Contents lists available at ScienceDirect

Food and Bioproducts Processing

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

Assessing bacterial recovery and efficacy of cold atmospheric plasma treatments

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ARTICLE INFO

Article history:
Received 12 March 2015
Received in revised form 20 July 2015
Accepted 25 July 2015
Available online 1 August 2015

Keywords:
Cold atmospheric plasma
Optical density
E. coli
Growth
Kinetic parameters
Lettuce

ABSTRACT

Cold atmospheric plasma (CAP) is a non-thermal decontamination technology capable of generating groups of antimicrobial agents including; photons, electrons, positively and negatively charged ions, atoms, free radicals and excited or non-excited molecules. CAP treatments (70 kV, 50 Hz) at different times (i.e. 15, 30, 60, 90 s) were applied to a broth system inoculated either with *Escherichia coli* NCTC 12900, *E. coli* ME 9062 or *E. coli* knockout mutants of *soxS*, *rpoS* and *dnaK* genes. Optical emission spectra were performed and the main antimicrobial reactive substances generated by CAP treatments were found to be N_2, N_2^+, NO, H_α and He. After treatments, reduction levels of the studied bacteria were up to 0.5 log CFU/mL, no showing statistical differences. Moreover, the kinetic growth parameters were calculated by applying the 2-fold dilution method. μ_{max} decreased in all bacteria when the time of CAP treatment increased. *E. coli dnaK* was the most sensitive mutant to plasma, showing no recovery after CAP of 60 and 90 s. In addition, CAP challenge test treatments were applied on butter head lettuce inoculated with *E. coli* NCTC 12900 with CAP treatments of 60 and 90 s reducing bacteria levels by more than 1.5 log CFU/cm^2.

1. Introduction

In the last 10 years, fresh vegetable production has increased by over 30% (FAOSTAT). Changes in consumer trends and the application of new preservation technologies, such as modified atmosphere packaging, were some of the main reasons for this increment. This has led to the development of a new range of ready-to-eat produce.

Recent outbreaks linked to fresh-cut vegetables were attributed to the introduction of pathogens throughout the production chain. The main transmission route of these pathogens for vegetables is through faecal contamination (originating from manure enriched soil and irrigation water), cross contamination and food handling (Warriner et al., 2009). Therefore, ready to eat products are no longer considered low risk in terms of food safety (Bhagwat and Matthews, 2006). Thus, there is a need to produce fresh products of high quality without putting consumers' health at risk.

Hence, the development of novel decontamination technologies assuring a high level of decontamination is essential for the food industries. The application of novel dry decontamination technologies is particularly desired. Cold atmospheric plasma (CAP) is a non-thermal emerging antimicrobial decontamination technology, which was developed in the 1990s.

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http://dx.doi.org/10.1016/j.fbp.2015.07.011

CAP is generated by the excitation of different gases through the application of discharges at room temperature and atmospheric pressure (Fernández and Thompson, 2012). Hereafter, different antimicrobial substances including UV photons, reactive oxygen species such as hydrogen peroxide, hydroxyl radical, superoxide, singlet oxygen, atomic oxygen and ozone and reactive nitrogen species such as peroxynitrite, nitric oxide and nitrite are generated (Rød et al., 2012). These antimicrobial substances may result in microbial inactivation through several mechanisms. For example exposure to UV can cause DNA modifications and consequently erroneous cell replication, while charged particles have a role in membrane rupture due to electrostatic forces. Moreover, reactive oxygen and nitrogen species can induce oxidation of membranes (particularly those rich in lipids), amino acids and inhibit cell respiration (Ragni et al., 2010). In order to defend against this oxidative stress, bacteria have different repair mechanisms. Bacteria response against elevated concentrations of O_2^- is regulated by the *SoxRS* gene. *SoxRS* modulates the expression of manganese superoxide dismutase (encoded by *sodA*), the DNA repair enzyme endonuclease IV (*nfo*) and O_2^- resistant isozymes of fumarase (*fumC*) and aconitase (*acnA*). The lack of the *SoxS* gene plays an important role in the expression of the *SoxRS* gene due to the expression of the *SoxRS*'s regulon (Storz and Imlayt, 1999). The gene *rpoS* encodes the transcription factor σ^S which is related to the expression of a large number of genes involved in cellular responses to a diverse number of stresses (i.e., starvation, osmotic stress, acid shock, cold shock, heat shock, oxidative DNA damage and transition to stationary phase) (Loewen et al., 1998). The *DnaK* gene is a key factor during peroxidative stress mainly through the protection of proteins from oxidative damage (Patil et al., 2011; Echave et al., 2002) and the degradation of misfolded proteins (Farr and Kogoma, 1991).

The aim of this study was to estimate the growth parameters of *Escherichia coli* and some of its mutants when recovered under optimal temperature conditions following the application of an electric barrier discharge CAP system (operating at 70 kV and 50 Hz). Additionally, the efficacy of CAP as a decontamination process was assessed by carrying out challenge microbial tests on the surface of lettuce leaves as an example of a fresh produce food testing.

2. Materials and methods

2.1. Plasma equipment and diagnostics

A similar CAP system as described by Pankaj et al. (2014) was used to carry out this study. The plasma discharge was generated between two circular aluminium plate electrodes (outer diameter = 158 mm) over perspex dielectric layers (10 mm thickness), with distance between them equal to 3 cm. The applied voltage to the electrodes was obtained from a step-up transformer (Phoenix Technologies, Inc., USA) using a variac. The input of 230 V, 50 Hz was given to the primary winding of high voltage step-up transformer from the mains supply.

The antimicrobial substances generated were identified using Optical Emission Spectroscopy (OES) (Edmund Optics UV–VIS Enhanced Smart CCD Spectrometer) at room temperature for experiments carried out with both the broth system and the lettuce samples. OES was carried out using an Edmund Optics UV Enhanced Smart CCD Spectrometer. The OES technique is based on the integration of measured signals over a line of sight observation. OES is a non-intrusive diagnostic technique, which is commonly used to detect light emitted by excited species from plasma discharges. Experiments were carried out to investigate species intensity. All spectra were recorded at 70 kV RMS and 120 W input power. For the broth system experiments, samples were placed at the corner of an aseptically cleaned polypropylene container (210 mm × 230 mm × 20 mm) and were treated by an indirect (i.e., not within the plasma stream) at CAP treatment conditions of 70 kV and 50 Hz for treatment times of 15, 30, 60, 90 s. Indirect CAP treatments were applied for the inoculated broth system in order to ensure that there is some microbial survival and the bacteria recovery could be assessed. For the experiments with lettuce, samples were placed in the centre of an aseptically cleaned polypropylene container (210 mm × 230 mm × 20 mm) and were treated by a direct CAP treatment (i.e., within the plasma stream) (70 kV, 50 Hz) at different times (15, 30, 60, 90 s).

2.2. Bacteria strains and inoculum preparation

E. coli O157:H7 NCTC 12900 was obtained from the microbiology stock culture of the School of Food Science and Environmental Health, Dublin Institute of Technology while *E. coli* ME 9062 and the *E. coli* mutants *ΔsoxS*, *ΔrpoS*, *ΔdnaK* were obtained from the National BioResource Project, Japan (NIG, Japan) (Baba et al., 2006). The studied bacteria were maintained in beads and kept in vials at −70 °C. The stock cultures were re-activated by inoculation onto Tryptic Soya Agar (TSA) plates, which were incubated for 24 ± 2 h at 37 °C, in order to obtain single colonies that were stored at 4 °C for a maximum period of one month. The inoculum was prepared by selecting a single colony from the stock culture and incubating it in 10 mL of Tryptic Soya Broth without dextrose (TSB-D) at 37 °C for 24 ± 2 h. After incubation, 10 mL of the suspension was centrifuged (10,000 × *g*) for 10 min and washed with 10 mL of Maximum Recovery Diluent (MRD) twice, resulting in a concentration of $10^{8–9}$ CFU/mL. Finally, the inoculum was 10 fold diluted to 10^4 CFU/mL before inoculation of the studied products.

2.3. Experiments in broth

2.3.1. Sample preparation

Equal volume (150 μL) of the original inoculum (10^4 CFU/mL) of the different bacteria was loaded into a microtiter plate (Startdest, Nümbrecht, Germany). From the original inoculum, the bacteria were diluted into MRD following the 2-fold dilution method until a final concentration of 10^2 CFU/mL was achieved. Each experiment was replicated twice and each inoculum was assessed in duplicate in each microtiter plate.

2.3.2. Microbial analysis for reduction studies

After the broth system was treated by CAP, as described before, samples were stored for 1 h at room temperature before analysis (Ziuzina et al., 2013) to provide sufficient time for the action of the plasma generated reactive species. Treated samples were plated out on TSA in order to determine the reduction levels. Plates were incubated at 37 °C for 24 h. Three repetitions of each bacteria at the studied CAP treatments were carried out. Separate experiments were performed for the kinetic studies.