Food Microbiology 81 (2019) 108–114

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Inactivation of conidia from three *Penicillium* spp. isolated from fruit juices by conventional and alternative mild preservation technologies and disinfection treatments

Masja Nierop Groot[a], Tjakko Abee[b], Hermien van Bokhorst-van de Veen[a,*]

[a] Wageningen Food & Biobased Research, Wageningen University and Research, P.O. Box 17, 6700 AA, Wageningen, The Netherlands
[b] Laboratory of Food Microbiology, Wageningen University and Research, P.O. Box 17, 6700 AA, Wageningen, The Netherlands

ARTICLE INFO

Keywords:
Penicillium
Spore resistance
Food spoilage
Surface decontamination
Minimal processing
Shelf life control

ABSTRACT

Fungi are able to grow on diverse food products and contribute to food spoilage worldwide causing food loss. Consumers prefer freshly squeezed fruit juices, however, the shelf life of these juices is limited due to outgrowth of yeast and fungi. The shelf life of pulsed electric field (PEF) treated juice can be extended from 8 days up to a few weeks before spoilage by moulds becomes apparent. Conidia produced by three *Penicillium* ssp. (*Penicillium expansum*, *Penicillium buchwaldii* and *Penicillium bialowiezense*), previously isolated from spoiled PEF treated fruit juice and smoothie, were characterized for resistance towards selected mild physical processing techniques in orange juice and toward sanitizers on surfaces. The results show that *Penicillium* spp. conidia are susceptible to mild heat, high pressure pasteurization (HPP), PEF, cold atmospheric plasma (CAP), UV, and chemical sanitizers chlorine dioxide and hypochlorite albeit with different susceptibility. Treatment with mild heat, HPP, PEF, or chlorine dioxide reduced conidia by more than 5 log. For hypochlorite, UV, and CAP the reduction was between 1 and 3 log. Together, this study provides data for the development of intervention strategies to eliminate spoilage mould conidia in fruit juices.

1. Introduction

Worldwide, 25% of the food is estimated to be lost due to spoilage (Bondi et al., 2014; Raso and Álvarez, 2016). Exact numbers are not readily available but it has been estimated that 5–10% of all food loss is caused by fungal growth (Pitt and Hocking, 2009a). Fungi are able to grow on diverse food products, including cereals, meat, milk, fruit, vegetables, nuts, and fats, and products thereof (Filtenborg et al., 1996). Fungal spoilage encompasses decay of foods including the development of off-flavours, acidification, discolouring, and disintegrating (Filtenborg et al., 1996; Pitt and Hocking, 2009a). In addition, formation of mycotoxins, like patulin, alternariols, and citrinin make the food unsafe for consumption. Food preservation methods such as thermal pasteurization and sterilization reduce the fungal spoilage largely but compromise sensory and nutritional characteristics of the products and are therefore less desired by consumers. As a result, food processors search for mild preservation methods that can ensure or even enhance the shelf life of high quality foods, without affecting the quality and safety of the food products or using chemical preservatives.

An example of a food product that is susceptible to spoilage by fungi is fruit juice. Freshly squeezed fruit juices are preferred by consumers for their aroma and nutrient content. However, the shelf life of fresh, untreated juice is limited (< 9 days when stored at 4 °C (Timmermans et al., 2011; Toepfl, 2011)), due to outgrowth of mainly yeast. PEF is a mild pasteurization process that inactivates yeasts resulting in an increase in shelf life of fruit juices up to 2–3 weeks (Timmermans et al., 2016; Toepfl, 2011). However, the inactivation of yeast in the juice, combined with the shelf life extension provides outgrowth opportunities for mould spores that either survive the PEF conditions or following recontamination of the juice after processing (Timmermans et al., 2016).

Moulds typically isolated from PEF treated fruit juices and smoothies are *Penicillium expansum, Penicillium buchwaldii*, and *Penicillium bialowiezense* [(Timmermans et al., 2016) and unpublished data]. *P. expansum* is a typical spoilage mould of apples and pears and products thereof (Amiri et al., 2005; Filtenborg et al., 1996; Pitt and Hocking, 2009b). Also from other types of fruit including tomatoes, strawberries, avocados, mangoes, and grapes, *P. expansum* has been isolated (Pitt and Hocking, 2009b), indicating that it can occupy a wide variety of different fruits. *P. bialowiezense* is a filamentous fungi commonly found in spoiled dairy products and, together with *Penicillium commune*, represents 20% of the total spoilage isolates (Garnier et al.,

* Corresponding author.
E-mail address: hermien.vandeveen@wur.nl (H. van Bokhorst-van de Veen).

https://doi.org/10.1016/j.fm.2018.06.004
Received 4 December 2017; Received in revised form 21 March 2018; Accepted 8 June 2018
Available online 08 June 2018

Abbreviations

CAP	cold atmospheric plasma
CFU	colony forming units
HPP	high pressure pasteurization
MEA	malt extract agar
MIC	minimal inhibitory concentration
PEF	pulsed electric field
PPS	peptone physiological salt
SS	stainless steel

2017). In addition, *P. bialowiezense* has been isolated from (forest) soil and was also identified as predominant mould in indoor dust (Scott et al., 2008). *P. buchwaldii* has been isolated from wheat, (processing factory) air, soil, and from several foods including strawberries (Frisvad et al., 2013). This indicates that these *Penicillium* species can occupy various environmental niches representing different routes for contamination in the food production chain.

In this study, the efficacy of mild preservation technologies and disinfection treatments on conidia of three *Penicillium* spp. isolated from fruit juice and fruit smoothie was assessed. Mild processing technologies investigated include HPP, mild heat treatment, and PEF treatment and, for surface disinfections, oxidative chemicals, UV, and nitrogen CAP were tested.

2. Materials and methods

2.1. Penicillium spp. culture conditions and conidia isolation

Two *Penicillium* strains were isolated as dominating spoilage moulds from PEF processed strawberry smoothies at the end of shelf life (Timmermans et al., 2016). The isolates were identified based on sequence data in combination with phenotypic characters (Westerdijk Fungal Biodiversity Institute, KNAW-CBS, Utrecht, The Netherlands) and identified as *P. expansum* Link and *P. buchwaldii*. *P. bialowiezense* K.M. Zalessky was isolated from a PEF treated (10–15 kV/cm) apple-pear-raspberry juice obtained from retail after 35 days storage at 7 °C. Identification of the strain was performed as described above.

Conidia were prepared by inoculating the strains on malt extract agar (MEA; Oxoid) plates containing trace elements (0.01 g/L of $ZnSO_4{\cdot}7H_2O$ and 0.005 g/L of $CuSO_4{\cdot}5H_2O$). After 7 days incubation at 21 °C, the plates were flooded with 10 ml water containing 0.01% (v/v) Tween80 (Sigma) and conidia and mycelium were harvested using a Drigalski spatulum. Subsequently, debris was removed by filtration through sterile glass wool, purity was checked by phase contrast microscopy, and conidia were counted using a haemocytometer (Burker-Turk; 0.100 mm, 0.04 mm^2). Before heat, HPP, and PEF experiments, conidia were centrifuged (2000 × *g*, Centrifuge 5810R, Eppendorf, Hamburg, Germany) for 15 min at 4 °C and the pellet was washed twice with water containing 0.01% Tween80 and eventually dissolved in orange juice (Appelsientje, The Netherlands). For each experiment, fresh conidia were prepared.

2.2. Inactivation of conidia by heat, HPP, UV, CAP, and PEF

All experiments were performed on two separate days (biological duplicate) and in duplicate (technical replicates) for all three *Penicillium* species, unless stated otherwise. Surviving conidia were serially diluted in peptone physiological salt (PPS) and 100 µL aliquots were plated on MEA plates and incubated at 21 °C to determine colony forming units (CFU).

Heat inactivation of conidia was determined following the procedure described by Berendsen et al. (2015) for bacterial spores (Berendsen et al., 2015). In short, 135 µL of conidial suspension in sterile (15 min at 121 °C) commercial orange juice (pH 3.8, Appelsientje, The Netherlands) was transferred to a 200 µL capillary (Micropipettes, Blaubrand intraMARK, Wertheim, Germany). The capillaries with conidia were sealed by heating using a gas burner and placed in ice water until use. Heat treatment was performed in a 58 °C oil bath for up to 50 s. After incubation, a capillary was taken out and immediately cooled in ice water with chlorine (prepared by diluting household bleach 16 times) for 5 min. Capillaries were transferred aseptically to a sterilized glass tube containing 5 mL sterile PPS and a 2 cm long magnetic stirrer. The tube was closed and the capillary was broken by using a vortex and conidia were released in the PPS solution. This resulted in the ~6 $\log_{10}$ mL^{-1} initial load for CFU determination as described above.

HPP was conducted using sterilized (15 min at 121 °C) orange juice (pH 3.8, Appelsientje, The Netherlands) inoculated with conidia (~8 $\log_{10}$ mL^{-1}). One mL conidial suspension was heat sealed (Sealmaster Magneta, Audion Elektro BV, Weesp, The Netherlands) in sterile plastic bags (1.5 by 6 cm) made from stomacher bags (lab system classic 400, Seward, Worthing, United Kingdom). The bags were placed in a laboratorium scale HPP unit filled with demi water at room temperature (~22 °C) (Resato, Assen, The Netherlands) for treatment (400 MPa for 0, 1, 3, or 5 min holding time). The final pressure was reached after 30 s and final temperature during treatment was maximal 43 °C. After treatment, the bags were directly cooled in ice-water, aseptically opened using pair of sterilized scissors, and conidia suspensions were collected for CFU determination as described above.

UV and nitrogen CAP treatments were performed with ~8 $\log_{10}$ mL^{-1} conidia in water containing 0.01% Tween80 according to Van Bokhorst-van de Veen et al. (2015). In short, 10 µL conidia were spotted on GSWP filters (mixed cellulose membranes with 0.22 µm pore sizes and 47 mm diameter, Merck, Darmstadt, Germany) that were mounted on sterile Petri dishes. Conidia were allowed to dry for 1 h on the filters in a laminar flow cabinet before exposure to UV or cold plasma. Conidia were exposed to either 0, 10, 20, 50, or 100 mJ/cm^2 UV generated in a UV Crosslinker (CL-1000, 254 nm, UVP, Upland, USA) or for 0, 5, 10, 15, 20, or 30 min exposed to nitrogen plasma generated from a commercially available nitrogen plasma jet (15 standard litre min^{-1} N_2; CP121 Plasma Demonstrator, OMVE, Schalkwijk, The Netherlands), as described elsewhere (Mastwijk et al., 2009), at room temperature. As a control for CAP treatment, conidia were exposed to non-ionized N_2 for 30 min. After UV or CAP treatment, the filter was aseptically removed from the Petri dish and placed in a sterile 50 mL tube containing 10 mL 0.1% (w/v) peptone and 0.1% (v/v) Tween80 and vortexed vigorously for 10 s. Surviving conidia were determined as described above.

The PEF system used was a continuous-flow system as described by Timmermans et al. (2014). In short, pasteurized (30 min at 98 °C) and sieved orange juice (pH 3.8, Appelsientje, The Netherlands) was inoculated with *P. expansum* or *P. bialowiezense* conidia (~8 $\log_{10}$ mL^{-1}). The inoculated juice was pumped through a 6 mm silicone hose at a flow rate of 15 mL/min. Before PEF treatment, the juice was preheated in continuous flow through a heat spiral that was immersed in a water, and inoculated juice was preheated to 39 ± 1 °C for *P. expansum* and to 41 ± 1 °C for *P. bialowiezense*. Next, the juice was pumped through a co-linear PEF treatment device, comprising two vertically positioned treatment chambers with a diameter of 1.0 mm and a gap of 2.0 mm each, resulting in a residence time of 12.5 µs in the treatment chambers at a flow rate of 15 mL/min. For PEF treatment, a fixed electric field strength of 17 kV/cm and monopolar pulses of 2 µs were used. The highest frequency was applied first at 785 Hz and resulted in a maximum temperature of 63–64 °C, followed by 650 Hz (T_{max} 59–60 °C), 470 Hz (T_{max} 55–56 °C), 330 Hz (52 °C), and 120 Hz (T_{max} 46–47 °C). The temperature at the outlet was measured using a 0.3 mm thermocouple type K (TM-914C, Lutron, Taiwan). The juice reached a maximum temperature of 62 °C when PEF was on (at 785 Hz). No holding section was included, so directly after leaving the PEF treatment

chambers (typically within 2 s), the juice was pumped through a cooling spiral of 2 mm diameter and 500 cm length that was cooled on melting ice. After cooling, samples were collected under aseptic conditions. For each frequency, two samples were taken for conidia quantification as described above. As a control, when all PEF-treatments were carried out, inoculated orange juice was pumped through the system and CFU were determined as described above.

2.3. Inactivation of conidia by hypochlorite and chlorine dioxide

For disinfectant treatment of conidia of the three *Penicillium* spp., the NEN-EN 13697 standard (2015) was used as protocol, with minor modifications (see below). Conidia were attached on non-porous surfaces, specifically stainless steel (SS), in the absence or presence of sucrose as interfering substance suggested by the NEN-EN 13697 standard for the beverage and soft drink industries. Chlorine dioxide was prepared from tablets (Dutrion, Lifarma B.V., Baexem, The Netherlands) as described by Banach et al. (2017) and prepared one day before the experiment and stored in dark at 4 °C (Banach et al., 2017). Sodium hypochlorite was freshly prepared from a stock containing 4.00–4.99% free chlorine (Sigma-Aldrich). SS coupons (18 by 22 mm) were cut and cleaned as described elsewhere (Castelijn et al., 2013). Fifty µL containing 1.5–5.0 × 10^7 CFU/mL conidia in water containing 0.01% Tween80 with or without 10 g/L sucrose was spotted on a SS coupon. When dried, 100 µL disinfectant solution, containing 500 ppm free chlorine from hypochlorite, 500 ppm chlorine dioxide, or water (control) was added and incubated for 5 min at 7 °C. Ten mL 20 g/L sodium thiosulfate (Sigma-Aldrich) was used as the neutralizer as suggested by the NEN method. Instead of glass beads, 2 g of sterile sea sand was added to the neutralizer to remove the conidia from the coupon. Chlorine dioxide and free chlorine (from hypochlorite) concentrations were measured using a colorimetric assay in a Dulcometer (Prominent, Germany) and pH was measured (FiveEasy pH meter, Mettler Toledo, Columbus, USA) just before the addition of the chlorine dioxide or hypochlorite solutions to the conidia. CFU were determined as described above.

2.4. Data analysis

Reduction in CFU was plotted as $\log_{10} N/N_0$ where N is CFU/mL (heat, HPP, or PEF), CFU/coupon (hypochlorite or chlorine dioxide), or CFU/filter (UV or CAP) after treatment and N_0 is CFU/mL, CFU/coupon, or CFU/filter before treatment. The *D*-value (decimal reduction time: the time required to kill 90% of the exposed microorganisms, i.e. 1 $\log_{10}$ reduction) and standard error of heat-treated conidia (see above) were determined by the linear least squares curve fitting method using the LINEST function in Excel (Excel 2010). R^2 were 0.87, 0.81, and 0.98 for *P. buchwaldii, P. bialowiezense, and P. expansum*, respectively. The *D*-values were calculated from the resulting regression and are the negative reciprocal of the slope.

3. Results and discussion

3.1. Inactivation of conidia of three Penicillium spp. in fruit juice during mild heat or high pressure treatments

Firstly, two pasteurization processes applied commercially for fruit juices were tested, namely mild heat treatment and HPP (Fig. 1). For the heat treatment, a relatively mild temperature (58 °C) was chosen that is known to have minimal effect on aroma and taste of the juice (Timmermans et al., 2011). *P. bialowiezense* conidia were the most sensitive to heat treatment and showed 5.4 log reduction after 10 s exposure to 58 °C in orange juice, while *P. buchwaldii* and *P. expansum* conidia showed 0.9 and 1.6 log reduction, respectively, under these conditions (Fig. 1A). The calculated *D*-values and standard deviation are 0.09 ± 0.01 min for *P. expansum*, 0.16 ± 0.04 min for *P. buchwaldii*, and 0.03 ± 0.01 min for *P. bialowiezense* in orange juice (pH 3.8) at 58 °C. The thermal resistance of conidia from *Penicillium* spp. in acid matrices is determined in several other studies. *P. expansum* conidia in apple juice (pH 3.25) showed D-values of 1.14 and 0.61 min at 56 °C and 60 °C respectively (Salomao et al., 2009). Other studies report $D_{60°C}$ -values for a *Penicillium citrinum* juice spoilage isolate of 0.010, 0.016, and 0.009 min in citrate buffers of pH 3.0, 3.5, and 4.0, respectively (Shearer et al., 2002). For *P. roqueforti*, another juice spoiler isolate, the $D_{60°C}$ -values are 0.201 (pH 3.0), 0.238 (pH 3.5), and 0.290 min (pH 4.0) in citrate buffer (Shearer et al., 2002). Present data and literature data show a high diversity in thermal inactivation of conidia from different *Penicillium* species in juice and acidic buffers.

For all three *Penicillium* species tested, conidia were readily inactivated by a mild HPP treatment resulting in > 7 log reduction after HPP treatment at 400 MPa with a holding time of 5 min (Fig. 1B). Conidia from *P. bialowiezense* were most sensitive to HPP showing already 6 log reduction after 1 min holding time (Fig. 1B). A few other HPP inactivation studies for *Penicillium* spp. conidia have been reported in literature. Conidia of *Penicillium roqueforti* in water decreased over 6 log at 400 MPa for 10 min at 20 °C (Martinez-Rodriguez et al., 2014). Other studies show inactivation of 6 log conidia from *P. expansum* at 350 MPa for 20 min applied at room temperature in apple juice with pH 3.3 and in broccoli juice with pH 6.6 (Merkulow et al., 2000) and 6 log inactivation conidia from *P. roqueforti* at 400 MPa for 20 min in cheese slurry (O'Reilly et al., 2000). In conclusion, *Penicillium* spp. conidia are readily inactivated at relative mild HPP processing settings (< 500 MPa at RT) in several food products including fruit juices.

3.2. Inactivation of conidia of three Penicillium spp. attached on a surface during chemical, nitrogen cold atmospheric plasma, and UV treatments

Next, the inactivation of conidia from *Penicillium* spp. by three surface treatments, namely chemical disinfectants, UV, and CAP was tested. In industry, the disinfection step during sanitation should be applied when all soil is removed via an appropriate cleaning step. In the

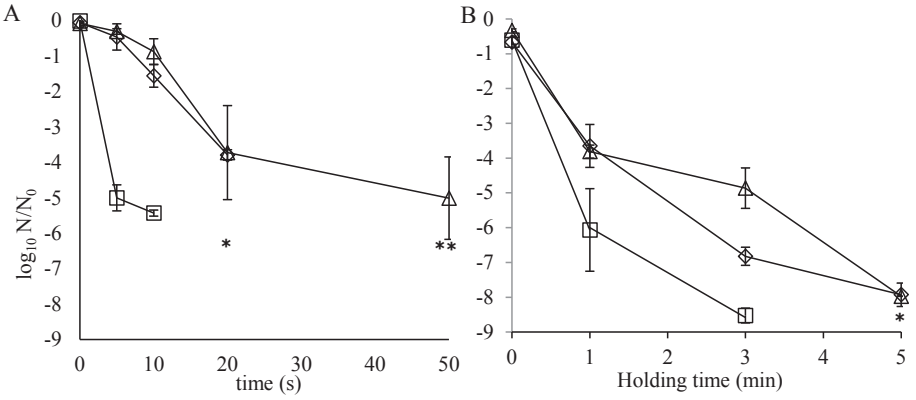

Fig. 1. Inactivation of *P. expansum* (diamonds), *P. bialowiezense* (squares), and *P. buchwaldii* (triangles) conidia in orange juice treated with heat (58 °C, panel A) and HPP (400 MPa; B). Asterisk indicates that conidia levels were below detection level [*P. bialowiezense* at 20 and 50 s in panel A (detection limit −6.3 log) and at 5 min in panel B (detection limit −8.7 log); *P. expansum* at 50 s in panel A (detection limit −6.0 log).

experimental setup of the chemical disinfectants, sucrose was also added to the conidia solution as interfering substance to mimic improper removal of fruit product during cleaning. Hypochlorite and chlorine dioxide were chosen as chlorine based disinfectants typically applied in practice, including disinfection of processing plants (Al-Zenki et al., 2012; Kakurinov, 2014). Exposure to approximately 500 ppm, as measured just before incubation, free chlorine from sodium hypochlorite or chlorine dioxide showed different inactivation of conidia on SS coupons after 5 min at 7 °C (Fig. 2A). Addition of only water instead of disinfectant (as a control) did not inactivate conidia (data not shown). Chlorine dioxide was more effective against the tested conidia showing > 5 log reduction for all three *Penicillium* species (Fig. 2B). Conidia of *P. bialowiezense* showed 2.6 log reduction when exposed to free chlorine from hypochlorite in the absence of sucrose (Fig. 2A). These conidia were more sensitive to hypochlorite compared to conidia of *P. expansum* and *P. buchwaldii*, which showed less than 1 log reduction (Fig. 2A). Limited data is available in literature for the effect of chlorine dioxide on *Penicillium* spp. conidia. A recent study showed over 5 log inactivation of *Penicillium* sp. conidia in PBS using 2 ppm chlorine dioxide for 5 min at 27 °C. The inactivation was shown temperature dependent since only 1.5 log conidia were inactivated at 10 °C and 15 °C (Wen et al., 2017). For *P. expansum*, aqueous solutions containing 3 ppm ClO_2 inactivated 1.4 to 2.0 log conidia in solution after 30 s incubation (Okull et al., 2006; Roberts and Reymond, 1994). Minimal inhibitory concentration (MIC; lowest concentration inhibiting visible growth) of free chlorine from sodium hypochlorite added for *P. expansum* conidia was determined by an apple juice agar dilution assay as 1000 ppm at 25 °C (using 10^4 conidia per plate) (Venturini et al., 2002). When subjected for 2 min to an aqueous hypochlorite solution, a MIC of 50 ppm free chlorine was detected for *P. expansum* conidial suspension with a 10^6 conidia/mL load at 25 °C (Cerioni et al., 2013). This study shows that conidia of *P. bialowiezense* are more resistant against free chlorine from hypochlorite compared to *P. expansum* and *P. buchwaldii* conidia in the absence of sucrose and that chlorine dioxide is more effective compared to free chlorine.

In the USA, use of 100–200 ppm free chlorine and ClO_2 (and related oxychloro species) are permitted for sanitizing equipment and utensils that are used for food processing without a subsequent rinsing step but with adequate draining of the surfaces (Beuchat et al., 1998; Kakurinov, 2014). Therefore, when 500 ppm ClO_2 is applied in industry, the decontaminated material should be rinsed with water of proper quality. In the EU, the use of biocides is regulated in the directive 98/8/EC and since 2013, Regulation (EU) 528/2012 “concerning making available on the market and use of biocidal products” is in place. This regulation implies that for biocides not (yet) registered in the regulation (EU) 528/2012, national law is leading. Latter is still the case for chlorine dioxide. In The Netherlands, the Board for the Authorisation of Plant Protection Products and Biocides (Ctgb) is authorised to assess whether plant protection products and biocidal products are safe for humans, animals, and the environment in accordance with international agreements and criteria laid down in legislation. For the product category “food and feed area disinfectants” (PT04), several products that contain ClO_2 as active compound (after on-site generation) were found in the Ctgb database. Concentrations of ClO_2 prescribed by the manufactures range from 0.5 to 3000 ppm of the approved products, depending on the application.

Contrary to ClO_2, on European level, active chlorine released from hypochlorite is approved as biocide in the food and feed area [regulation (EU) 2017/1273]. When used, the food producer should take into account the maximum residue levels (MRLs) that are allowed in food or feed products according to regulation (EC) 396/2005 are not exceeded. In The Netherlands, approved products containing hypochlorite to generate free chlorine, working concentrations prescribed by manufactures of free chlorine range between 120 and 1890 ppm to disinfect fungi on food contact surfaces.

Overall, in the presence of sucrose, chlorine dioxide is less effective on conidia of the three species: ClO_2 inactivated between 3.5 and 4.4 log conidia (Fig. 2B). Compared to its absence, sucrose showed limited interference with free chlorine from hypochlorite for conidia of *P. expansum* and *P. bialowiezense*; 0.7 and 2.1 log reduction was detected, respectively, (Fig. 2A). Remarkably, for conidia of *P. buchwaldii,* inactivation was enhanced in the presence of sucrose with chlorine from hypochlorite; 1.8 log reduction was detected in presence of sucrose compared to 0.9 log in the absence of sucrose (Fig. 2A). It is known that sugars can trigger conidial germination of for instance *Aspergillus niger*, *Botrytis cinerea*, and *Fusarium oxysporum* (Mahuku and Goodwin, 1998). It remains to be determined whether the presence of sucrose could trigger *P. buchwaldii* conidia germination thereby loosing part of their resistance against free chlorine from hypochlorite.

Exposure to UV is also applied by industry to decontaminate surfaces (Bintsis et al., 2000). Conidia of the three species were exposed to UV with energy of 100 mJ/cm^2, resulting in reduction by 4.4 log (for *P. bialowiezense*), 2.4 log (*P. buchwaldii*), and 2.3 log (*P. expansum*) (Fig. 3A). Studies describing the effect of UV on *Penicillium* spp. conidia are limited in scientific literature. Moreover, the available studies are difficult to compare with each other due to differences in matrices. For example, *Penicillium italicum* conidia spread on agar plates were reduced below detection levels (> 4.8 log CFU/plate) with UV treatments of 420 mJ/cm^2 and for *Penicillium digitatum* this was 299 mJ/cm^2 (Gunduz and Pazir, 2013). Furthermore, UV treatment (263 mJ/cm^2) inactivated about 4 log *Penicillium pinophilum* conidia in mineral water (Watanabe et al., 2010). The present study showed on cellulose filter > 4 log reduction of *P. bialowiezense* conidia but with lower UV dose (100 mJ/cm^2; Fig. 3A). It seemed that all three dose-response curves have two phases, in which the inactivation rate of the first phase

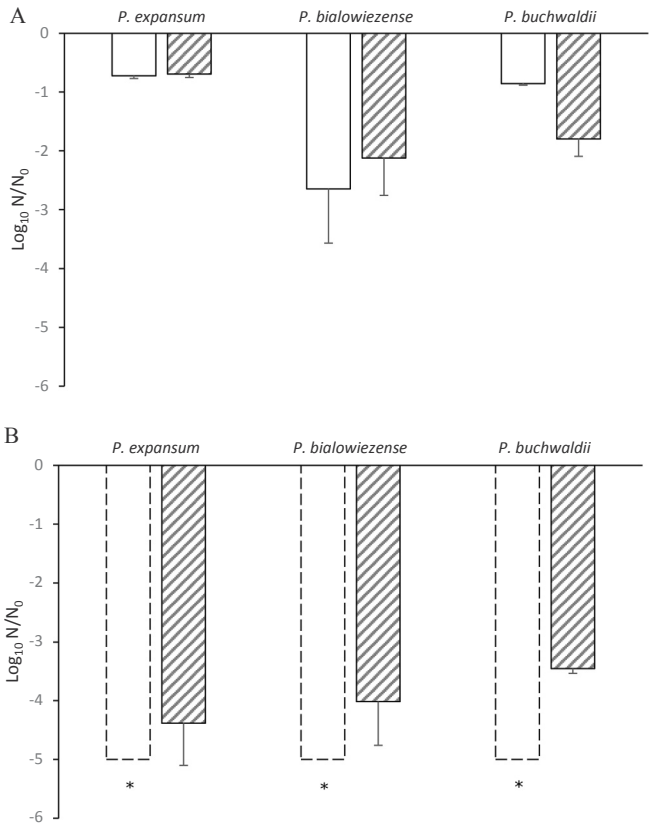

Fig. 2. Inactivation of *P. expansum*, *P. bialowiezense*, and *P. buchwaldii* conidia on SS coupons by 500 ppm free chlorine from hypochlorite (panel A) and 500 ppm chlorine dioxide (B) in the absence (open bars) or presence (diagonal lined bars) of sucrose after 5 min incubation at 7 °C. Asterisks and dashed bars indicate that conidia levels were below detection level (−5.0 log; all data points of chlorine dioxide in the absence of sucrose).

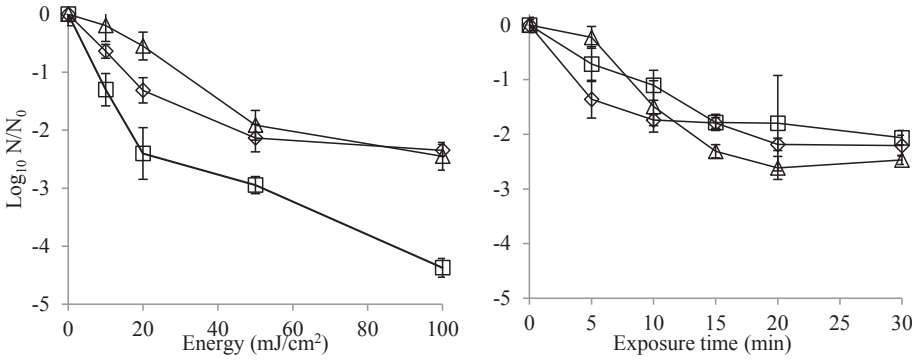

Fig. 3. Inactivation of *P. expansum* (diamonds), *P. bialowiezense* (squares), and *P. buchwaldii* (triangles) conidia with UV (254 nm; panel A) and CAP (B).

is faster (up to 20 mJ/cm^2 for *P. bialowiezense* and up to 50 mJ/cm^2 for *P. buchwaldii* and *P. expansum*) compared to the second phase (between 20 or 50 and 100 mJ/cm^2). Possible explanations for this observation could be that conidia can be shielded against UV due to clumping and/or shading of other conidia (Coohill and Sagripanti, 2009) and/or that a subpopulation is present that resists higher UV doses (Coohill and Sagripanti, 2008). Actual parameters that affect the efficacy of the UV treatment remain to be determined. Overall, for UV treatment, conidia of *P. bialowiezense* were more sensitive compared to *P. buchwaldii* and *P. expansum*.

The effect of nitrogen CAP as a novel technique to disinfect surfaces was tested on conidia. Nitrogen CAP was shown previously effective against bacterial spores (Van Bokhorst-van de Veen et al., 2015); however, limited data is available for the effectiveness of CAP on mould spores. CAP application generates a non-thermal ionised gas, which is a mixture of electrons, ions and/or free radical species that cause damage to microorganisms (Chizoba Ekezie et al., 2017; De Geyter and Morent, 2012). Due to the relative low penetration depth in foods, CAP is less suitable for juice decontamination, but is effective as surface decontamination method. When conidia of the *Penicillium* spp. isolates spotted on filters were exposed to nitrogen CAP, comparable inactivation profiles were obtained (Fig. 3B), while exposure to only nitrogen gas did not result in inactivation (data not shown). After 20 min CAP exposure, conidial reductions were 2.6, 2.2, and 1.8 log for *P. buchwaldii*, *P. expansum*, and *P. bialowiezense*, respectively. Extended treatment did not result in further reduction in conidia (Fig. 3B). It cannot be excluded that, as for UV, a fraction of the conidia clumped or were layered in such a way that they were shielded from the CAP. Another explanation can be that a subpopulation is more resistant. Notably, CAP exposure induced some particular morphology changes compared to untreated or nitrogen only treated conidia including extrusions from the walls of plasma-treated *P. expansum* conidia signifying damage (data not shown). Other studies show CAP inactivation of *Penicillium* spp. conidia, including *Penicillium* MS1982 on seed surface: 3 log within 20 min using a low pressure cold plasma system with air (Selcuk et al., 2008) and *P. digitatum* on cover glass: 4 log using an oxygen/argon high-density non-equilibrium plasma system for 7 min (Hashizume et al., 2013; Iseki et al., 2010). In addition, *Penicillium crustosum* conidia in PBS (pH 7.4) were 4 log inactivated using a direct current discharge for 25 min with air as feeding gas (Souskova et al., 2011) and aerosolised *P. expansum* conidia were 2 log inactivated using an air corona discharge for 150 min (Liang et al., 2012). However, comparison between different systems is difficult due to variations in systems, settings, type of gas, and treatment temperature (Niemira, 2012). No difference in the inactivation of conidia of the three *Penicillium* species was observed using atmospheric pressure nitrogen plasma jet system in this study.

3.3. Inactivation of conidia of *P. expansum* and *P. bialowiezense* with pulsed electric field

Conidia of *P. expansum*, which are relatively heat-resistant, and conidia of *P. bialowiezense*, which are relatively heat-sensitive, were exposed to PEF treatment to determine inactivation efficacy of PEF. A variation in the number of pulses, resulting in variation in outlet temperature (T_{out}), was introduced while all other settings were kept unchanged thereby mimicking an industrial setting. A reduction in conidia of 4.3 log was observed for *P. bialowiezense* at T_{out} of 55 °C, while *P. expansum* conidia showed only 0.4 log reduction at T_{out} of 56 °C (Fig. 4). More intense conditions further inactivated conidia of both species and ≥7 log reduction at T_{out} of 60 and 63 °C for *P. bialowiezense* and *P. expansum* conidia, respectively (Fig. 4). These data indicate that *P. expansum* conidia are more resistant to PEF treatment than *P. bialowiezense* conidia. Furthermore, according to SEM analysis, it appeared that some *P. expansum* PEF treated conidia were compressed (data not shown). Evrendilek et al. (2008) reported that PEF treatments of 34 kV/cm with 3 μs pulses and 163 μs treatment time or 218 μs treatment time

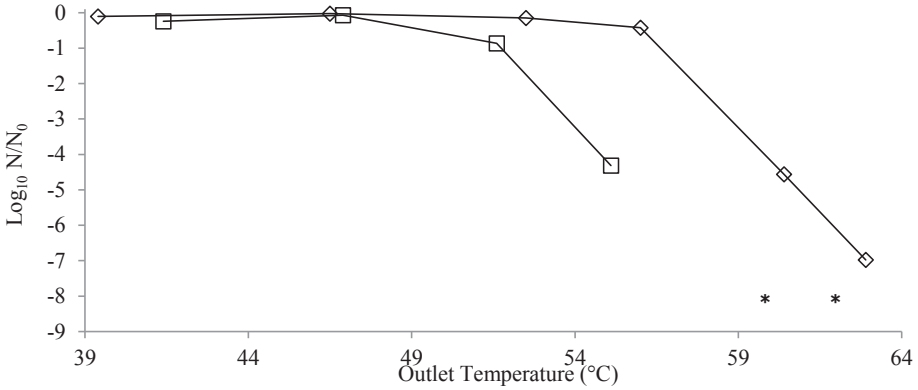

Fig. 4. Inactivation of *P. expansum* (diamonds) and *P. bialowiezense* (squares) conidia in orange juice treated with PEF. Representative data from two experiments. Asterisks indicate that conidia levels were below detection level (−8.0 log; *P. bialowiezense* at 60 and 62 °C).

with 3 μs pulses at a field strength of 17 kV/cm on sour cherry juice, apricot nectar, or peach nectar could fully inactivate *P. expansum* conidia germination and tube elongation, based on microscopic observations of approximately 200 conidia for each treatment (Evrendilek et al., 2008). Our results show that conidia of *P. expansum* and *P. bialowiezense* that were isolated as spoilage microorganisms from PEF treated smoothies and juices can be inactivated by PEF treatment, albeit to different levels (Fig. 4). Since the transit time to leave the PEF treatment chamber to the cooling section is approximately 2 s, heat inactivation plays a negligible (for *P. expansum*) or minor role (for *P. bialowiezense*) in inactivation. Based on the *D*-value determined for *P. bialowiezense*, 2 s exposure to 58 °C would result in approximately 1 log inactivation by heat only, compared to ≥6 log inactivation by a PEF treatment with T_{out} is 58 °C. *P. expansum* and *P. bialowiezense* strains used in this study were isolated from PEF treated fruit drinks (smoothie and juice, respectively). When *P. expansum* conidia are present in the untreated juice via contamination of ingredients, they are likely to survive PEF-treatment. For *P. bialowiezense* conidia this route of contamination in the final product is less expected due to their susceptibility to PEF treatment. However, since aseptic filling follows PEF processing, conidia may also be introduced in the juice after treatment via recontamination from air and/or equipment. To reduce *Penicillium* spp. conidia in fruit juices, several alternatives to PEF can be proposed. For instance, high pressure treatment of packaged juice seems promising, although processing time and costs will increase compared to PEF processing because this is a batch process. An alternative possibility can be a combination of PEF and mild heat treatment of the juice (hurdle approach) and thoroughly cleaning of processing equipment using chlorine dioxide. Further research can elucidate the effectiveness of such combined processing and cleaning strategies.

4. Conclusions

This study showed the effect of mild heat, alternative preservation technologies (high pressure and PEF), and surface disinfection methods (UV, nitrogen CAP, chlorine dioxide, and free chlorine from hypochlorite) on conidia of three *Penicillium* spp. The data showed substantial variation in the reduction of conidia by most of the tested treatments. Moreover, the conidia of the three species varied substantially in resistance towards mild heat, HPP, UV, hypochlorite, and free chlorine, whereas for CAP and chlorine dioxide the reduction of conidia of all three species was at comparable level. In juice, all technologies (mild heat, high pressure, and PEF) could inactivate over 5 log conidia of the *Penicillium* species. Of the surface technologies tested, chlorine dioxide was most effective resulting in over 5 log reduction of *Penicillium* conidia in this study. In general, *P. bialowiezense* conidia appeared more sensitive compared to *P. expansum* and *P. buchwaldii* requiring less intense intervention strategies. The data provided in this study can be used for design of intervention strategies that aim to eliminate mould conidia from fruit juice products and fruit juice production environments.

Acknowledgements

We greatly acknowledge the technical assistance of Zhu Feng (Wageningen Food and Biobased Research), Hennie Mastwijk (OMVE The Netherlands), Jaap Hulstein, Mariette Helmond, and Louise Nederhoff (Wageningen Food and Biobased Research).

This research is part of the Public-Private Partnership Mild Preservation of Food Products (TKI-AF-12148) and partly financed by the Dutch Government via the Topsector Agri&Food.

References

Al-Zenki, S., Al-Omirah, H., Sidhu, J.S., 2012. Microbial Safety and Sanitation of Fruits and Fruit Products, Handbook of Fruits and Fruit Processing. Wiley-Blackwell, pp. 333–351.

Amiri, A., Cholodowski, D., Bompeix, G., 2005. Adhesion and germination of waterborne and airborne conidia of *Penicillium expansum* to apple and inert surfaces. Physiol. Mol. Plant Pathol. 67, 40–48.

Banach, J.L., van Bokhorst-van de Veen, H., van Overbeek, L.S., van der Zouwen, P.S., van der Fels-Klerx, H.J., Groot, M.N.N., 2017. The efficacy of chemical sanitizers on the reduction of *Salmonella Typhimurium* and *Escherichia coli* affected by bacterial cell history and water quality. Food Contr. 81, 137–146.

Berendsen, E.M., Zwietering, M.H., Kuipers, O.P., Wells-Bennik, M.H.J., 2015. Two distinct groups within the *Bacillus subtilis* group display significantly different spore heat resistance properties. Food Microbiol. 45, 18–25.

Beuchat, L.R., Team, W.H.O.F.S., Nations, F.a.A.O.o.t.U., 1998. Surface Decontamination of Fruits and Vegetables Eaten Raw: a Review. Available from: http://www.who.int/iris/handle/10665/64435.

Bintsis, T., Litopoulou-Tzanetaki, E., Robinson, R.K., 2000. Existing and potential applications of ultraviolet light in the food industry - a critical review. J. Sci. Food Agric. 80, 637–645.

Bondi, M., Messi, P., Halami, P.M., Papadopoulou, C., de Niederhausern, S., 2014. Emerging microbial concerns in food safety and new control measures. BioMed Res. Int. 2014, 251512.

Castelijn, G.A., Parabirsing, J.A., Zwietering, M.H., Moezelaar, R., Abee, T., 2013. Surface behaviour of *S. Typhimurium, S. Derby, S. Brandenburg* and *S. Infantis*. Vet. Microbiol. 161, 305–314.

Cerioni, L., Lazarte, M.D., Villegas, J.M., Rodriguez-Montelongo, L., Volentini, S.I., 2013. Inhibition of *Penicillium expansum* by an oxidative treatment. Food Microbiol. 33, 298–301.

Chizoba Ekezie, F.G., Sun, D.W., Cheng, J.H., 2017. A review on recent advances in cold plasma technology for the food industry: current applications and future trends. Trends Food Sci. Technol. 69, 46–58.

Coohill, T.P., Sagripanti, J.L., 2008. Overview of the inactivation by 254 nm ultraviolet radiation of bacteria with particular relevance to biodefense. Photochem. Photobiol. 84, 1084–1090.

Coohill, T.P., Sagripanti, J.L., 2009. Bacterial inactivation by solar ultraviolet radiation compared with sensitivity to 254 nm radiation. Photochem. Photobiol. 85, 1043–1052.

De Geyter, N., Morent, R., 2012. Nonthermal plasma sterilization of living and nonliving surfaces. Annu. Rev. Biomed. Eng. 14, 255–274.

Evrendilek, G.A., Tok, F.M., Soylu, E.M., Soylu, S., 2008. Inactivation of *Penicillum expansum* in sour cherry juice, peach and apricot nectars by pulsed electric fields. Food Microbiol. 25, 662–667.

Filtenborg, O., Frisvad, J.C., Thrane, U., 1996. Moulds in food spoilage. Int. J. Food Microbiol. 33, 85–102.

Frisvad, J.C., Houbraken, J., Popma, S., Samson, R.A., 2013. Two new *Penicillium* species *Penicillium buchwaldii* and *Penicillium spathulatum*, producing the anticancer compound asperphenamate. FEMS Microbiol. Lett. 339, 77–92.

Garnier, L., Valence, F., Pawtowski, A., Auhustsinava-Galerne, L., Frotté, N., Baroncelli, R., Deniel, F., Coton, E., Mounier, J., 2017. Diversity of spoilage fungi associated with various French dairy products. Int. J. Food Microbiol. 241, 191–197.

Gunduz, G.T., Pazir, F., 2013. Inactivation of *Penicillium digitatum* and *Penicillium italicum* under in vitro and in vivo conditions by using UV-C light. J. Food Protect. 76, 1761–1766.

Hashizume, H., Ohta, T., Jia, F.D., Takeda, K., Ishikawa, K., Hori, M., Ito, M., 2013. Inactivation effects of neutral reactive-oxygen species on Penicillium digitatum spores using non-equilibrium atmospheric-pressure oxygen radical source. Appl. Phys. Lett. 103.

Iseki, S., Ohta, T., Aomatsu, A., Ito, M., Kano, H., Higashijima, Y., Hori, M., 2010. Rapid inactivation of *Penicillium digitatum* spores using high-density nonequilibrium atmospheric pressure plasma. Appl. Phys. Lett. 96.

Kakurinov, V., 2014. Food Safety Assurance Systems: Cleaning and Disinfection, Encyclopedia of Food Safety. Academic Press, Waltham, pp. 211–225.

Liang, J.L., Zheng, S.H., Ye, S.Y., 2012. Inactivation of *Penicillium* aerosols by atmospheric positive corona discharge processing. J. Aerosol Sci. 54, 103–112.

Mahuku, G.S., Goodwin, P.H., 1998. Influence of sucrose, mucin and xanthan gum on spore germination of ten different fungi. Eur. J. Plant Pathol. 104, 849–852.

Martinez-Rodriguez, Y., Acosta-Muniz, C., Olivas, G.I., Guerrero-Beltran, J., Rodrigo-Aliaga, D., Mujica-Paz, H., Welti-Chanes, J., Sepulveda, D.R., 2014. Effect of high hydrostatic pressure on mycelial development, spore viability and enzyme activity of *Penicillium Roqueforti*. Int. J. Food Microbiol. 168–169, 42–46.

Mastwijk, H.C., Wichers, H.J., Van Dijk, C., Schuten, H.J., 2009. Observation of the free hole gases at ambient conditions. In: Condensed Matter: strongly Correlated Electrons, pp. 14. arXiv:0711.4737v2.

Merkulow, N., Eicher, R., Ludwig, H., 2000. Pressure inactivation of fungal spores in aqueous model solutions and in real food systems. High Pres. Res. 19, 643–652.

Niemira, B.A., 2012. Cold plasma decontamination of foods. Annual Review of Food Science and Technology 3, 125–142 3.

O'Reilly, C.E., O'Connor, P.M., Kelly, A.L., Beresford, T.P., Murphy, P.M., 2000. Use of hydrostatic pressure for inactivation of microbial contaminants in cheese. Appl. Environ. Microbiol. 66 4890-+.

Okull, D.O., Demirci, A., Rosenberger, D., LaBorde, L.F., 2006. Susceptibility of *Penicillium expansum* spores to sodium hypochlorite, electrolyzed oxidizing water, and chlorine dioxide solutions modified with nonionic surfactants. J. Food Protect. 69, 1944–1948.

Pitt, J.I., Hocking, A.D., 2009a. Introduction, Fungi and Food Spoilage. Springer US, Boston, MA, pp. 1–2.

Pitt, J.I., Hocking, A.D., 2009b. *Penicillium* and Related Genera, Fungi and Food Spoilage. Springer US, Boston, MA, pp. 169–273.

Raso, J., Álvarez, I., 2016. Pulsed Electric Field Processing: Cold Pasteurization, Reference Module in Food Science. Elsevier.
Roberts, R.G., Reymond, S.T., 1994. Chlorine dioxide for reduction of postharvest pathogen inoculum during handling of tree fruits. Appl. Environ. Microbiol. 60, 2864–2868.
Salomao, B.C.M., Churey, J.J., Aragao, G.M.F., Worobo, R.W., 2009. Modeling *Penicillium expansum* resistance to thermal and chlorine treatments. J. Food Protect. 72, 2618–2622.
Scott, J.A., Wong, B., Summerbell, R.C., Untereiner, W.A., 2008. A survey of *Penicillium brevicompactum* and *P. bialowiezense* from indoor environments, with commentary on the taxonomy of the *P. brevicompactum* group. Botany 86, 732–741.
Selcuk, M., Oksuz, L., Basaran, P., 2008. Decontamination of grains and legumes infected with *Aspergillus* spp. and *Penicillum* spp. by cold plasma treatment. Bioresour. Technol. 99, 5104–5109.
Shearer, A.E.H., Mazzotta, A.S., Chuyate, R., Gombas, D.E., 2002. Heat resistance of juice spoilage microorganisms. J. Food Protect. 65, 1271–1275.
Souskova, H., Scholtz, V., Julak, J., Kommova, L., Savicka, D., Pazlarova, J., 2011. The survival of micromycetes and yeasts under the low-temperature plasma generated in electrical discharge. Folia Microbiol. 56, 77–79.
Timmermans, R.A., Nederhoff, A.L., Nierop Groot, M.N., van Boekel, M.A., Mastwijk, H.C., 2016. Effect of electrical field strength applied by PEF processing and storage temperature on the outgrowth of yeasts and moulds naturally present in a fresh fruit smoothie. Int. J. Food Microbiol. 230, 21–30.
Timmermans, R.A.H., Mastwijk, H.C., Knol, J.J., Quataert, M.C.J., Vervoort, L., Van der Plancken, I., Hendrickx, M.E., Matser, A.M., 2011. Comparing equivalent thermal, high pressure and pulsed electric field processes for mild pasteurization of orange juice. Part I: impact on overall quality attributes. Innovat. Food Sci. Emerg. Technol. 12, 235–243.
Toepfl, S., 2011. Pulsed Electric Field food treatment - scale up from lab to industrial scale. Procedia Food Science 1, 776–779.
Van Bokhorst-van de Veen, H., Xie, H., Esveld, E., Abee, T., Mastwijk, H., Nierop Groot, M., 2015. Inactivation of chemical and heat-resistant spores of *Bacillus* and *Geobacillus* by nitrogen cold atmospheric plasma evokes distinct changes in morphology and integrity of spores. Food Microbiol. 45, 26–33.
Venturini, M.E., Blanco, D., Oria, R., 2002. *In vitro* antifungal activity of several antimicrobial compounds against *Penicillium expansum*. J. Food Protect. 65, 834–839.
Watanabe, M., Masaki, H., Mori, T., Tsuchiya, T., Konuma, H., Hara-Kudo, Y., Takatori, K., 2010. Inactivation effects of UV irradiation and ozone treatment on the yeast and the mold in mineral water. J. Food Protect. 73, 1537–1542.
Wen, G., Xu, X., Huang, T., Zhu, H., Ma, J., 2017. Inactivation of three genera of dominant fungal spores in groundwater using chlorine dioxide: effectiveness, influencing factors, and mechanisms. Water Res. 125, 132–140.