Effects of Shading and Growth Phase on the Microbial Inactivation by Pulsed Light

Elizabeth CUDEMOS[1], Adriana IZQUIER[1], María S. MEDINA-MARTÍNEZ[2] and Vicente M. GÓMEZ-LÓPEZ[1]

[1]Instituto de Ciencia y Tecnología de Alimentos, Facultad de Ciencias and [2]Cátedra de Microbiología de Alimentos, Facultad de Farmacia, Universidad Central de Venezuela, Caracas, Venezuela

Abstract

Cudemos E., Izquier A., Medina-Martínez M.S., Gómez-López V.M. (2013): **Effects of shading and growth phase on the microbial inactivation by pulsed light**. Czech J. Food Sci., **31**: 189–193.

Pulsed light is an emerging technology that kills microorganisms using pulses of an intense broad-spectrum light. This work aimed to determine the effect of population density and microbial growth phase on its microbicidal efficacy. To this, *Pseudomonas fluorescens* cultures were grown, diluted to different population densities, flashed, plated, incubated, and enumerated, Also, cultures of *P. fluorescens, Bacillus cereus,* and *Saccharomyces cerevisiae* were taken at different growth phases, diluted to the same population density, flashed, plated, incubated, and enumerated. Microbial inactivation was lower at high densities, probably as the consequence of the shading effect, and higher at the exponential phase. This study sets the background information useful for scientists and industrial implementation. The population density and growth phase must be taken into account in the planning experiments and comparing the literature. On the industrial scale, heavily contaminated solids are not suitable for pulsed light (PL) treatment; while liquids should receive several PL flashes under the flow conditions that assure that all microorganisms receive a PL treatment, that should be also designed in function of the growth phase of the microbial contaminant.

Keywords: UV-C light; non-thermal methods; shading effect; decontamination; inactivation

Pulsed light (PL) is a non-thermal emerging technology to decontaminate surfaces and transparent liquids by killing microorganisms using pulses of an intense broad-spectrum light where its UV-C part is the most lethal (Gómez-López *et al.* 2007).

The shading effect is usually cited as a limiting factor of PL efficacy. It occurs when microorganisms are present one upon another, which causes that those at the top can be killed by PL but protect in turn the underlying microorganisms (Gómez-López *et al.* 2007). Its existence is claimed from the beginning of the use of PL as a germicidal method, however, just for specific microorganisms that readily absorb UV rays such as *Aspergillus niger* and not as a general limiting factor (Hiramoto 1984). Furthermore, the patent of Hiramoto (1984) also claimed that this phenomenon occurs with the conventional UV lamps and is overcome by the photothermal effect of PL. On the other side, Farrell *et al.* (2010) reported a population density effect in the inactivation curves of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, where tailing was only observed at very high initial sizes.

The susceptibility of microorganisms to inactivation by physical methods depends on several factors, which depend in turn on the specific method. The effect of the growth phase of the microor-

ganism applied is one of the factors classically studied, giving information specially useful for inoculation studies on laboratory and pilot plant scales, which provides the basic information for implementation in real scenarios. The growth phase effect on microbial inactivation has been identified also for other non-thermal technologies such as high pressure processing (Hayman *et al.* 2007; Saucedo-Reyes *et al.* 2009), supercritical carbon dioxide treatment (Ortuño *et al.* 2012), pulsed electric fields (Arroyo *et al.* 2010), and ultrasonic waves under pressure (Arroyo *et al.* 2011). With PL, Gómez-López *et al.* (2005) studied the inactivation of *Listeria monocytogenes* during the growth. Those experiments did not take into consideration that the population density increases, while the microorganisms advance in the growth phase, which is especially relevant with PL if the shadow effect exists. While those results are relevant from the point of view of a real scenario, where both events occur together, their separate study allows to identify the actual effect of the growth phase on PL efficiency. In this regard, Farrell *et al.* (2010) reported that 16-h cultures of *Escherichia coli* are more sensitive to PL than 24-h cultures. Therefore, the aim of this study was to determine the effect of the shading and microbial growth phase on the efficacy of PL.

MATERIAL AND METHODS

Microbial culture. Three microorganisms were used, all purchased at the internationally certified culture collection Centro Venezolano de Colecciones de Microorganismos, a member of the World Federation of Culture Collections, WCDM 815. They were *Bacillus cereus* CVCM 461, *Pseudomonas fluorescens* CVCM 627, and *Saccharomyces cerevisiae* CVCM 1629. The bacteria were cultured in Nutrient Broth (HiMedia, Mumbai, India) at 37°C for *B. cereus* and 30°C for *P. fluorescens*, and the yeast was cultured in Potato Dextrose Broth (HiMedia, Mumbai, India) at 30°C. The strains were activated by three successive cultures in/at the respective broths and incubation temperatures.

PL equipment. PL processing was performed using a XeMaticA-1XL system (SteriBeam GmbH, Kehl, Germany), which had been described previously (Izquier & Gómez-López 2011). The emission spectrum is shown in Figure 1. The experiments were carried out applying a single pulse with a fluence of 0.35 J/cm^2 measured at the sample level, according to the system built-in photodiode readings. That fluence was selected after preliminary experiments looking for a fluence strong enough to cause lethality and weak enough to obtain countable results in the microbial determinations.

Population density test. The culture of *P. fluorescens* was grown to ca. 9 log CFU/ml, and then serially diluted in peptone (HiMedia, Mumbai, India) water to 4 log CFU/ml. 6 ml aliquots of dilutions 2, 4, 6, 8, and 9 log CFU/ml were transferred to sterile Petri dishes in quadruplicate, two remaining untreated as controls while the other two were flashed. Later, 1 ml aliquots were with-
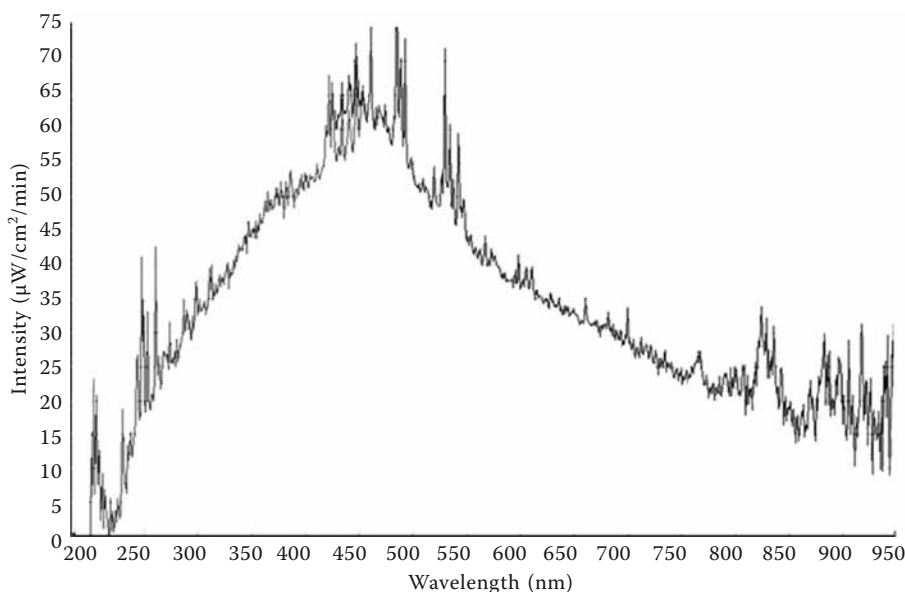

Figure 1. Emission spectrum of used xenon lamp working at 2.5 kV. Courtesy of Steribeam Systems

drawn from these samples, serial dilutions were performed and the samples were spread plated on Nutrient Agar (HiMedia, Mumbai, India). The test was performed three times.

Phase growth tests. The activated cultures were grown for 12 h before inoculating 1 ml aliquots in 100 ml broths, and the incubation was carried out with agitation in an orbital shaker. Samples of 3 ml were withdrawn at different incubation times and their optical density was measured at 600 nm in a spectrophotometer (UV-2501PC; Shimadzu, Kyoto, Japan) in order to construct the growth curves. Nine sampling times were used, three in the course of the latency phase, three in the course of the exponential phase, and the remaining three in the course of the stationary phase. Simultaneously, four 0.1 ml samples were taken (two for controls and two for flashing), serially diluted, and spread plated in Nutrient Agar (Hi Media, Mumbai, India) for bacteria or Potato Dextrose Agar for the yeast. Two plates were directly incubated, and the other two flashed and then incubated. Preliminary tests were performed to set up the times when samples should be taken (the growth curves were known) and to set up the serial dilutions in order to receive plates with 25–250 CFU.

Data analysis. Inactivation was calculated as log CFU/ml without flashing – log CFU/ml after flashing. The data were analysed by one-way ANOVA and Duncan test with $P < 0.05$ using a statistical software (Statgraphics Centurion XVI; StatPoint Technologies, Warrenton, USA).

RESULTS AND DISCUSSION

The effect of the population density on PL efficacy was studied with *P. fluorenscens* suspended in the nutrient broth. The cultures were grown until stationary phase and then diluted to get specific counts, this method excluded a possible effect of the growth phase on the results. Significant ($P < 0.05$) differences were found between the inactivation levels as a function of the population density (Figure 2). The cultures with 7.7 and 8.8 log CFU/ml initial populations decreased in their population by ca. 0.5 log CFU/ml, while those with 6.1 and 4.1 log CFU/ml initial population by more than 1 log CFU/ml. The culture with 2 log CFU/ml was completely inactivated and the result is not shown in the figure. The results show that with the increase of the population density, the PL inactivation efficiency decreased. This finding is explained by the
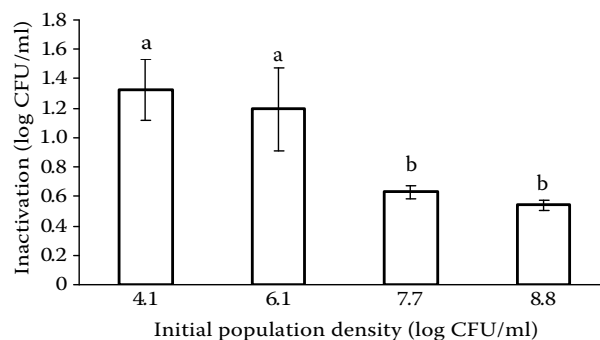

Figure 2. Effect of population density on the PL inactivation of *P. fluorescens* cells

shading effect, where the cells located at the top of the sample become inactivated and protect the underlying cells from PL illumination. Farrell *et al.* (2009) reported similar results for the inactivation of clinically relevant yeasts.

The effect of the growth phase on PL efficiency was studied in three microorganisms, a Gram-negative bacterium (*P. fluorescens*), a Gram-positive bacterium (*B. cereus*), and a yeast (*S. cerevisiae*) (Figures 3A–C). For *P. fluorescens*, the inactivation was 0.8 log in the exponential phase, 0.6 log in the latency phase, and 0.3 in the stationary phase. For *B. cereus*, the inactivation was > 0.6 log cycles in the exponential phase, 0.5 log in the lag phase, and 0.3 log in the stationary phase. For *S. cerevisiae*, the inactivation was 0.35 in the log phase, 0.2 log in the lag phase and 0.15–0.20 in the stationary phase. The experimental set-up where the cultures were diluted to similar initial counts ruled out the population density effect.

A general trend can be observed in the growth phase tests; the cells in the log phase are more susceptible to PL than the cells in the lag phase, and the latter are in turn more susceptible than the cells in the stationary phase. Previously, Farrell *et al.* (2010) reported that the resistance of *E. coli* to PL inactivation was higher in 24-h cultures versus 16-h cultures. Two inactivation mechanisms may have played a role in the lethal effects observed in this work, the photochemical effect (DNA damage), and the photophysical one (structural damage). The third possible mechanism, the photothermal effect, has been proposed for fluences higher than that used in this work (0.5 J/cm^2 > 0.35 J/cm^2). During the exponential phase, microbial cells undergo mitosis, which involves changes, specifically in their DNA, and structural changes in general. Therefore, the disturbance in DNA and cell structure in general caused by PL might be difficult to overcome by
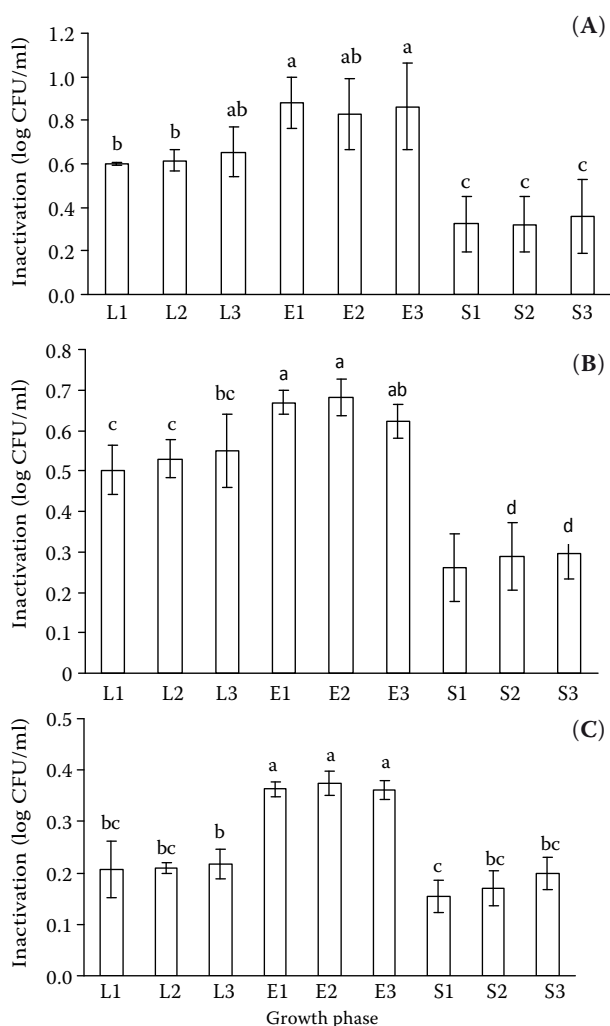
with high fluences. The same precautions have to be taken into account when microorganisms are either in the lag phase or in the stationary phase; furthermore, the difficulties for decontamination are enhanced when microorganisms present on the target have grown there and find them selves in the two worst conditions, at high population density and in the stationary phase. At laboratory level, these effects have to be taken into account when comparing the susceptibility of different microorganisms to PL, and when testing PL efficacy against microorganisms inoculated on foods.

Figure 3. Effect of growth phase on the PL inactivation of (**A**) *P. fluorescens*, (**B**) *B. cereus*, and (**C**) *S. cerevisiae*

L – latency phase; E – exponential phase; S – stationary phase; numbers are to differentiate different sampling times along the same phase

the microorganism, and any repairing mechanism can be, if not inactivated by PL itself, at least difficult to activate during such a critical moment of the cell life. In contrast, the lag and stationary phases do not involve such demanding activities from the cells, and would be comparatively less susceptible to PL.

These results suggest that the degree of contamination of the item and the growth phase of the microorganism have to be taken into account for industrial implementation of PL. For solid targets such as food surfaces and food-contact surfaces, heavily contaminated items will undergo limited decontamination. For fluid targets such as water, brines, syrups, clear juices, or waste streams, highly contaminated items should be mixed and treated

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Corresponding author

Dr. Vicente M. Gómez-López, CEBAS-CSIC, Campus Universitario de Espinardo, Murcia 30100, Spain; E-mail: vgomez@cebas.csic.es, vicente2709@yahoo.es