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Impact of cold plasma on *Citrobacter freundii* in apple juice: Inactivation kinetics and mechanisms

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ABSTRACT

Various studies have shown that cold plasma is capable of inactivating microorganisms located on a variety of food surfaces, food packaging materials and process equipment under atmospheric pressure conditions; however, less attention has been paid to the impact of cold plasma on microorganisms in liquid foodstuffs.
The present study investigates cold plasma's ability to inactivate *Citrobacter freundii* in apple juice. Optical emission spectroscopy (OES) and temperature measurements were performed to characterise the plasma source. The plasma-related impact on microbial loads was evaluated by traditional plate count methods, while morphological changes were determined using scanning electron microscopy (SEM). Physiological property changes were obtained through flow cytometric measurements (membrane integrity, esterase activity and membrane potential). In addition, mathematical modelling was performed in order to achieve a reliable prediction of microbial inactivation and to establish the basis for possible industrial implementation.
C. freundii loads in apple juice were reduced by about 5 log cycles after a plasma exposure of 480 s using argon and 0.1% oxygen plus a subsequent storage time of 24 h. The results indicate that a direct contact between bacterial cells and plasma is not necessary for achieving successful inactivation. The plasma-generated compounds in the liquid, such as H_2O_2 and most likely hydroperoxy radicals, are particularly responsible for microbial inactivation.

1. Introduction

The development of new preservation technologies is based on a growing demand for natural and nutritious foods that are attractive and flavourful. Conventional thermal treatments, such as pasteurisation or ultra-high temperature processing, ensure a high level of product safety, but they cannot fulfil the aforementioned prerequisites. In addition, food products with added chemical preservatives are hardly marketable today.

New processes for the preservation of perishable foodstuffs are required in order to satisfy this demand. Besides emerging technologies, such as PEF (pulsed electric fields), UHP (ultra high pressure) and different types of irradiation, non-thermal atmospheric pressure plasma (also known as "cold plasma") has entered the field of food science and technology. The technological progress in this field allows us to treat heat-sensitive matrices at room temperature and atmospheric pressure (Ehlbeck et al., 2011; Schlüter et al., 2013).

Numerous studies are now investigating the impact of cold plasma on microorganisms. Some years ago, studies into the decontamination of inorganic materials were the particular focus of research, but recently increasing interest has been paid to the application of cold plasma on the surfaces of foods (e.g. Vleugels et al., 2005; Critzer et al., 2007; Niemira and Sites, 2008; Perni et al., 2008; Song et al., 2009; Ragni et al., 2010; Schnabel et al., 2012; Fröhling et al., 2012b; Fernández et al., 2013) and on liquids (e.g. Ikawa et al., 2010; Oehmigen et al., 2011; Hayashi et al., 2013). However, very little literature is available regarding the plasma treatment of liquid foodstuffs. The only studies published in this area are research into the inactivation of *Escherichia coli* O157:H7 in apple juice by a pulsed, non-thermal plasma system (Montenegro et al., 2002) and the treatment of milk by an atmospheric plasma corona discharge system (Gurol et al., 2012). Montenegro et al. (2002) were able to inactivate more than 5 log units of *E. coli* O157:H7 at a frequency of less than 100 Hz with 4000 pulses of 9000 V peak voltage without obvious temperature increase. Gurol et al. (2012) found that their corona discharge system, equipped with a 9 kV AC power supply and two tungsten electrodes, significantly reduced the number of *E. coli* cells in different types of milk (whole, semi-skimmed and skimmed) by about 4 log cycles after 20 min, while having negligible effects on pH and colour.

This study investigates the plasma-induced inactivation of *Citrobacter freundii* in apple juice. The facultative anaerobic, Gram-negative and long rod-shaped bacterium belongs to the family of *Enterobacteriaceae*, and can be found in water, food and the intestinal tracts of animals and humans (Wang et al., 2000). As an opportunistic pathogen, *C. freundii* is responsible for a number of significant opportunistic infections, such

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as infections of the respiratory tract, urinary tract and blood (Whalen et al., 2007).

A range of approaches was used in order to learn more about the plasma-related impact. Besides determining the effect of different process parameters such as plasma exposure time and oxygen to argon ratio in the process gas, also the role of subsequent storage times has been studied. Physiological property changes were obtained using flow cytometric measurements (membrane integrity, esterase activity and membrane potential), and further to this mathematical modelling was carried out to gain a reliable prediction of microbial inactivation and to establish the basis for possible industrial implementation.

2. Material & methods

2.1. Microorganisms and treatment media

C. freundii (isolate no. 0613, department's collection) was stored in Roti-Store® cryo-vials (Carl-Roth, Karlsruhe, Germany) at −80 °C. One cryo bead was added to 20 ml ST1 broth (Oxoid Ltd., Basingstoke, UK) and incubated on a shaker at 120 rpm for 24 h at 30 ± 0.1 °C.

An aliquot of this broth (91 μl in 9 ml Ringer's solution and then 20 μl of this dilution in 20 ml of the second-step broth) was then used to inoculate the final broth. This was again incubated at 30 ± 0.1 °C for 24 h, to obtain the microorganisms in their stationary growth phase (approximately 7.5×10^9 CFU/ml). Fifty ml of the treatment medium was inoculated with 0.5 ml of the second-step broth, resulting in a final concentration of approximately 7.5×10^7 CFU/ml. Commercially available pasteurised apple juice (Granini, Nieder-Olm, Germany) as well as phosphate-buffered saline (PBS, 0.05 M) were used for the treatment medium. PBS contained 137 mM NaCl, 2.7 mM KCl, 40.6 mM Na_2HPO_4 and 7.1 mM KH_2PO_4, adjusted to pH 7.0 with HCl, and was finally filtered with a 0.2-μm membrane filter. All reagents were provided by Carl Roth GmbH + CO. KG, Germany.

Viable cell counts were principally determined by the drop plating method using ST1-Agar (Merck, Darmstadt, Germany) three hours after the treatment. In order to determine the impact of subsequent storage times, the samples were also analysed directly (0 h) and 24 h after plasma exposure, using 0.1% oxygen in the feeding gas, since preliminary tests revealed this oxygen concentration as the most efficient one. Plates were finally incubated for 24 h at 37 ± 0.1 °C.

2.2. Mathematical modelling of inactivation kinetics

The inactivation kinetics obtained were modelled with GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool, version 1.6), a freeware Add-Inn for Microsoft® Excel. This tool enables the testing of different types of microbial survival models, such as log-linear, Weibull and biphasic regressions. Five statistical measures support the choice of the best fit. Following Ratkowsky (2004), the root mean square error (RMSE) value was chosen as an indicator for the goodness-of-fit of the models tested.

2.3. Cold plasma equipment and treatment conditions

Cold plasma was generated at atmospheric pressure by a plasma jet (kINPen 09; neoplas tools GmbH, Greifswald Germany), which has already been described by Weltmann et al. (2009). The device consists of the plasma jet itself (length: 170 mm; diameter: 20 mm; weight: 170 g), a gas flow controller (Multi Gas Controller 647C; MKS Instruments, Andover, USA) and a DC power supply (Precision DC Power Supply PPS-12008; Voltcraft, Conrad Electronic SE, Hirschau, Germany).

The plasma jet was fixed to a plotter device, which allowed for accurate motion control and control of the distance between the nozzle outlet and sample. The functioning principle is based on capacitive high-frequency excitation (1.1 MHz). The process gases were argon 5.0 (purity ≥ 99.999%) as well as mixtures of argon and 0.025–0.1% oxygen, and the plasma exposure time was varied between 0 and 480 s. All gases were obtained from Air Liquide (Düsseldorf, Germany). The gas flow rate was 5 slm (standard litre per minute, 8.44375 $(Pa^*m^3)/s$), and the power supply was operated at a voltage of 65 V and a resonance balancing of 0.05 A. The distance between nozzle outlet and sample was set to 10 mm.

Each experiment was performed at least twice on two different days. Two millilitres of the sample was transferred into a small glass vial, which was placed under the nozzle outlet so that the sample was directly exposed to the plasma. Continuous mixing of the samples was provided due to the gas flow. For each parameter, at least six 2-ml samples were treated and subsequently combined in order to obtain sufficient quantities for the following analysis. Cooling was applied before and immediately after the treatment. The inactivation of microorganisms was evaluated in quadruplicate by calculating the log reduction in viable cell counts compared to the untreated sample.

2.4. Flow cytometric measurements

To investigate the influence of plasma on cell membrane potential of *C. freundii*, 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$) was applied. *C. freundii* cells were stained with 5(6)-carboxyfluorescein diacetate (cFDA) and PI in combination to investigate the metabolic activity and membrane integrity of the cells after plasma treatment. Additionally, *C. freundii* cells were stained with thiazole orange (TO) and propidium iodide (PI) in combination to gain further insight into the membrane integrity of the cells as well as RNA damage after plasma treatment. $DiOC_2(3)$, cFDA and TO were provided by Sigma-Aldrich, Germany and PI was provided by Life Technologies GmbH, Darmstadt, Germany.

For the flow cytometric measurements, a 10-ml sample was required for each plasma exposure time and treatment parameter. The samples were centrifuged at 3220 ×*g* and 10 °C for 15 min. The supernatant was then discarded and the cell pellet was resuspended in 250 μl of 0.05 M PBS (pH 7). The samples were transferred into 1.5-ml reaction tubes and centrifuged at 7000 ×*g* and 10 °C for 5 min. The supernatant was discarded and the cell pellet was resuspended in 100 μl of 0.05 M PBS (pH 7). Each of the staining procedures described below was performed in triplicate.

In the next step, 10 μl of the bacterial suspension was diluted in 490 μl of 0.05 M PBS (pH 7.0), and 420 nM TO as well as 30 μM PI (final concentrations) were added and incubated for 10 min at room temperature in the dark before the flow cytometric measurements were made.

The staining procedure, to measure membrane potential of the cells, was modified from Novo et al. (1999). Accordingly, 10 μl of the suspension were diluted in 490 μl PBS containing 20 μM D-glucose and 30 μM $DiOC_2(3)$ (final concentration), and then incubated for 15 min in the dark at room temperature. Afterwards, the suspension was centrifuged at 7000 ×*g* and 10 °C for 5 min. The pelleted material was resuspended in PBS to a cell density of ~10^6 cells per ml and immediately measured in the flow cytometer.

The remaining suspension was mixed with 0.83 mM cFDA (final concentration). The cells were incubated for 45 min at 37 °C in a water bath to allow for hydrolyses of cFDA to the fluorescent carboxyfluorescein (cF) by intracellular esterases. Afterwards, the surplus cFDA was removed by centrifugation at 7000 ×*g* and 10 °C for 5 min. The pelleted material was resuspended with PBS to a cell concentration of ~10^6 cells per ml. 30 μM (final concentration) of the PI was then added and allowed to penetrate into the permeabilised cells for 10 min at 4 °C in the dark before the flow cytometric measurements were taken.

All experiments were performed using a Cytomics FC 500 flow cytometer (Beckman Coulter, Germany) equipped with a 20 mW argon ion laser emitting at a wavelength of 488 nm. The field stop was set to 1–8°. The discriminator to reduce background noise was set on the side scatter (SS = 2). The fluorescence signal of TO, cF, and green ($DiOC_2(3)$) was collected in the FL1 photomultiplier in the