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Sanitization of packaging and machineries in the food industry: Effect of hydrogen peroxide on ascospores and conidia of filamentous fungi

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ABSTRACT

In the food industry, sterilization of packaging and filling machines by hydrogen peroxide (HP) is a widespread practice. Its effectiveness is usually tested by means of inactivation tests on selected test microorganisms that were any case chosen without taking into account that food products could be also spoiled by microorganisms presumably resistant to HP. For this reason, the aim of this work was to assess the resistance of different ascospore-forming moulds (*Talaromyces bacillisporus, Aspergillus hiratsukae, Chaetomium globosum*) to HP, in order to find the most resistant to this kind of chemical stress, and to compare their resistance with that registered for other moulds, including test microorganism *Aspergillus brasiliensis* ATCC 16404. Tests were carried out from 50 to 60 °C on spores or conidia, depending on the strain, either by immersing inoculated strips (aluminium, tin-plate, HDPE, PET) in HP, or by directly inoculating cells in the sanitizing medium. In both tests, *T. bacillisporus* proved the most resistant strain, followed by *A. hiratsukae*, *C. globosum and A. brasiliensis* at all temperatures tested. In test without a supporting material, D values of *T. bacillisporus* varied from 6 to 23 s. In test with metallic or plastic strips, D values of *T. bacillisporus* were higher on plastic materials, compared to those obtained on metallic ones up to 53 °C, whereas at higher temperatures D values proved similar. For *A. hiratsukae*, D values were similar if different materials were compared, except for D_{50} on aluminium and HDPE, which proved slightly higher (3.1–3.4 s) than those obtained on tin-plate and PET (2.7–2.8 s). Analogously, ascospores of *C. globosum* behaved in a similar way if different materials were compared, except for D_{50} values that varied in a wide range (from 2.9 s on tin-plate to 4.0 s on HDPE). *A. brasiliensis* was rapidly inactivated by the synergistic effect of heat and hydrogen peroxide, so for this strain it was not possible to calculate any D value. Based on the results obtained in this paper, tested ascospore-forming moulds proved to be sensibly more resistant to HP than other heat-sensitive strains tested, their D values always being significantly higher, regardless of the strain considered and the supporting material assessed. Ascospore-forming moulds could be furtherly investigated, as for practical purposes they seemed most suitable as target microorganisms than heat-sensitive microorganisms such as *Aspergillus brasiliensis* ATCC 16404, their use during bio-validations of sanitizing processes on machineries used for refrigerated products (pH > 4.5) or non-refrigerated acid products (pH ≤ 4.5) leading to more performing results.

1. Introduction

Within an aseptic processing, packaging and machinery sterilization is aimed to the inactivation of the microbial population that can contaminate both packaging and machine interiors prior to filling (Featherstone, 2015; Kumar et al., 2008). It can be performed using heat (e.g., steam and hot water, dry heat) or chemical sanitizers (e.g., hydrogen peroxide, peracetic acid or a mixture of both), whereas physical methods (e.g., ultraviolet radiations) are actually used for packaging devices only (VDMA, 2005). Among these methods, sterilization by hydrogen peroxide is a widespread practice, since this molecule is a very effective antimicrobial agent, decomposing readily to oxygen and water with release of heat (Finnegan et al., 2010). When used by food industries on either packaging devices or hygienic filling machines, it is applied in its stabilized form at concentrations ranging from 10% to 40% by spraying or dipping the materials to be sterilized and then heating at temperatures ranging from 55 °C to about 75 °C. Its effectiveness is usually checked by means of inactivation tests on selected microorganisms: *Aspergillus niger ATCC 6275* or *Aspergillus brasiliensis* ATCC 16404 (conidia), and *Bacillus subtilis SA 22* or *Bacillus atrophaeus* ATCC 9372 (spores). In particular, when a sanitization process concerning pasteurized products (pH > 4.5) distributed in the

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cold chain or acid products (pH ≤ 4.5) distributed outside the cold chain must be assessed, *Aspergillus niger* ATCC 6275 or *Aspergillus brasiliensis* ATCC 16404 are usually applied as test microorganisms for bio-validation processes (VDMA, 2005). This choice was probably made because they had been considered as the most resistant microbial forms that could occur on packaging devices or in industrial environments and that are able to grow also at low pH and temperatures. Nevertheless, in making this choice, it has not been taken into account that these two black aspergilli, despite being representative for all those anamorphic Fungi that can contaminate packaging materials or machineries, are sensitive to either thermal or chemical shocks. In addition to this, this *status quo* does not contemplate the possibility that packing devices and filling machines used for the above-mentioned products could be contaminated by stress-resistant moulds, too. Among them are heat-resistant moulds (HRM). These microorganisms can produce ascospores characterized by a dormant state called "quiescence" that can only be broken if shocks occurred. This state allow them not only to survive to stressing conditions during food pasteurization, but also to germinate and spoil a comprehensive range of food products (Dijksterhuis, 2007; Rico-Munoz, 2017). In last decades they have just sporadically been searched and isolated from mechanical devices such as palletizer, filler or cooling tunnels, and from packaging materials such as PET bottles, laminated paperboard, stretch wraps or slip sheets (Delgado et al., 2012b; Rico-Munoz et al., 2007; Rico-Munoz, 2017; Sato and Takano, 2000), their responsibility in contaminating packaging or machineries and then spoiling acid products being probably underestimated. Consequently, also their resistance to chemical sanitizers applied for packaging and machineries sterilization has been scarcely documented, the only papers concerning this topic being those by Delgado et al. (2012a) and Dijksterhuis et al. (2018) for resistance to activated sodium chlorite, chlorine dioxide or iodine, respectively. On the contrary, the remaining articles are focused on conidia-forming species (Olivier Bernardi et al., 2018) or on fungal genus *Chaetomium*, whose ascospores proved heat-sensitive, but resistant to chemical agents (Nakayama et al., 2013; Sato and Takei, 2000).

For this reason, the aim of this work was: (i) to assess the resistance of two different HRM to hydrogen peroxide on different materials, in order to find the most resistant to this kind of chemical stress, (ii) to compare HRM resistance to hydrogen peroxide with that registered for *Chaetomium globosum* and some conidia-forming moulds, including *Aspergillus brasiliensis* ATCC 16404 that is actually indicated as one of the target microorganisms for bio-validations of sanitization processes by hydrogen peroxide.

2. Materials and methods

2.1. Microorganisms

This study was carried out on different fungal strains:

- *Aspergillus hiratsukae* (≡ *Neosartorya hiratsukae*) SSICA 3913, isolated from a spoiled tea beverage;
- *Talaromyces bacillisporus* SSICA 10915, isolated from heat-treated blueberries;
- *Mucor circinelloides* SSICA 12986, isolated from spoiled tomato pulp in aseptic bags;
- *Penicillium solitum* SSICA 18608, isolated from the environment of an apple juice production plant;

In addition to the above-mentioned strains, one *Aspergillus brasiliensis* ATCC 16404 and one *Chaetomium globosum* ATCC 6205 were used as reference strains.

Strains were identified according to Samson et al. (2007) for *Aspergillus* isolate with *Neosartorya* morphs, Samson et al. (2010) for *Mucor* and *Penicillium* isolates, and Yilmaz et al. (2014) for *Talaromyces* isolate. The examination of microscopic structures was performed by a Differential Interference Contrast (DIC) Microscope (Eclipse 80i, Nikon, Tokyo, Japan). Apart from *T. bacillisporus*, that was easily recognizable by means of their phenotypic characteristics, the identity of all other SSICA strains was confirmed by means of molecular techniques. For the *Aspergillus* strain with *Neosartorya* ascospores, genomic DNA was extracted using CetylTrimethyl Ammonium Bromide (CTAB) and the partial β-tubulin gene were amplified using the primers N2F (5′-GGC TCTGGCCAGTAAGTTCG-3′) and N2R (5′-TTGTCACCGTTGGCCTA GTA-3′), as described by Yaguchi et al. (2012). The resulting amplicons were purified using the QIAquick PCR 163 purification Kit (Qiagen, Hilden, Germany), and sequenced by MACROGEN Europe (Amsterdam, The Netherlands). For all other strains, genomic DNA was extracted using Cetyl-trimethyl Ammonium Bromide (CTAB) following the protocol of Graham et al. (1994) and the nrDNA ITS1–5.8S–ITS2 were amplified using the universal primers ITS1F/ITS4. PCR amplifications were performed following the parameters by White et al. (1990) for the ITS region and carried on the sequencing of the purified samples. All sequences obtained in this study were compared to those available in the GenBank database (www.ncbi.nlm.nih.gov/Genbank).

2.2. Preparation of fungal suspensions

Since *Aspergillus brasiliensis* and *Penicillium solitum* are known as anamorphic Fungi reproducing asexually, conidia were used to prepare fungal suspensions. Each purified strain was spread on Malt Extract Agar (MEA; OXOID, Cambridge, UK) and incubated at 25 °C for 7 days. It was then collected into a 0.1% (v/v) Tween 80 solution, filtered through sterile glass wool and counted on MEA supplemented with 0.01% chlortetracycline (Sigma-Aldrich, St. Louis, MO, USA). Filtered suspensions were then stored at −20 °C (*P. solitum*) or 0 °C (*A. brasiliensis*) until use.

Since Mucor circinelloides had been found in the air of industrial plants only in its asexual form (conidia called sporangiospores), sporangiospores were used to prepare fungal suspensions. The strain was spread on Malt Extract Agar (MEA; OXOID, Cambridge, UK) and incubated at 25 °C for 7 days. It was then collected into a 0.1% (v/v) Tween 80 solution, filtered through sterile glass wool and counted on MEA supplemented with 0.01% chlortetracycline (Sigma-Aldrich, St. Louis, MO, USA). Filtered suspensions were then stored at 0 °C until use.

Since *Talaromyces*, *Aspergillus* with *Neosartorya* morphs and *Chaetomium* are recognized as ascospore-forming moulds that could be found in the processing environment, their ascospores were used to prepare fungal suspensions. Each purified strain was spread on Potato Dextrose Agar (PDA, Oxoid, Cambridge, UK) and incubated at 30 °C for 60 days to enhance ascospore production and to increase resistance (Conner and Beuchat, 1987; Dijksterhuis and Teunissen, 2004; King and Whitehand, 1990; Tournas and Traxler, 1994). Mycelium and ascoma were collected into a 0.1% (v/v) Tween 80 solution containing sterile glass beads (3 mm diameter). Each suspension was shaken for 5 min using a mixer (Vortex, Continental Instruments), and filtered through sterile glass wool. It was then checked to confirm it was a single spore suspension using a Differential Interference Contrast (DIC) Microscope (Eclipse 80i, Nikon, Tokyo, Japan). Filtered suspensions were then stored at −20 °C until use.

2.3. Hydrogen peroxide inactivation tests

A pure stabilized 40% hydrogen peroxide solution (Carlo Erba, Milan, Italy) was used without any dilution at temperatures ranging from 25 °C to 60 °C, in order to evaluate the efficacy of hydrogen peroxide with or without temperature contribution. Once opened, it was maintained at 4 °C up to six months, according to manufacturer's instructions. Each time it was used, hydrogen peroxide solution has been checked by means of a Chemets Hydrogen Peroxide Test Kit (CHEMetrics, Midland, USA), in order to be sure to work at a proper concentration. A frozen catalase from bovine liver powder (4323 units/

mg protein; Sigma-Aldrich, St. Louis, MO, USA) was diluted in a peptone salt solution (8.5 g/l NaCl, 1 g/l tryptone), in order to obtain a final concentration equal to 0.233 g/l. It was then filtered by means of a presterilized Vacuum-Driven Disposable Filtration System (Express™ PLUS 0.22 µm, Millipore, Billerica, MA, USA) and maintained at 4 °C until use. Such concentration was needed to neutralize the residual amount of hydrogen peroxide present on each strip and was previously determined (data not shown).

2.3.1. Tests without a supporting material

Tests were performed by inoculating 0.02 ml of each fungal suspension in 50-ml PYREX® round-bottom sterile tubes with screw cap containing 0.2 ml of pre-heated hydrogen peroxide. For each temperature tested, the come-up time was previously calculated by means of a Platinum-sensor probe (Delta HOM, Padua, Italy). Hydrogen peroxide effect was evaluated at 25 °C for all strains and at 50 °C, 55 °C and 60 °C for *A. brasiliensis* ATCC 16404 and ascospore-forming strains up to a maximum of 60 s. At each treatment time, hydrogen peroxide was neutralized with 9.8 ml of the catalase solution. Appropriate decimal dilutions were then plated on MEA supplemented with 0.01% chlortetracycline and colonies were counted after incubation at 25 °C up to five days (for heat-sensitive strains) or at 30 °C up to 10 days (for *Aspergillus* and *Talaromyces* strains). Each time/temperature combination was tested at least twice.

2.3.2. Tests on a supporting material

Different laminated materials (aluminium, tin-plate, PET, HDPE) were cut into strips (5 × 50 mm) that were sterilized by dipping in a 96% ethanol solution. Strips were then separately inoculated with 0.020 ml of each conidial or spore suspension and kept for 1 h under sterile conditions, in order to allow them to dry.

Tests were performed by dipping strips in a 100-ml glass beaker containing the hydrogen peroxide solution, immersed into a stirring water bath (SB 15, Falc, Treviglio, Italy) equipped with a Platinum-sensor probe (Delta HOM, Padua, Italy) for a continuous temperature check. Hydrogen peroxide effect was evaluated at 25 °C for all strains and at 50 °C, 53 °C, 57 °C and 60 °C for *A. brasiliensis* ATCC 16404 and ascospore-forming strains up to a maximum of 30 s. After each treatment, strips were rapidly removed from the heated hydrogen-peroxide solution and transferred in 50-ml PYREX® round-bottom sterile tubes with screw cap containing 10 ml of the neutralizing solution. Appropriate decimal dilutions were then plated on MEA supplemented with 0.01% chlortetracycline and colonies were counted after incubation at 25 °C up to five days (for heat-sensitive strains) or at 30 °C up to 10 days (for *Aspergillus* and *Talaromyces* strains). Each time/temperature combination was tested at least twice.

2.4. Modeling the thermal death rate curves

Since ascospores usually survive in a dormant state that can only be broken with a sub-lethal treatment called “activation” (Beuchat, 1986; Dijksterhuis, 2007), inactivation curves are usually characterized by a linear activation phase and then, after a maximum number of countable ascospores occurred, by a shoulder preceding an accelerating death rate. In this paper, hydrogen peroxide did not cause a real activation of the spores from *Aspergillus* with *Neosartorya* morphs, *Talaromyces* and *Chaetomium*, except for trials with *T. bacillisporus* and *A. hiratsukae* directly inoculated in the sanitizing medium, where a slight increase of the countable ascospores was observed. Only in these cases, the activation phase was the not taken into account for the determination of the thermal parameter, D_T.

2.5. Statistical analysis

Microsoft ® Excel 2013 (Microsoft, Redmond, WA, USA) was used for graphical elaboration of thermal reduction data that were presented as mean values ± standard deviation (SD). The linear regression function enabled the determination of the D_T values, defined as the time required to provide a 1-log reduction in the population of a given microorganism at a given temperature, and the calculation of z values, defined as the degree of temperature required to bring a 10-fold reduction in a given D_T value.

For those combinations that displayed a marked non-linear behavior in their inactivation phase, GInaFiT (Geeraerd and Van Impe INActivation Model Fitting Tool, version 1.7), a freeware Add-inn tool for Microsoft® Excel, was used for calculation of 1D or 4D values and for statistical analysis (https://cit.kuleuven.be/biotec/software/GinaFit). Both linear- and nonlinear-models have been applied as a comparison. The goodness of fit of the models was assessed using the automatically reported regression coefficient (R^2) and a Root Mean Sum of Squared Error (RMSSE). The kinetic parameters and the maximum specific inactivation rate (kmax) were obtained from each model. The 1Dvalues were calculated as 2.303/kmax for each combination, as also described by Lahou et al. (2015) (Table 3).

3. Results and discussion

3.1. Hydrogen peroxide inactivation tests

Tests were carried out on conidia (specialized, asexual propagules) or ascospores (stress-resistant, sexual structures), depending on the strain tested. This choice has been done since, during bio-validations of packaging and machineries in industrial plants, conidia of the asexual fungus *Aspergillus brasiliensis ATCC 16404* are used to assess the effectiveness of a given sanitizer. Since one of our goals was to make a comparison between the above-mentioned fungus and other species contaminating industrial environments in the operative conditions applied during bio-validations, this is the reason why we tested different kinds of structures for different strains.

Tests with or without a supporting material were carried out at 25 °C on both heat-sensitive and stress-resistant strains, giving divergent results. For *Penicillium solitum*, *Aspergillus brasiliensis* and *Mucor circinelloides*, also at lower times (up to 30 s) conidia were partially or totally inactivated without either good reproducibility or repeatability in the analyses, standard deviations calculated being in most cases equal to logarithmic values obtained and regression coefficients being very low (from 0.22 to 0.69). For ascospore-forming strains, reproducibility or repeatability were excellent, but ascospores proved not to be affected by hydrogen peroxide, their concentration being unvaried even after 600 s, compared to positive controls (data not shown). The lack of reproducible results with conidia could be due to the presence of a thinner cellular membrane that makes *Penicillium*, *Aspergillus* and *Mucor* strains more sensible to chemical stresses, or by their inability to encode transcription factors required for stress tolerance (e.g. heat shocks or hydrogen peroxide), such that observed in *Aspergillus oryzae* by Sakamoto et al. (2008). On the contrary, the strong resistance of ascospore-forming strains to hydrogen peroxide could be attributed to the accumulation in their membrane of trehalose and mannitol that proved essential for prolonged stabilization of cells against stress (Wyatt, 2014), but it could also be due to the fact that hydrogen peroxide becomes active at higher temperatures (50 °C or more, according to Burckett St. Laurent et al., 2007).

3.1.1. Tests without a supporting material

Since experiments at 25 °C do not allow us to build proper inactivation curves and to calculate thermal death parameters for *Penicillium* and *Mucor* strains, further tests were carried out at temperatures up to 60 °C only on *A. brasiliensis* ATCC 16404 and ascospore-forming moulds. For all strains, it was possible to find a linear correlation between heating time and logarithm of surviving conidia or ascospores (Fig. 1). Consequently, the regression analysis on the best fit has been carried out and the decimal reduction times for each

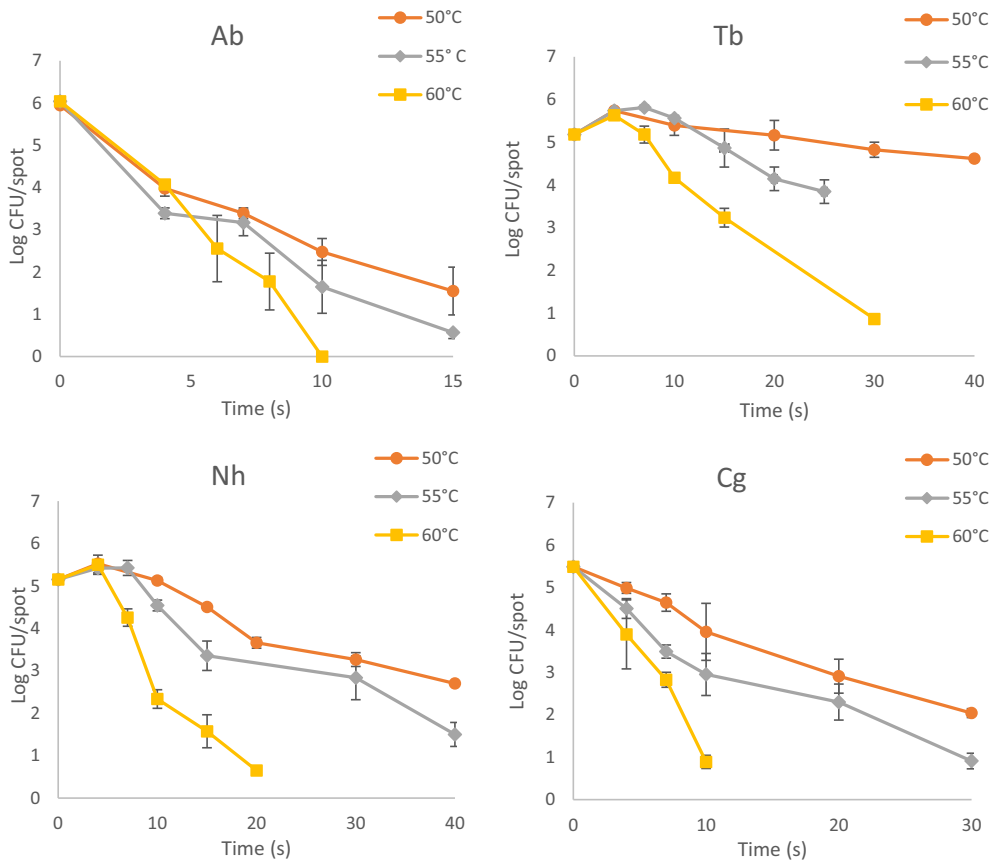

Fig. 1. Inactivation curves for conidia or spores directly immersed in heated hydrogen peroxide (50–60 °C). (Nh) *A. hiratsukae SSICA 3913*; (Ab) *A. brasiliensis ATCC 16404;* (Tb) *T. bacillisporus SSICA 10915;* (Cg) *C. globosum ATCC 6205*. Vertical error bars indicate standard deviation for mean values.

Table 1
Death rate parameters and hydrogen-peroxide resistance values for strains tested (Ab = *A. brasiliensis*; Nh = *A. hiratsukae*, Tb = *T. bacillisporus*, Cg = *C. globosum*), directly inoculated in a 40% hydrogen peroxide solution.

Strain	Temperature (°C)	Activation time (s)	R^2	Mean D value (s)	Standard deviation	z value (°C)
Ab	50	0	0.96	3.9	0.2	
	55	0	0.94	3.1	0.1	nd*
	60	0	0.99	1.7	0.1	
Nh	50	4	0.95	12.3	0.1	
	55	4	0.93	9.5	0.6	18
	60	4	0.93	3.4	0.0	
Tb	50	4	0.94	22.7	1.5	
	55	4	0.96	9.8	1.9	16
	60	4	0.99	5.5	0.0	
Cg	50	0	0.98	9.0	2.8	
	55	0	0.93	7.2	0.3	nd*
	60	0	0.98	2.2	0.0	

nd* data concerning *A. brasiliensis* and *C. globosum* were not reported, since the synergistic effect of heat and hydrogen peroxide could give back non-linear kinetics, supposing a total inactivation within few seconds at temperatures higher than 60 °C for the above-mentioned strains.

temperature have been obtained (Table 1). As Fig. 1 and Table 1 show, *T. bacillisporus* proved the most resistant strain, followed by *A. hiratsukae*, *C. globosum* and *A. brasiliensis* at all temperatures tested. Furthermore, a regression analysis on heating temperatures and logarithms of D values has been carried out and the z values for HRM were calculated (Table 1), similar values being found for both *Talaromyces* and *Aspergillus* strains.

These data could be compared with those obtained by Delgado et al. (2012a) who observed D_{60} values ranging from 2.1 to 4.9 s, respectively for 1-month-old spores of *Paecilomyces variotii* (≡*Byssochlamys spectabilis*) and *Aspergillus fischeri* (*Neosartorya fischeri* in the above-mentioned text) treated at 60 °C with a 40% hydrogen peroxide solution. Even if a direct comparison with strains tested in the above-mentioned paper is not possible, our D_{60} values seemed slightly higher, maybe due to our longer incubation times before harvesting of spores for suspension (60 days) that enhanced their resistance (Conner and Beuchat, 1987; Dijksterhuis and Teunissen, 2004; King and Whitehand, 1990; Tournas and Traxler, 1994).

Furthermore, a comparison of the results obtained with HRM was made with those obtained treating *Bacillus atrophaeus DSM* 675 (=ATCC 9372) that is one test microorganism that can be used as an alternative to *A. brasiliensis* ATCC 16404 and *B. subtilis* SA 22 during bio-validations of hygienic filling machines of Class IV or V for liquid and viscous foods (VDMA, 2005; VDMA, 2008), respectively. At 55 °C, the comparison allowed us to register a lower resistance to hydrogen peroxide of the *Bacillus* species, its D value (4.9 s) being half the one registered for both *T. bacillisporus* (9.8 s) and *A. hiratsukae* (9.5 s). Despite this, the z value of *B. atrophaeus* proved consistently higher (29 °C) than those calculated for *T. bacillisporus* (16 °C) and *A. hiratsukae* (18 °C), *B. atrophaeus* becoming the most resistant microorganism at temperatures higher than 60 °C, that were those actually used in the industrial practice (see additional information).

3.1.2. Tests on a supporting material

As for tests without a supporting material, since experiments at 25 °C do not allow us to build proper inactivation curves and to calculate thermal death parameters for *Penicillium* and *Mucor* strains, further tests were carried out at temperatures ranging from 50 °C to 60 °C only on *A. brasiliensis* ATCC 16404 and ascospore-forming

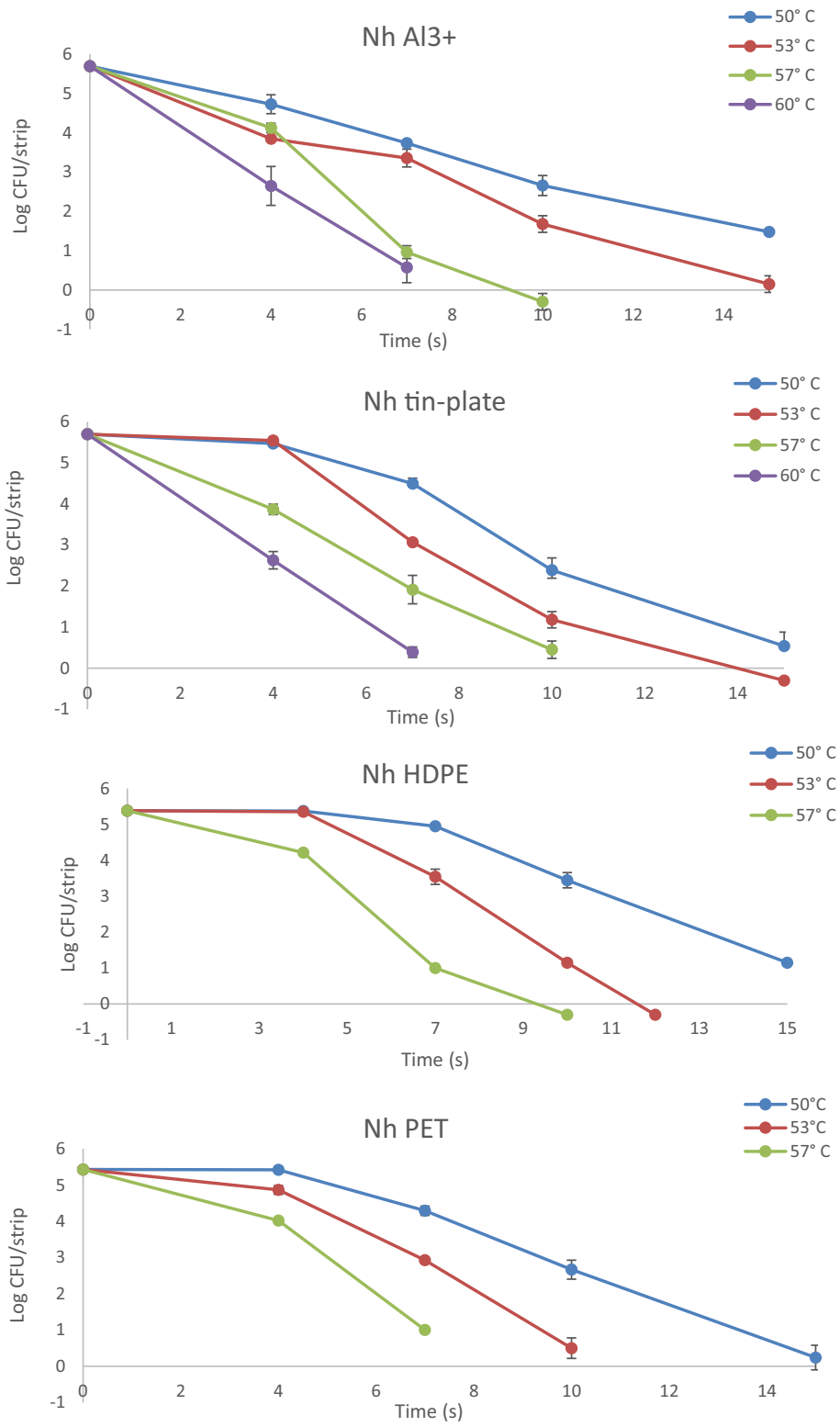

Fig. 2. Inactivation curves for spores inoculated on metallic or plastic strip and immersed in heated hydrogen peroxide (50–60 °C). 2.1 (Nh) *A. hiratsukae SSICA 3913*; 2.2 (Tb) *T. bacillisporus SSICA 10915;* 2.3 (Cg) *C. globosum ATCC 6205*. Vertical error bars indicate standard deviation for mean values.

moulds.

Tests with *A. brasiliensis* ATCC 16404 resulted in a total inactivation of treated conidia at any temperature and time tested, except for the shorter treatment time (4 s), when sporadic survivals were observed (data not shown). Consequently, the production of inactivation curves and the calculation of a D_T was not possible.

Differently, tests with ascospore-forming moulds resulted in both reproducible and repeatable data, death rate curves and corresponding parameters being respectively shown in Fig. 2 and Table 2.

Most of the combinations showed a linear trend and excellent regression coefficients (R^2). Nevertheless, on HDPE and at lower temperatures *T. bacillisporus* and *A. hiratsukae* showed a non-linear

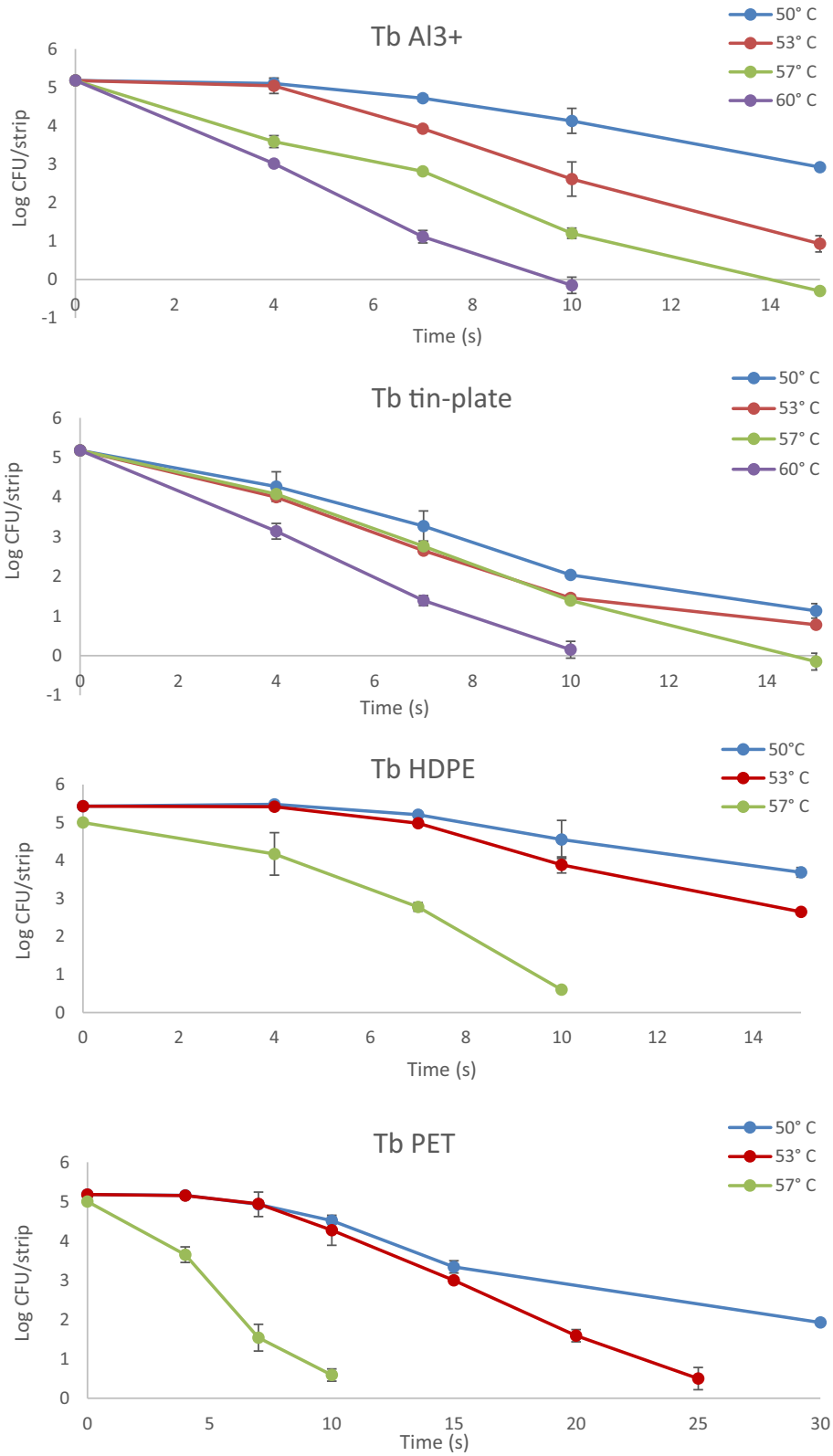

Fig. 2. (*continued*)

behavior in their inactivation phase, thus resulting in lower regression coefficients (0.86–0.89). For this reason, for the above-mentioned combinations the linear (Bigelow and Esty) model used for data elaboration was compared with non-linear models by means of the GInaFiT add-inn tool, in order to highlight any significant difference in D values, as already done by Berni et al. (2017). When the Bigleow and Esty model was used, D values of *A. hiratsukae* ranged between 1.6 and 3.4 s. Similarly, D values of *T. bacillisporus* ranged between 2.4 and 8.2 s. When non-linear models were applied, 1D values of both HRM were lower: D_T of *A. hiratsukae* ranged between 0.9 and 2.1 s, whereas D values of *T. bacillisporus* ranged between 1.6 and 4.7 s. Results showed that non-linear models provided the best fit to data, with high R^2 values

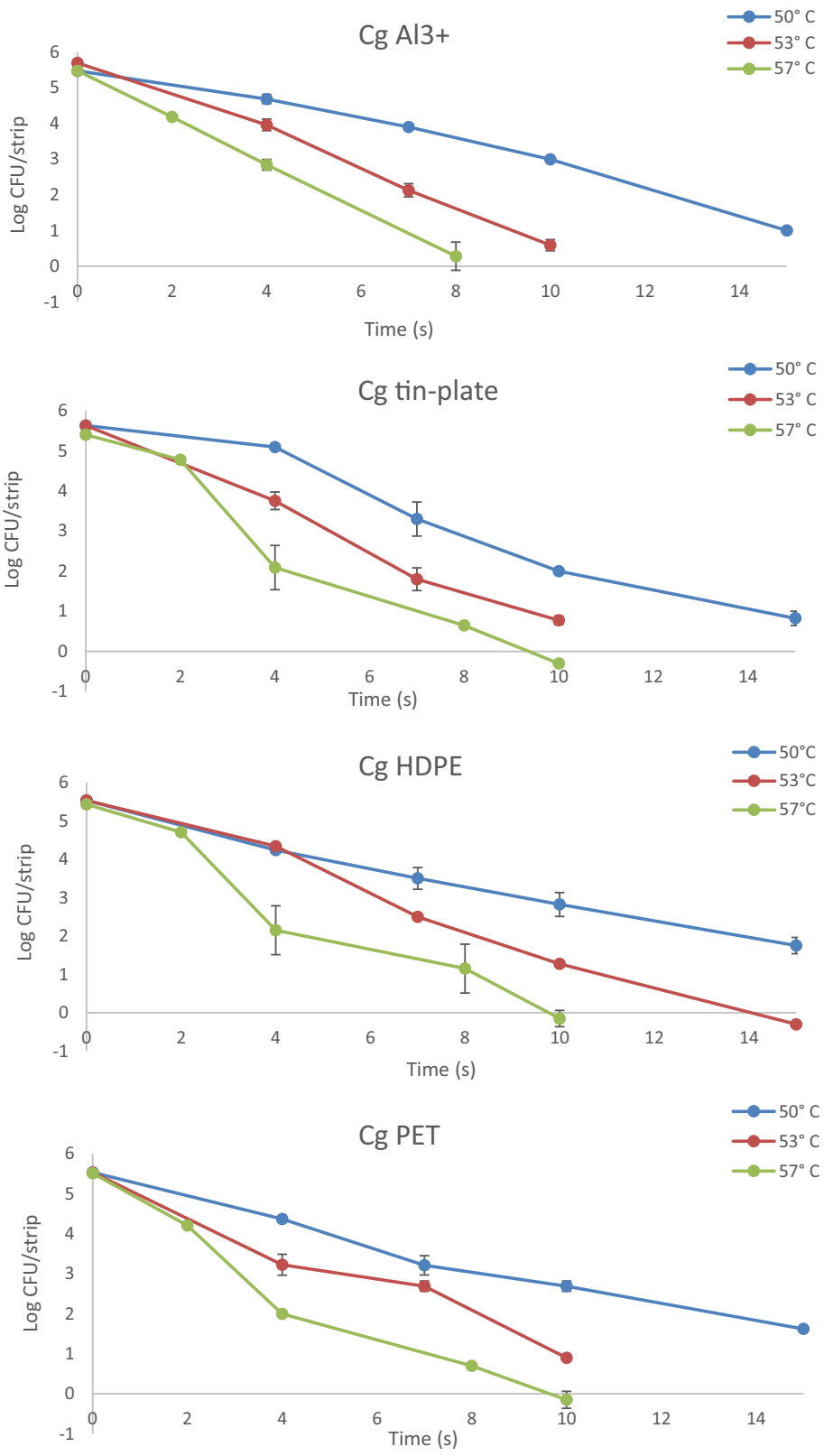

Fig. 2. (*continued*)

and relatively low RMSSE values. In particular, non-linear models provided a slightly higher correlation than linear models (the lowest R^2 was equal to 0.93) and lower response magnitude (Table 3). Nevertheless, when 4D values were compared, results were similar.

As Fig. 2 and Table 2 show, a greater hydrogen-peroxide resistance was registered on all materials and at all the temperatures tested for *T. bacillisporus*, followed by *A. hiratsukae* and *C. globosum*. For *T. bacillisporus*, D values at 50 °C and 53 °C were higher on plastic materials, compared to those obtained on metallic ones, whereas at higher temperatures D values proved similar. For *A. hiratsukae*, D values were similar if different materials were compared, except for D_{50} on aluminium and HDPE, which proved slightly higher (3.4 and 3.1 s) than those

Table 2
Thermal death rate parameters and hydrogen-peroxide resistance values for ascospore-forming strains (Nh = *A. hiratsukae*, Tb = *T. bacillisporus*, Cg = *C. globosum*) treated with 40% hydrogen peroxide on different materials (laminated aluminium, tin-plate, HDPE, PET).

Supporting material	Strain	Temperature (°C)	Activation time (s)	R^2	D value (s)	Standard deviation	z-Value (°C)
Aluminium	Nh	50	0	0.99	3.5	0.1	23
		53	0	0.99	2.7	0.1	
		57	0	0.96	1.6	0.0	
		60	0	1.00	1,35	0.1	
	Tb	50	0	0.91	6.5	0.2	20
		53	0	0.94	3.3	0.1	
		57	0	0.99	2.7	0.0	
		60	0	1.00	1.9	0.1	
	Cg	50	0	0.98	3.4	0.1	nd*
		53	0	1.00	2.0	0.1	
		57	0	1.00	1.7	0.1	
Tin-plate	Nh	50	0	0.93	2.7	0.1	43
		53	0	0.93	2.2	0.0	
		57	0	1.00	1.9	0.0	
		60	0	1.00	1.3	0.0	
	Tb	50	0	0.98	3.5	0.0	41
		53	0	0.96	3.2	0.0	
		57	0	1.00	2.7	0.1	
		60	0	1.00	2.0	0.1	
	Cg	50	0	0.96	2.9	0.1	nd*
		53	0	0.99	2.0	0.0	
		57	0	0.95	1.6	0.0	
HDPE	Nh	50	0	0.86	3.4	0.1	22
		53	0	0.89	2.0	0.0	
		57	0	0.94	1.6	0.0	
	Tb	50	0	0.87	8.1	0.2	13
		53	0	0.89	5.1	0.2	
		57	0	0.93	2.4	0.1	
	Cg	50	0	1.00	4.0	0.2	nd*
		53	0	0.99	2.3	0.0	
		57	0	0.95	1.8	0.0	
PET	Nh	50	0	0.91	2.8	0.2	30
		53	0	0.90	2.1	0.1	
		57	0	0.92	1.6	0.0	
	Tb	50	0	0.96	8.4	0.0	14
		53	0	0.95	4.9	0.1	
		57	0	0.98	2.7	0.0	
	Cg	50	0	0.98	3.9	0.1	nd*
		53	0	0.97	2.3	0.0	
		57	0	0.95	1.8	0.0	

nd* data concerning *C. globosum* were not reported, since the synergistic effect of heat and hydrogen peroxide could give misleading results, supposing a total inactivation within few seconds at temperatures higher than 60 °C for the above-mentioned strains.

Table 3
Thermal death rate parameters for *A. hiratsukae* (Nh) and *T. bacillisporus* (Tb) on HDPE, obtained using the Bigelow and Esty model or the best fitting model by means of the GInaFiT Add-inn tool.

	T (°C)	Linear model						Best fitting model					
		kmax	R^2	RMSSE	1D value (s)	4D value (s)	Ref.	kmax	R^2	RMSSE	1D value (s)	4D value (s)	Ref.
Nh	50	0.67	0.86	0.6909	3.4	13.8	a	1.12	1.00	0.1107	2.1	14.6	b
	53	1.15	0.89	0.8514	2.0	8.0	a	1.87	1.00	0.0934	1.2	9.8	b
	57	1.40	0.94	0.6285	1.6	6.6	a	2.51	1.00	0.0001	0.9	6.7	b
Tb	50	0.28	0.82	0.3310	8.2	ND	a	0.49	0.93	0.2197	4.7	ND	b
	53	0.46	0.84	0.4887	5.0	ND	a	0.75	0.97	0.2347	3.0	ND	b
	57	0.97	0.95	0.4124	2.4	9.5	a	1.42	0.96	0.4250	1.6	9.6	b

Note. ND = not determined due to software inability to automatically report a time needed to reach 4 logarithmic count reductions.
References: a. [W.D. Bigelow and J.R. Esty. 1920. The thermal death point in relation to typical thermophilic organisms. Journal of Infectious Diseases, 27, 602-617]. b [A.H. Geeraerd, C.H. Herremans and J.F. Van Impe. 2000. Structural model requirements to describe microbial inactivation during a mild heat treatment. International Journal of Food Microbiology, 59, 185–209].

obtained on tin-plate and PET (2.7 and 2.8 s). Analogously, ascospores of *C. globosum* behaved in a similar way if different materials were compared, except for D_{50} values that proved to vary in a wide range (from 2.9 s on tin-plate to 4.0 s on HDPE).

Furthermore, a regression analysis on heating temperatures and logarithms of D values has been carried out and the z values for HRM were calculated (Table 2). A linear correlation between heating temperatures and logarithms of D_T values was registered for heat-resistant strains. For each of them, z-values obtained were comparable on the materials tested, except for *T. bacillisporus* and *A. hiratsukae* on tin-plate, where they both showed a greater resistance to hydrogen peroxide also at higher temperatures, their z-values being respectively

equal to 41 °C and 43 °C.

A comparison between results obtained with or without a supporting material allowed us to register a stronger resistance and a better repeatability in tests carried out on conidia or ascospores directly inoculated in the sanitizing medium. This could be due to the fact that tests without a supporting material were carried out in a confined environment (50-ml PYREX® round-bottom sterile tubes) where all conidia or ascospores were recovered when neutralizing agent was added to hydrogen peroxide. On the contrary, tests on metallic or plastic strips were carried out in a beaker where a mechanical detachment and a further dispersion of the spotted inoculum in the sanitizing medium was observed after a first oxidative effect of hydrogen peroxide. In particular, this phenomenon was registered on plastic (HDPE and PET) strips, where the detachment was more marked when higher temperatures were approached (see Fig. 2).

4. Conclusions

Within an aseptic plant, vegetative cells are not a matter of concern for food producers, since the lack of stress-resistant structures make them liable to sanitizing treatments usually applied during sanitization of packaging and machineries. On the contrary, spore-forming microorganisms could be a real problem, since they proved very resistant to both chemical and thermal shocks. Among these, *Bacillus* spp. could be supposed to be the most resistant microbes to such kind of stresses. Nevertheless, their inactivation is critical only in sterilized products (e.g. milk, egg products, meat-based sauces), where they can grow within variable time intervals, whereas their growth is considered negligible in acid products (e.g. fruit juices, flavored drinks) or pasteurized refrigerated products (e.g. Extended Shelf Life milk products), since most of them proved not capable to grow both at refrigeration temperatures and at low pH values. On the contrary, heat-resistant moulds proved able to grow under a wide pH interval or in some cases also at refrigeration temperatures, thus being therefore considered as the real target for sanitization processes of packaging and machineries used for the above-mentioned product categories.

Recent findings about HRM presence on packaging devices and in industrial plants have lead us to reconsider the state of art concerning test microorganisms used in bio-validation processes. We assumed that ascospores from genera such as *Talaromyces* or *Aspergillus* (with *Eurotium*-type or *Neosartorya*-type ascomata) could be more resistant to a sanitizing chemical agent such as hydrogen peroxide and could better fit for real measurement when a bio-validation process was carried out.

Based on the results obtained in this paper, our strains of *Aspergillus* spp. with *Neosartorya* morphs and *Talaromyces* proved to be sensibly more resistant to hydrogen peroxide than *C. globosum* and *A. brasiliensis*, their D values always being significantly higher, regardless of the strain considered and the supporting material assessed. Despite a limited number of strains and fungal species assessed in this study, HRM could be furtherly investigates, as for practical purposes they seemed most suitable as target microorganisms than vegetative microorganisms such as *Aspergillus niger* or *Aspergillus brasiliensis*, their use during bio-validations of sanitizing processes on machineries used for refrigerated products (pH > 4.5) or non-refrigerated acid products (pH ≤ 4.5) leading to more performing results.

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Declaration of competing interest

The authors have no conflict of interest to declare.

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