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Multifactorial fungal inactivation combining thermosonication and antimicrobials

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Abstract

The combined effect of simultaneous application of heat treatments, low frequency ultrasound (20 kHz) at different amplitudes, on *Aspergillus flavus* and *Penicillium digitatum* spore viability suspended in laboratory broth formulated at different a_w (0.99 or 0.95) and pH (5.5 or 3.0), with or without vanillin or potassium sorbate were evaluated. An ultrasonic horn (13 mm) was submerged into the broth, heat and ultrasound was continuously applied. Survival curves were obtained, and D and z values calculated. For a_w 0.99, increasing ultrasound amplitude and reducing pH resulted in reduced D values. At constant a_w, D values decreased with pH reduction. At constant pH, D values were lower for a_w 0.99 than for 0.95. In general, D values were lower for thermosonication treatments than for thermal treatments. When potassium sorbate or vanillin was added and thermosonication treatment was applied at increased amplitude, lower D values were obtained.

Keywords: Ultrasound treatments; Fungal inactivation; Antimicrobial agents; Multifactorial preservation

1. Introduction

Food preservation emerging technologies, such as pulsed electric fields, high hydrostatic pressure, short-wave ultraviolet irradiation, and low frequency ultrasound are being evaluated in order to inactivate microorganisms while significantly reduce or completely eliminate the amount of heat required. These processes could be, for the most part, less energy-intensive and therefore more cost-efficient and environmentally friendly than conventional thermal processing (Piyasena, Mohareb, & McKellar, 2003). The use of ultrasound within the food industry has been subject of research for many years (Earnshaw, 1998; Earnshaw, Appleyard, & Hurst, 1995; Mason, 1990; Mason, Paniwnyk, & Lorimer, 1996; Piyasena et al., 2003; Zenker, Heinz, & Knorr, 2003). Heat combined with ultrasound is considered to reduce process temperatures and processing times, for pasteurization or sterilization processes that achieve the same lethality values as with conventional processes (Mason et al., 1996; Villamiel, van Hamersveld, & de Jong, 1999). Reduction of the temperature and/or processing time should result in improved food quality (Piyasena et al., 2003; Zenker et al., 2003). First introduced by Ordoñez, Aguilera, Garcia, and Sanz (1987), ultrasound applicability was predicted for the support of conventional thermal treatments, based on the possible synergy between low frequency

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ultrasound and heat for bacterial inactivation (Zenker et al., 2003).

Low frequency ultrasound refers to pressure waves with a frequency of 20 kHz or more (Butz & Tauscher, 2002; Suslick, 1988). Generally, ultrasound equipment uses frequencies from 20 kHz to 10 MHz. Higher-power ultrasound at lower frequencies (20–100 kHz), which is referred to as "power ultrasound", has the ability to cause cavitation that could be used in food processing to inactivate microorganisms (Povey & Mason, 1998). Ultrasound is known to disrupt biological structures and when applied with sufficient intensity has the potential to cause cell death (Harvey & Loomis, 1929; Hughes & Nyborg, 1962; Williams, Stafford, Callely, & Hughes, 1970). Ultrasonic waves generate gas bubbles in liquid media, which produce a high temperature- and pressure increase when they immediately burst (Vollmer, Everbach, Halpern, & Kwakye, 1998).

The bactericidal effect of ultrasound has been evaluated (Pagán, Mañas, Alvarez, & Condón, 1999; Pagán, Mañas, Raso, & Condón, 1999; Sala, Burgos, Condón, López, & Raso, 1995; Scherba, Weigel, & O'Brien, 1991; Wrigley & Llorca, 1992) and it is widely recognized that cell disruption during the application of ultrasound is caused by the cavitation effect (Butz & Tauscher, 2002; Kinsloe, Ackerman, & Reid, 1954; Piyasena et al., 2003; Scherba et al., 1991). Intracellular micro-mechanical shocks may disrupt cellular structural and functional components up to the point of cell lysis (Guerrero, López-Malo, & Alzamora, 2001). When the bubbles produced during ultrasonic treatment collapse, the compression/expansion cycles generated are thought to be responsible for cell disruption (Harvey & Loomis, 1929; Scherba et al., 1991). The effect of quick alternating pressures produced during cavitation disrupts microbial structures and causes the cell wall to break down. At high intensities, low frequency, an ultrasonic wave generates intense pressure, shear and temperature gradients within a material, which can physically disrupt its structure (McClements, 1995). The high temperatures produced during cavitation may also have some effect, but as these temperature changes occur momentarily, only the liquid in the immediate surroundings is heated and therefore only a small number of cells are affected (Sala et al., 1995).

Inactivation of mold spores using ultrasonic treatment was studied by Jiménez-Munguía, Arce-García, Argaiz, Palou, and López-Malo (2001), boiling chips and air bubbles were added to the treatment medium (Sabouraud broth) to determine their effect on the inactivation, both aids enhanced the effect of the cavitation from the sonication, reducing D values. This enhanced cavitation effect could be applied to sonication treatment processes to increase efficiency. Ultrasound critical processing factors are the nature of the ultrasonic waves, exposure time, type of microorganism, volume of food to be processed, composition of the food, and temperature (Hoover, 2000).

Resistance of different species to ultrasound differs widely as a result from the specific effect of ultrasound on the cell wall and differences in the cell wall structures among species (Earnshaw, 1998). Bacterial spores are much more resistant than vegetative ones and fungi are more resistant in general than vegetative bacteria (Alliger, 1975). Since molds and yeasts are in general more resistant to high intensity ultrasound (Earnshaw et al., 1995), and not enough information about mold spores is available, *Aspergillus* and *Penicillium* were chosen as representative species. In terms of microbial resistance more research is necessary about the potential enhancement of the ultrasound in combination with other preservation factors, among them antimicrobials, especially for fungi. The purpose of this research was to evaluate the simultaneous application of heat treatments (45, 47.5, 50, 52.5, 55, 57.5 or 60 °C), and low frequency ultrasound (20 kHz) at different amplitudes (60, 90 or 120 μm), on *Aspergillus flavus* or *Penicillium digitatum* spore viability when suspended in a laboratory broth formulated at selected a_w (0.99 or 0.95), pH (5.5 or 3.0), and with or without vanillin or potassium sorbate.

2. Materials and methods

A. flavus (ATCC 16872) and *P. digitatum* (LMU-DLA-2) obtained from Universidad de las Américas, Puebla Food Microbiology Laboratory were cultivated on potato-dextrose agar slants (PDA; Merck, Mexico) for 10 days at 25 °C and the spores harvested with 10 ml of 0.1% Tween 80 (Merck, Mexico) solution sterilized by membrane (0.45 μm) filtration.

Thermal and ultrasonic treatments were carried out in a double wall cylindrical vessel (4.5 cm internal diameter, 8.5 cm height) in which water was circulated with a refrigerated bath (Model 1268-24, Cole-Parmer, Chicago, IL) to attain 45, 47.5, 50, 52.5, 55, 57.5 or 60 °C. The refrigerated bath temperature was set as lower as needed in each thermosonication treatment to maintain constant temperature in the vessel. Ultrasound at 20 kHz was applied with a 13 mm diameter probe (CPX-400, Cole-Parmer, Chicago, IL) operating continuously at an amplitude of 60, 90 or 120 μm. The probe tip was maintained at 4 cm from the vessel bottom (Jiménez-Fernández, Palou, & López-Malo, 2001). Three trials were performed for every treatment.

For each treatment, the spore suspension was inoculated ($\approx 10^4$ spore/ml) into sterile broth prepared with Sabouraud glucose 2% broth adjusted by sucrose addition to a_w 0.99 or 0.95 and by incorporation of citric acid to pH 5.5 or 3.0. Potassium sorbate or vanillin was added in such concentrations that were not

inhibitory to the evaluated molds. For *A. flavus* 500 ppm of potassium sorbate or vanillin were tested while 500 ppm vanillin or 75 ppm potassium sorbate were evaluated in the case of *P. digitatum*. In every case the broth was heated to the desired temperature before treatment. At fixed intervals during treatments, samples were taken and serially diluted (0.1% peptone water). Surviving viable mold spore counts were determined immediately after treatment by surface plating on potato-dextrose agar. Two plates were used for each dilution and were incubated at 27 °C for 3–5 days. Analysis of variance of treatment effect on *A. flavus* or *P. digitatum* decimal reductions and two-sample *t* test (test of the difference between means) on *z* values were performed. The Statistica™ software (Statsoft™, Tulsa, OK) was used to analyze experimental results.

3. Results and discussion

Multifactorial *A. flavus* and *P. digitatum* spore inactivation by the combined effects of heat, ultrasound and antimicrobials (Fig. 1), as well as for thermal inactivation followed first order reaction kinetics. Decimal reduction times (*D* values) were calculated from the survival curve slopes. Significant differences ($p < 0.05$) in the heat resistance between molds were observed, being *A. flavus* spores more resistant to the evaluated multifactorial conditions than *P. digitatum*. For *P. digitatum*, temperatures higher than 52.5 °C applied for short times were sufficient to inactivate spores, thus thermal effects were evaluated in a lower temperature range than for *A. flavus*. Decimal reduction times for *A. flavus* varied from 37.6 to less than 0.5 min (Table 1), while for *P. digitatum*, *D* values varied from 78.7 to less than 2.0 min (Table 2), lower *D* values were obtained for treatments that combined thermosonication at high amplitudes with the presence of antimicrobial agents. In the case of *A. flavus*, when combining temperatures of 57.5 or 60 °C, high ultrasound amplitudes or the presence of potassium sorbate or vanillin, very short treatments were sufficient to eliminate the initial inoculated spores so we reported values less than 0.5 min (Table 1).

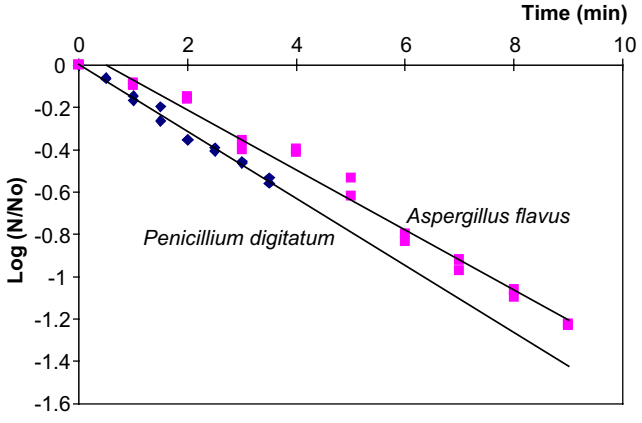

Fig. 1. *Aspergillus flavus* and *Penicillium digitatum* spore survival curves suspended in broth adjusted to a_w 0.95 and pH 3.0 during thermosonication at 120 μm and 52.5 °C.

For both studied molds, *D* values for multifactorial treatments were in several cases significantly smaller ($p < 0.05$) than *D* values obtained for thermal treatments alone. In the evaluated temperature range, simultaneous application of heat and ultrasound at amplitude of 90 or 120 μm decreased spore resistance, being more noticeable at temperatures ⩽57.5 °C for *A. flavus* and ⩽50 °C for *P. digitatum*. Effectiveness of thermal treatments was greatly increased with the simultaneous application of ultrasound; similar observations have been previously reported (Guerrero et al., 2001; López-Malo, Guerrero, & Alzamora, 1999; Sala et al., 1995; Wrigley & Llorca, 1992). However, at temperatures around 60 (for *A. flavus*) or 52.5 °C (for *P. digitatum*), the benefits of ultrasound application were reduced (Tables 1 and 2), probably as a result of an increased thermal effect and a reduced intensity of cavitation. Other authors (Raso, Palop, Pagán, & Condón, 1998; Raso, Pagán, Condón, & Sala, 1998) reported that cavitation intensity and thus the lethal effect of ultrasound depends on pressure, amplitude of ultrasound waves and temperature.

At pH 5.5, reduced a_w exerted a protective effect since *D* values were significantly ($p < 0.05$) higher. A reduced pH exerts a synergistic effect during thermosonication, reducing *D* values. For thermal treatments, a protective effect of reduced a_w (0.95) was observed at both evaluated pHs.

Presence of vanillin during thermosonication reduced *D* values, being more important at temperatures ⩽55 (for *A. flavus*) or ⩽47.5 °C (for *P. digitatum*). Addition of 75 or 500 ppm of potassium sorbate improved thermosonication effects on spore inactivation especially at wave amplitude ⩽90 μm. Ultrasound can be used to enhance the effect of heat or other antimicrobial treatments (Beuchat, 1981a, 1981b; Mason et al., 1996; McClements, 1995; Sala et al., 1995), since if ultrasound is combined with other preservation techniques such as heat or antimicrobials, then the microbial cells undergo an attack from two or three directions, which probably enhances microbial disruption and then inactivation (Alzamora, Guerrero, López-Malo, & Palou, 2003). Spore-forming microorganisms appear to be much more resistant to ultrasound than vegetative cells, and fungi are more resistant in general than vegetative bacteria (Alliger, 1975). Ultrasonic treatments, at levels that do not adversely modify nutritional and sensory properties of the food, are unable to effectively kill every microorganism present. This limitation suggests that ultrasound could be more effective when used in combination with other stress factors in a multifactorial approach, as

Table 1
Decimal reduction time (D, min) *for Aspergillus flavus* inactivation during thermal and thermosonication treatments at selected ultrasound wave amplitudes, water activities (a_w), pHs, with or without 500 ppm of vanillin (Vi) or potassium sorbate (KS)

T (°C)	pH	Ultrasound amplitude (μm)	a_w 0.99			a_w 0.95		
			Without antimicrobial	500 ppm KS	500 ppm Vi	Without antimicrobial	500 ppm KS	500 ppm Vi
52.5	5.5	120	6.20	3.23	6.55	12.00	3.77	10.28
		90	16.28	3.97	6.83	29.02	25.12	10.58
		60	28.18	8.13	7.08	37.63	28.04	25.93
		0	30.42	–[a]	–	36.07	–	–
	3.0	120	6.15	2.64	5.57	5.90	2.75	4.96
		90	6.77	2.48	9.27	7.51	3.15	9.31
		60	30.24	4.30	8.40	30.99	5.12	22.68
		0	31.70	–	–	33.34	–	–
55.0	5.5	120	5.06	1.09	4.94	5.85	<0.5	5.47
		90	6.26	4.13	5.11	6.10	2.80	5.68
		60	7.44	5.37	5.38	7.34	4.53	6.56
		0	17.40	–	–	23.74	–	–
	3.0	120	5.15	0.92	4.74	3.39	<0.5	3.74
		90	5.34	1.58	5.95	5.73	2.80	4.29
		60	5.89	3.88	6.45	9.40	4.53	4.51
		0	11.04	–	–	22.89	–	–
57.5	5.5	120	1.59	0.71	1.45	2.98	<0.5	2.61
		90	1.93	1.84	1.66	4.63	2.86	4.18
		60	3.72	2.80	1.73	4.67	3.16	4.26
		0	4.55	–	–	8.75	–	–
	3.0	120	1.94	<0.5	1.75	2.41	<0.5	1.78
		90	2.14	1.17	1.99	5.48	<0.5	3.68
		60	2.34	2.57	1.94	6.21	2.78	3.68
		0	4.36	–	–	8.48	–	–
60.0	5.5	120	1.20	<0.5	<0.5	1.24	<0.5	<0.5
		90	1.59	<0.5	<0.5	2.00	<0.5	<0.5
		60	2.46	<0.5	<0.5	2.11	<0.5	<0.5
		0	2.60	–	–	2.49	–	–
	3.0	120	0.80	<0.5	<0.5	2.18	<0.5	<0.5
		90	0.82	<0.5	<0.5	2.25	<0.5	<0.5
		60	1.67	<0.5	<0.5	2.21	<0.5	<0.5
		0	1.63	–	–	2.28	–	–

[a] Not tested.

observed in our results. Accordingly, numerous studies reported in the literature have combined ultrasound with heat (thermosonication) or with heat under pressure (manothermosonication) for inactivation of some pathogenic and spoilage microorganisms (García, Burgos, Sanz, & Ordoñez, 1989; Guerrero et al., 2001; Raso, Palop, et al., 1998; Raso, Pagán, et al., 1998).

In our laboratories, sensitivity of *Saccharomyces cerevisiae* cells to the action of ultrasound combined with moderate temperatures, addition of preservatives (synthetic and/or naturally occurring ones) and control of pH with citric acid was examined (Guerrero et al., 2001; Guerrero, Tognon, & Alzamora, 2001a; Guerrero, Tognon, & Alzamora, 2001b). The addition of 500 ppm vanillin significantly improved the inactivation effect of thermosonication treatments against *S. cerevisiae* cells at pH 3.0. The effect was synergistic with respect to the thermosonication treatments alone. This synergy was even greater when the medium was supplemented in equal proportions (500 ppm) with ethylene diamine tretra acetic disodium salt or EDTA (Guerrero et al., 2001b). EDTA and vanillin would share at least one common mechanism of action altering microbial cell permeability, permitting the loss of macromolecules from the interior and facilitating penetration of the phenolic compound into the cell and its interaction with enzymes and proteins (Conner & Beuchat, 1984). Both these antimicrobials would cooperate with ultrasound on yeast inactivation. Microbial cell alterations reported in the literature involved disruption of subcellular particles during sonication with fine membrane fragmentation (Alliger, 1975), internal cavitation as well as internal microstreaming, with modification of the cellular structure (Ciccolini, Taillandier, Wilhem, Delmas, & Strehaiano, 1997), as well as fragmented cells and marked irregularity in density (Kinsloe et al., 1954). Observations obtained with transmission electron microscopy on the structural effects of ultrasound in *S. cerevisiae* cells showed that thermosonication produced several modifications, from cytological disruption of

Table 2
Decimal reduction time (D, min) for *Penicillium digitatum* inactivation during thermal and thermosonication treatments at selected ultrasound wave amplitudes, water activities (a_w), pHs, with or without 500 ppm of vanillin (Vi) or 75 ppm potassium sorbate (KS)

T (°C)	pH	Ultrasound amplitude (μm)	a_w 0.99			a_w 0.95		
			Without antimicrobial	75 ppm KS	500 ppm Vi	Without antimicrobial	75 ppm KS	500 ppm Vi
45.0	5.5	120	29.89	14.39	23.25	37.30	24.24	29.41
		90	35.47	16.90	24.38	41.33	27.97	30.52
		60	43.92	20.22	34.72	54.76	32.70	35.91
		0	62.74	–[a]	–	78.69	–	–
	3.0	120	20.01	12.74	19.45	22.72	13.24	17.45
		90	22.46	16.23	19.68	26.82	17.23	25.59
		60	36.65	18.15	31.55	38.64	18.83	32.14
		0	50.35	–	–	66.39	–	–
47.5	5.5	120	26.74	11.96	19.82	32.70	20.49	26.47
		90	28.24	13.46	21.66	35.47	25.14	30.16
		60	35.63	18.86	29.90	47.83	29.10	32.92
		0	51.53	–	–	62.94	–	–
	3.0	120	17.59	10.87	17.12	19.80	11.00	14.28
		90	21.15	12.96	18.10	22.40	13.76	20.90
		60	27.41	15.69	20.37	26.10	16.69	25.12
		0	33.42	–	–	54.52	–	–
50.0	5.5	120	9.59	7.15	8.57	15.61	10.13	13.95
		90	11.56	8.48	8.91	17.65	12.47	14.83
		60	14.70	11.65	10.77	20.57	15.53	16.75
		0	25.42	–	–	54.17	–	–
	3.0	120	6.03	4.05	5.84	7.41	5.71	10.36
		90	8.63	4.39	7.95	9.38	7.54	11.99
		60	12.01	6.87	8.59	13.20	10.47	13.95
		0	22.01	–	–	24.29	–	–
52.5	5.5	120	5.33	4.19	6.47	11.48	7.85	11.78
		90	7.66	4.20	6.82	15.29	9.28	12.92
		60	9.85	5.19	7.20	18.57	10.72	15.48
		0	13.30	–	–	34.35	–	–
	3.0	120	3.81	1.98	3.05	6.31	3.30	5.08
		90	4.80	2.92	4.26	7.43	7.85	6.08
		60	6.95	4.62	5.07	8.80	6.28	6.68
		0	9.54	–	–	13.97	–	–

[a] Not tested.

organelles till puncturing of the cell wall and breakage of plasma membrane (Guerrero et al., 2001).

Decimal reduction time dependence on temperature exhibited a significantly ($p < 0.10$) greater z value in several of the multifactorial inactivation treatments (Tables 3 and 4). The obtained z values reflect the relative resistance of *A. flavus* and *P. digitatum* to different destructive temperatures indicating that spore sensitivity to changes in the heating temperature is, in general, lower for the combined process than for thermal treatments. García et al. (1989) observed that simultaneous application of heat and ultrasound drastically reduced (72–99%) decimal reduction times for the inactivation of *B. subtilis* spores, with lower differences in D values between

Table 3
Aspergillus flavus inactivation dependence on temperature (z, °C) for the evaluated conditions

pH	Ultrasound amplitude (μm)	a_w 0.99			a_w 0.95		
		Without antimicrobial	500 ppm KS	500 ppm Vi	Without antimicrobial	500 ppm KS	500 ppm Vi
3.0	0	5.85			6.36		
	60	5.99	22.39	7.86	6.91	18.85	6.33
	90	7.92	15.32	7.48	15.73	49.54	12.40
	120	8.11	5.48	9.94	17.31	–[a]	11.20
5.5	0	6.60			6.38		
	60	7.19	10.80	8.15	6.33	5.27	6.37
	90	7.06	14.92	8.12	6.94	5.30	12.39
	120	9.46	7.56	7.64	7.69	–[a]	8.40

[a] Insufficient data to calculate z value.

Table 4
Penicillium digitatum inactivation dependence on temperature (z, °C) for the evaluated conditions

pH	Ultrasound amplitude (μm)	a_w 0.99			a_w 0.95		
		Without antimicrobial	75 ppm KS	500 ppm Vi	Without antimicrobial	75 ppm KS	500 ppm Vi
3.0	0	10.64			10.49		
	60	9.90	11.68	9.06	11.24	15.31	10.86
	90	10.42	9.24	10.63	12.19	19.45	11.82
	120	9.51	8.76	8.68	11.92	11.93	14.31
5.5	0	10.74			21.83		
	60	10.72	12.62	10.02	14.08	14.48	17.99
	90	10.48	12.41	12.22	15.64	14.35	17.50
	120	9.28	13.66	12.31	13.47	14.09	17.01

treatments as temperature increased. Calculated z-values for *B. subtilis* spore inactivation were approximately 2-fold higher for the combined treatment. This behavior may be attributed to the changes in the properties of suspension solution during heating and its consequence on cavitation (Mason et al., 1996). At large amplitudes, the bubble may grow so large on rarefaction that time available for collapse is insufficient (Mason et al., 1996; Sala et al., 1995). Therefore, depending on liquid properties (vapor pressure, surface tension, and viscosity) cavitation effect can be diminished with larger amplitudes. In our case, by setting the amplitude, the ultrasonic vibrations at the probe tip were fixed to the desired level. The amount of power required, maintaining constant amplitude varies depending on the resistance to the movement at the probe tip (Guerrero et al., 2001; López-Malo et al., 1999). Lethality of ultrasonic treatments is highly influenced by the amplitude of ultrasonic wave (Raso, Palop, et al., 1998; Raso, Pagán, et al., 1998).

4. Conclusions

The use of ultrasound on its own in the food industry for fungal inactivation is presently unfeasible. However, combination of ultrasound, antimicrobials and heat shows considerable promise. The future of ultrasound in the food industry for fungicidal as well as bactericidal purposes lies in multifactorial processes combining antimicrobials, reduced pH and/or a_w with thermosonication, manosonication and/or manothermosonication, as they are more energy-efficient and result in the reduction of D values when compared to conventional heat treatments. These multifactorial processes need to be further studied to generate the basis of feasible ultrasonic pasteurization treatments.

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