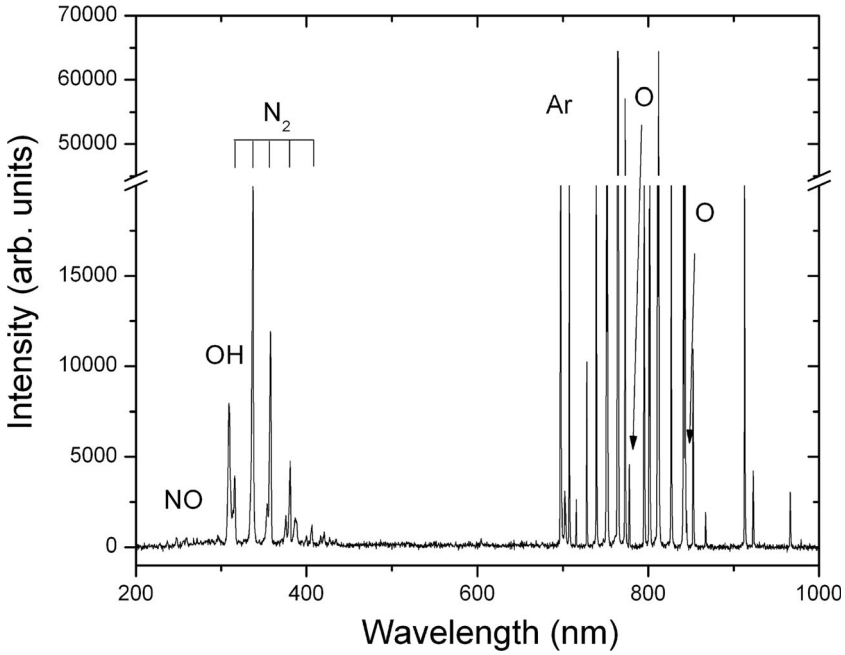

Fig. 2 Optical emission spectroscopy (OES) measurements at different wavelengths

Table 4 Analysis of variance for the effect of high power ultrasound (amplitude (*A*), treatment time (*B*) and temperature (*C*)) on viable yeast cell count (*N*); analysis of variance for the effect of non-thermal gas-phase plasma treatment (gas flow (X_1), treatment time (X_2), sample volume (X_3)) on viable yeast cell count (*N*) of *Candida* spp. and *Rhodotorula* spp. at 95 % confidence level

Source	*Candida* spp.		*Rhodotorula* spp.	
	F ratio	*p* value	*F* ratio	*p* value
A—amplitude	0.90	0.3786	0.23	0.6474
B—treatment time	24.03	0.0027	33.03	0.0012
C—temperature	49.92	0.0004	69.94	0.0002
AA	0.16	0.7034	0.43	0.5374
AB	0.16	0.6993	0.02	0.8989
AC	0.16	0.6993	0.00	0.9821
BB	1.08	0.3394	2.57	0.1601
BC	10.22	0.0187	15.88	0.0072
CC	0.75	0.4208	5.38	0.0596
X_1—gas flow	0.00	0.9709	0.02	0.8921
X_2—treatment time	16.69	0.0065	43.39	0.0006
X_3—sample volume	18.79	0.0049	77.35	0.0001
X_1X_1	0.02	0.9063	9.63	0.0210
X_1X_2	0.53	0.4957	2.37	0.1746
X_1X_3	0.15	0.7078	5.65	0.0551
X_2X_2	3.97	0.0935	7.76	0.0318
X_2X_3	0.91	0.3777	18.13	0.0053
X_3X_3	0.00	0.9818	3.28	0.1203

Durbin-Watson statistic (*Candida* spp., US)=2.23525; Durbin-Watson statistic (*Rhodotorula* spp., US)=2.13951; Durbin-Watson statistic (*Candida* spp., CP)=2.12744; Durbin-Watson statistic (*Rhodotorula* spp., CP)=1.43495

ROS such as hydroxyl radical ($^{\cdot}OH$), superoxide anion radical ($^{\cdot}O_2^-$) and singlet molecular oxygen ($^1O_2$) were detected by electron spin resonance (ESR) spectroscopy. The generation of ROS is believed to be one of the underlying mechanisms for the fungicidal activity of plasma.

Ryu et al. (2013) have investigated the influence of environmental factors (surrounding media) on the efficiency of plasma inactivation of *Saccharomyces cerevisiae*. Yeast cells treated with plasma in water showed the most severe damage in viability and cell morphology as well as damage to membrane lipids, and genomic DNA. Cells in saline were less damaged compared to those in water, and those in yeast extract, peptone and dextrose (YPD) were least impaired. Levels of hydroxyl radical ($OH^{\cdot}$) produced by plasma were the highest in water and the lowest in YPD. This may have resulted in differential inactivation of yeast cells in water, saline and YPD by plasma.

The reduction in the number of yeast cells (*N*) after US treatments and CP is displayed by response surface methodology (RSM) using the Statgraphics Centurion software. Statistical calculations were done at 95 % confidence level using ANOVA. From Table 4, the influence of treatment factor as well as quadratic influence of each and combined factor are shown. From the analysis of variance for the effect of high power ultrasound (amplitude (*A*), treatment time (*B*) and temperature (C)) on the number of viable yeast cells (*N*) of *Candida* spp. and *Rhodotorula* spp., statistically significant effect has treatment time (*B*) and temperature (C) and their combined effect (*B*C). For the effect of cold gas-phase plasma treatments (gas flow (X_1), treatment time (X_2) and sample volume (X_3)) on the number of viable yeast cells (*N*) of *Candida* spp. and *Rhodotorula* spp., statistically significant effect for reduction of *Candida* spp. has treatment time (X_2) and sample volume (X_3). For the reduction of *Rhodotorula* spp., statistically significant effect has treatment time (X_2), sample volume (X_3) and quadratic effect of each parameter (gas flow X_1^2 or X_1X_1), X_2X_2 and combined effect of treatment time (X_2) and sample volume (X_3) X_2X_3.

Table 5 Polynomial fit models for the effect of high power ultrasound (US) (amplitude (*A*), treatment time (*B*) and temperature (*C*)) on viable yeast cell count (*N*) and for the effect of non-thermal gas-phase plasma treatment (CP) (gas flow (X_1), treatment time (X_2), sample volume (X_3) on viable yeast cell count (*N*) of *Candida* spp. and *Rhodotorula* spp.). Optimal values of amplitude (*A*), treatment time (*B*) and temperature (*C*) for high power ultrasound treatment and of gas flow (X_1), treatment time (X_2) and sample volume (X_3) for non-thermal gas-phase plasma treatment of yeast suspension using a response surface models

Response	Model	Optimal values	Predicted log CFU mL^{-1}
Candida spp. 86 (US)	$=6.87448-0.0398621\ A-0.28773\ B+0.0513405\ C+0.000181683\ A^2-0.00101449\ A{\cdot}B+0.000152174\ A{\cdot}C+0.0433525\ B^2-0.0115\ B{\cdot}C-0.000812069\ C^2$	*A*—109.65 μm *B*—9.00 min *C*—59.99 °C	0.443
Rhodotorula spp. 74 (US)	$=5.25024-0.0404839\ A-0.466865\ B+0.132845\ C+0.000202874\ A^2+0.000236111\ A\ B+0.00000625\ A\ C+0.0497318\ B^2-0.0106458\ B\ C-0.00161853\ C^2$	*A*—89.70 μm *B*—8.99 min *C*—60.0 °C	0.305
Candida spp. 86 (CP)	$=-8.74792+2.41863\ X_1+6.25543\ X_2+0.489625\ X_3+0.886881\ X_1^2-0.751331\ X_1\ X_2-0.407421\ X_1\ X_3-0.898842\ X_2^2+0.246698\ X_2\ X_3-0.0107454\ X_3^2$	X_1—1.21 L min^{-1} X_2—5.0 min X_3—2.0 mL	2.154
Rhodotorula spp. 74 (CP)	$=-2.59249+11.0918\ X_1+1.28965\ X_2+0.022914\ X_3-7.97109\ X_1^2+0.567419\ X_1X_2+0.87579\ X_1X_3-0.447124\ X_2^2+0.392345\ X_2X_3-0.290543\ X_3^2$	X_1—1.25 L min^{-1} X_2—5.0 min X_3—2.0 mL	2.630

The Durbin-Watson (DW) statistics is presented in Table 4. Generally, the values can range from 0 to 4. As a general rule of thumb, the residuals are uncorrelated if the Durbin-Watson statistic is approximately 2. A value close to 0 indicates a strong positive correlation, while a value of 4 indicates a strong negative correlation. Serial correlation, sometimes also called autocorrelation, defines how any value or variable relates to itself over a time interval. Results in Table 4 show that the Durbin-Watson number is above 2, except for *Rhodotorula* spp. after CP treatments, where it is below 2. A positive serial correlation is associated with DW values below 2 and a negative serial correlation with DW values above 2.

According to the RSM model, the reduction in the number of yeasts can be described by a predicted mathematical model for the count of *Candida* spp. and *Rhodotorula* spp. The estimated effects of each operating variable and an analysis of variance for the model are presented in Table 5.

From Fig. 3a, b, the effect of treatment factors and a fixed value of treatment time of 3 min can be compared after US treatments and CP treatments. The strong influence of temperature in the reduction of the number of both *Candida* spp. and *Rhodotorula* spp. is shown in the figure. The greatest reduction is at temperature of 60 °C. As mentioned before, a statistically significant effect on the reduction in the number of viable yeast cells has treatment time (*B*), temperature (C) and their combined effect (*B*C). For ultrasound treatment for 3 min at 40 °C, the number of *Rhodotorula* spp. was higher than at 20 and 60 °C. This could be explained by the fact that there is higher free radical production at lower temperatures. Free radicals are also responsible for the inactivation of microorganisms. At higher temperatures, there is a combined thermosonication inactivation of microorganisms and, due to a cushioning effect of vapour, less influence of radicals on the reduction in the number of viable yeast cells.

From Fig. 3c, d, the influence of gas flow on the reduction in the number of viable yeast cells can be observed at a fixed value of treatment time at 3 min. For *Candida* spp., the least gas flow and the lowest sample volume lead to the greatest reduction in yeast number, whereas for *Rhodotorula* spp., the highest flow and the lowest sample volume lead to the greatest reduction in the number log CFU mL^{-1}.

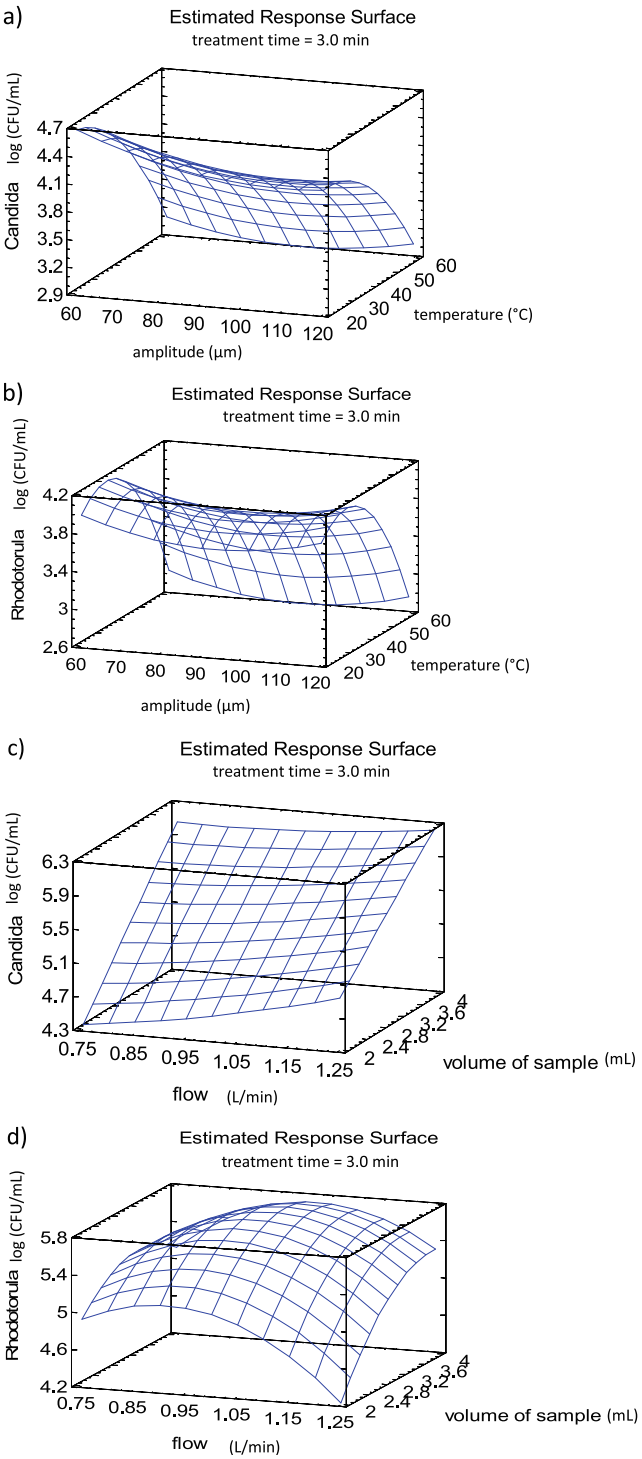

Fig. 3 Response surface plots for count (log CFU mL^{-1}) of *Candida* spp. 86 and *Rhodotorula* spp. 74 after high power ultrasound treatment (amplitude (*A*), treatment time (*B*) and temperature (*C*)) (**a**, **b**) at a constant temperature of 40 °C and after non-thermal gas-phase plasma treatment (gas flow (X_1), treatment time (X_2) and sample volume (X_3)) at a constant volume of sample (3 mL) for plasma treatment (**c**, **d**)

Conclusion

The effect of high power ultrasound and cold gas-phase plasma treatments on viable yeast cell count (*N*) of *Rhodotorula* spp. 74 and *Candida* spp. 86 in pure culture was studied. The results show that high power ultrasound and cold gas-phase plasma causes a reduction in the number of yeasts, *Rhodotorula* spp. 74 and *Candida* spp. 86. The greatest reduction (total removal of viable cells) of yeasts is after ultrasound treatments at 60 °C (thermosonication) for 6 and 9 min. Generally, there is a greater reduction in viable yeast cell count (*N*) when 115 μm of amplitude was applied. For plasma treatments, the greatest reduction in the number of yeast cells

(*N*) was observed for the longest treatment time (5 min) and the lowest sample volume (2 mL) (CP12, CP13, RP12 and RP13). The effect of high power ultrasound treatments on microorganisms is attributed to the elevated temperature (60 °C), cavitation phenomenon and free radical formation. For plasma treatment, the inactivation by UV radiation resulted in DNA strand breaks and also plasma-immanent reactive species such as atomic oxygen and OH^- radicals. Both technologies show great potential in the future food preservation, but in combination with elevated temperature or other non-thermal technologies.

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