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ORIGINAL PAPER

Study of Pulsed Light-Induced Damage on *Saccharomyces cerevisiae* in Apple Juice by Flow Cytometry and Transmission Electron Microscopy

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Abstract This work analyzed the pulsed light (PL) (0.0–71.6 J/cm^2)-induced damage on *Saccharomyces cerevisiae* KE162 cells in peptone water (pH 3.5 or 5.6) and apple juice (pH 3.5) by applying flow cytometry (FCM) and transmission electronic microscopy. Cells were labeled with fluorescein diacetate (FDA) for detecting membrane integrity and esterase activity and with propidium iodide (PI) for monitoring membrane integrity. *S. cerevisiae* inactivation curves reached 6–7 log reductions (peptone water systems) and 3.9 log reductions (apple juice) after 60 s (71.6 J/cm^2) of PL exposure. FCM revealed the same damage pattern (although at different doses) in all media: at low doses, there was an increase in population in PI$^+$−FDA$^+$ quadrant, while at high doses, most of the population was located at quadrant PI$^+$–FDA$^-$, indicating that PL provoked rupture of the cytoplasm membrane allowing PI to penetrate cells and there was progressive loss of esterase activity. Comparison of conventional culture technique with FCM revealed the occurrence of certain cell subpopulations in peptone water with pH 3.5 which were stressed and lost their ability to grow in agar but still showed metabolic activity. Transmission electron microphotographs of PL-treated cells clearly indicated that various cell structures other than plasma membranes were altered and/or destroyed in a different degree depending on exposure time and type of medium.

Keywords Pulsed light · Flow cytometry · Microscopy · *Saccharomyces cerevisiae* · Apple juice

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Introduction

Pulsed light (PL) is a nonthermal emerging technology to decontaminate surfaces by killing microorganisms using short time pulses (100–400 μs) of a continuous broadband spectrum between 100 and 1,100 nm where its short ultraviolet light fraction (UV-C) is the most lethal (Gómez-López et al. 2007). PL used for food processing applications typically emits 1 to 20 flashes per second at an energy density in the range of about 0.01 to 50 J/cm^2 at the surface. In 1999, the use of pulsed light treatment was approved by the Food and Drug Administration of the United States in the production, processing, and handling of food (Federal Register 1999). Various studies have also shown that PL is cost effective, has high processing speed systems, lacks residual compounds, and has the ability to be incorporated in processing lines. The effectiveness of PL treatment highly depends on many factors related to the sample and to the equipment: the number of pulses, light source, distance of the sample from the lamp, food composition, and sample thickness (Wambura and Verghese 2011). One of the drawbacks of PL application is the generation of heat during prolonged treatments, a detrimental fact to the quality and nutritive value of the food that must be balanced by the implementation of a cooling system (Gómez et al. 2012).

PL has shown to inactivate vegetative cells and spores in a variety of food products like milk, infant food, corn meal, bread, bell peppers, cut apple, and meat (Butz and Tauscher 2002; Jun et al. 2003; Gómez-López et al. 2007;

Krishnamurthy et al. 2008a; Gómez et al. 2012; Choi et al. 2010). The mechanism of inactivation by PL is still a topic of discussion and many questions remain unanswered. Its main effects have been reported to be the microbial DNA damage by thymine dimmer formation (photochemical effect) (Wang et al. 2005) and/or localized overheating of microbial cells (photothermal effect) (Wekhof 2000) and/or structural damage caused by the pulsing effect (photophysical effect) (Krishnamurthy et al. 2008a). All these mechanisms probably coexist, and the relevance of each one would depend on the fluence imparted to the food and target microorganism (Gómez-López et al. 2007). Changes in the physiological state of microbial cells should be studied in order to get a better understanding of PL inactivation process. The plate count procedure remains being the classical method for the determination of viability in most of the industries and research laboratories. However, this technique has some drawbacks putting on evidence changes on cellular integrity or metabolic activities within the microbial population when a process of preservation is applied (Schenk et al. 2011). It is known that a proportion of metabolically active cells could not be cultured, failing to form visible colonies in the agar plate (Ananta et al. 2004). The ability of PL to injure cells at the sublethal level has already been reported in *Listeria innocua*, *Listeria monocytogenes*, and *Escherichia coli* (Woodling and Moraru 2005; Rajkovic et al. 2008; Van Houteghem et al. 2008; Pataro et al. 2011) in either static or continuous PL systems by incubating in selective or nonselective medium.

The application of flow cytometry (FCM) for research purposes has great potential for the assessment of the physiological response of microorganisms to various processes on the single cell level. FCM analyzes the whole populations of microbial cells (typically 5,000 to 10,000 cells) and offers the possibility to detect microorganisms at relatively low concentrations in a short time (100 to 1,000 cells per second) (Comas-Riu and Rius 2009). It also allows identifying and isolating subpopulations with differences regarding structure and functionality when they differ in light scatter or fluorescence characteristics (Ananta and Knorr 2009). Light scattering pattern provides insights on cell size and granularity. On the other side, FCM uses functional dyes to put on evidence possible functional and structural changes that are aimed at specific cellular targets like DNA, enzymes, internal pH, or cytoplasmic membrane which may be involved in cellular injury or compromised metabolic activity induced by a given process (Nebe von Caron et al. 2000). A combination of fluorescein diacetate (FDA) and propidium iodide (PI) could be used in double staining procedures. FDA is a lipophilic, nonfluorescent precursor that readily diffuses across membranes, being used for the evaluation of enzymatic activity and membrane integrity. In the intracellular compartment of metabolically active cells, FDA undergoes hydrolysis by enzymes with esterase activity into fluorescein (F), a polar membrane fluorescent green compound that is unable to diffuse out of the cell. Thus, only cells with intact membranes will remain fluorescent due to the presence of internal F. In contrast, PI is a nucleotide-binding probe, with positive charge, widely used for the assessment of membrane integrity, supposed to enter only cells with damaged membranes. Following loss of membrane integrity, PI diffuses into the cell and stains the DNA giving red fluorescence (Hewitt and Nebe-Von Caron 2001).

Fruit juices could be easily spoiled by yeast, *Saccharomyces cerevisiae* being the most responsible strain for its ethanol-tolerant condition (Fleet 1992). Moreover, it has been already reported that the effect of PL depends on the type of treated juice (Sauer and Moraru 2009; Pataro et al. 2011).

In this study, the physiological response of *S. cerevisiae* KE162 following exposure to different PL doses in apple juice or peptone water was studied by multiparameter flow cytometry. In the case of peptone water, the influence of pH was also investigated. FCM analysis was performed under dual staining with FDA and PI. Additionally, cell structure changes were evaluated by transmission electron microscopy (TEM) observations. Plate counts were also performed to establish the relationship between the viability detected by FCM and culturable or nonculturable populations.

Materials and Methods

Strain and Preparation of Inoculum

Experiments were performed using *S. cerevisiae* KE162. Initial inoculum was prepared by transferring a loopful of a fresh stock culture maintained in potato dextrose agar (PDA; Britania, Buenos Aires, Argentina) to an Erlenmeyer flask containing 20 mL of Sabouraud Broth (Sab; Britania). Incubation was performed at 27 °C for 24 h. All inocula were harvested by centrifugation (11,180×*g*, 5 min) (Labnet, USA), washed twice, and resuspended in peptone water 0.1 g/100 mL to give a cell density of 10^8 CFU/mL. For inoculation, 100 μL of the microbial suspension was added to 4.9 mL fruit juice or peptone water samples prior to PL treatment.

Preparation of Samples

Apple pasteurized juice, with no declared preservatives (CEPITA, Coca Cola, Argentina) (pH 3.5±0.1; 9.5 °Brix), and peptone water (0.1 g/100 mL; pH 3.5±0.1 or 5.6±0.1 adjusted with a 10 g/100 mL citric acid aqueous solution) (Britania) were used in this study.

PL Processing

PL treatments were performed with a RS-3000B Steripulse-XL system (Xenon Corporation, Woburn, MA, USA), which produced polychromatic radiation in the wavelength range of 200 to 1,100 nm. The PL device consisted of a RC-747 power/control module, a treatment chamber that housed a xenon flash lamp (nontoxic, mercury free), and an air cooling system attached to the lamp housing to avoid lamp overheating during operation. It generated high intensity pulsed light at a rate of 3 pulses per second and a pulse width of 360 μs. According to the specifications supplied by the manufacturer, each pulse delivered 1.27 J/cm^2 for an input of 3,800 V at 1.9 cm from the quartz window surface of the lamp. The different PL doses were obtained by altering the number of applied pulses. Fluence measurements were taken by a pyroelectric head model ED500 (Gentec Electro-Optics, Québec, Canada) connected to an oscilloscope model TDS 2014 (Tektronix, Beaverton, USA), with an aperture cover of 20.3 cm^2. Measurements were performed in triplicate.

For each PL treatment, 4.9 mL of refrigerated juice (~4 °C) or of peptone water was poured into a 100-mm diameter Petri dish to ensure that the entire dish surface was covered with sample to a depth of 1 mm. Inoculum was added and the Petri dish was placed at a distance of 10 cm from the quartz window in a bigger Petri dish containing flake ice to minimize temperature increase of the sample. Inoculated samples were exposed to irradiation for 2–60 s, corresponding to applied fluences between 2.4 and 71.6 J/cm^2. Untreated inoculated samples were used as controls. The temperature evolution of samples during PL treatment was monitored using a T-type thermocouple connected to a data logger Digi-Sense model 69202–30 (Barnant Company Division, Barrington, USA).

Viable Population Determined by Plate Count

Peptone water tenfold dilution aliquots were surface-plated onto PDA for *S. cerevisiae* using a spiral plater (Autoplate 4000, Spiral Biotech, USA). Two replicates were used, but when PL irradiation treatment resulted in low counts, three PL-treated replicates corresponding to a given treatment time were collected for subsequent analysis and up to 3 mL of fruit juice was directly pour-plated into each Petri dish, using four plates as replicates. Plates were incubated for 72 h at 27 °C. A counting grid was used for enumeration of colonies. Survival curves were generated from experimental data by plotting Log N/No (where N is the number of CFU per milliliter at a given time and No is the initial number of CFU per milliliter) versus time of treatment. The nonviable cells were expressed as the difference between Log No and Log N (Log No − Log N).

Staining Procedure and FCM Analysis

Application of double staining with FDA (Calbiochem, Darmstadt, Germany) and PI (Sigma Aldrich, St Louis, MO, USA) was used for FCM analysis. Nonirradiated stained cells and cells treated at 85 °C for 15 min and subsequently stained with FDA or PI, respectively, were used as controls to define negative and positive histogram regions. Moreover, a nonstained, nontreated control was employed for determining the autofluorescence of cells. Control and PL-treated cells were initially incubated with 0.5 μL FDA (5 mg/mL acetone) at 37 °C for 30 min. After this labeling, cells were centrifuged twice (29×10^3 g, 5 min) and suspended in 1 mL phosphate buffered saline solution (PBS buffer, pH 7.0). This step was followed by the addition of 0.5 μL PI (1 mg/mL sterile water) and by incubation at room temperature for 10 min in the dark. Following incubation with PI, samples were placed on ice in the dark until analysis (maximum of 1 h). Analysis of microorganisms was performed on a BD FACSAria II flow cytometer (BD; New Jersey, USA). Scatter and fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. Green fluorescence of cells stained with FDA was collected in the FL1 channel (525±15 nm), whereas red fluorescence of cells labeled with PI was collected in the FL2 channel (620±15 nm). Flow rate and cell concentration of the samples were adjusted to keep acquisition at 200 microorganism cells per second. A total of 20,000 events were registered per sample. Trials were replicated at least three times with three samples for each PL dose. The software WinMDI 2.8 was used to analyze flow cytometric data. For cell sorting assay, cells corresponding to quadrant 2 (F^+/PI^+) were sorted for selected times of treatment, at a threshold rate of 2,000 events/s until an acquisition of 100,000 events. Obtained aliquots were then cultured in PDA for 72 h to analyze possible recovery.

Data Analysis

Dot plots representing forward scatter light (FSC) versus side scatter light (SSC) were obtained during measurements. FSC is measured in the plane of the beam and gives relative information on cell size. SSC is measured at 90° to the beam and can provide information on cell granularity. Dot plot analysis of FL1 versus FL2 was applied to determine the fluorescence properties of the population. Table 1 describes the gate designation of stained cells. Residual esterase activity following PL treatments was calculated using Eq. (1), in which the post pulsed light enzyme activity of the population was set in relation to the activity of untreated cells. Only cells solely labeled by F (in gate 1) were considered in the activity (%$EA_{overall}$) of the whole population. For this

Table 1 Gate designation of microorganism cells stained with FDA and PI

Gate #	Fluorescence properties of cells collected in each gate	Possible involved cellular mechanism
1	F^+PI^-	Active esterase, intact membrane
2	F^+PI^+	Active esterase, membrane minimally damaged
3	F^-PI^-	Esterase activity not detectable (or F extruded out of the cells), intact membrane
4	F^-PI^+	Esterase activity not detectable, membrane compromised

purpose, the esterase activity of the population gated in quadrants #2 and #1 was taken into account.

$$\%\mathrm{EA} = \left(\frac{\#1_{\mathrm{PL}}}{\#1_{\mathrm{Ctrl}}}\right) \times 100 \quad (1)$$

$$\%\mathrm{EA}_{\mathrm{overall}} = \left(\frac{\#1_{\mathrm{PL}} + \#2_{\mathrm{PL}}}{\#1_{\mathrm{Ctrl}} + \#2_{\mathrm{Ctrl}}}\right) \times 100 \quad (2)$$

where %EA is the percentage of residual enzymatic activity in response to a particular irradiation treatment, $\#1_{\mathrm{PL}}$ and $\#2_{\mathrm{PL}}$ are the percentages of population in gates 1 and 2 following PL treatment, respectively, and $\#1_{\mathrm{Ctrl}}$ and $\#2_{\mathrm{Ctrl}}$ are the percentages of population in gates 1 and 2 prior to PL treatment, respectively. The increase in permeabilized cells (PI uptake) was calculated as the difference between Log $\mathrm{No}_{\mathrm{PI}}$ and Log N_{PL} (Log $\mathrm{No}_{\mathrm{PI}}$−Log N_{PL}), where $\mathrm{No}_{\mathrm{PI}}$ is the number of cells that had not taken up PI (i.e., cells that did not show red fluorescence) before treatment and N_{PL} is the number of cells that had not taken up PI (gate 4) following treatment.

Transmission Electron Microscopy

One milliliter of peptone water (pH 3.5) or commercial apple juice (pH 3.5) inoculated with *S. cerevisiae* and exposed to PL for 10, 40, and 60 s was centrifuged at 11,180×*g* for 5 min, and the supernatant was discarded. The yeast cells were resuspended in 2 mL of glutaraldehyde (2.5 g/100 mL) in 0.1 M phosphate buffer, pH 7.1 for 2 h at 4 °C, washed with 0.1 M phosphate buffer and post-fixed with $KMnO_4$ (2 g/100 mL) in 0.1 M phosphate buffer for 17 h at 4 °C, washed well with distilled water, dehydrated with alcohol series, and embedded in Epon 812 (Shell Chemical Company, USA), during 48 h (Bolondi et al. 1995). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a microscope JEOL microscope model JEM 1200 EX II at 80 kV (JEOL, Japan). Nontreated cells were fixed in a similar manner and served as a control.

Results and Discussion

Survival curves of *S. cerevisiae* in commercial apple juice and pH 3.5 or 5.6 peptone water processed by PL at different doses are shown in Fig. 1. During PL treatments, the temperature of the pre-refrigerated samples increased with time as a consequence of the absorption of light by the liquid but it never reached lethal values (T<20 °C) according to the adopted process design (data not shown).

Inactivation curves in apple juice exhibited an almost sigmoid shape with a rapid first decay (~0.7 log cycle) before 10 s (11.9 J/cm^2) of PL exposure, followed by a pronounced shoulder with a slight slope, and then after 40 s of PL treatment, a second significant decay appeared reaching 3.9 log reduction cycles after 60 s (71.6 J/cm^2). The downward concavity exhibited after 10 s (11.9 J/cm^2) would indicate an accumulative damage in the more resistant microorganisms, while before 10 s (11.9 J/cm^2), sensitive cells were quickly destroyed.

S. cerevisiae inactivation curves in peptone water systems were characterized by a pronounced fall (~5.5 log cycles) during the first seconds of PL treatment, with the inactivation slope being a little more pronounced at the lowest pH. No colonies were detected after 60 s (71.6 J/cm^2) of PL exposure (Fig. 1). The greater PL effectiveness reached in peptone water could be related to the absorption of light in the UV range. Apple juice has much higher absorbance of light than peptone water in the range

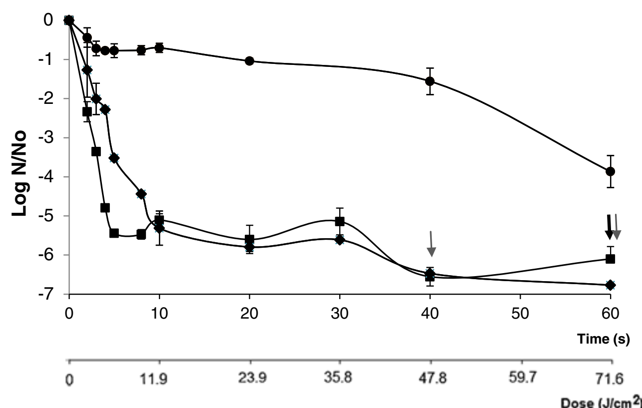

Fig. 1 Survival curves of *S. cerevisiae* in peptone water, pH 3.5 (*square*) or pH 5.6 (*diamond*), and apple juice (*circle*) treated with PL for 60 s (71.6 J/cm^2). Undetectable counts (<3 CFU/mL) for peptone water pH 3.5 (*gray arrow*) and pH 5.6 (*black arrow*). *Whiskers* Mean ± standard deviation of three replicates, ranging from 0.01 to 1 log cycle

of wavelengths from 200 to 400 nm, the region that is often credited as having the maximum bactericidal effect (Ferrario et al. 2012). It is well known that the disinfection process may be less effective in high absorption matrixes as contact between photons and microorganisms is impaired (Gómez-López et al. 2007).

Both pH and absorbance of the matrixes influenced PL efficiency (Fig. 1) but the last one was more relevant. The prevailing role of the liquid substrate absorption properties above its pH in the efficiency of PL treatment was also reported by Sauer and Moraru (2009), who observed that PL treatment (doses up to 13.1 J/cm^2) in Butterfield's phosphate buffer (pH 6.8) with low absorbance of UV light was more efficient than in apple juice and cider (pH~4.0), liquid substrates with high absorbance around λ of 254 nm.

Light scattering data for *S. cerevisiae* in peptone water systems and apple juice before and after PL exposure at the highest dose assayed (60 s, 71.6 J/cm^2) are shown in Fig. 2. Untreated *S. cerevisiae* cells (Fig. 2 a1, a2, and a3) in all media produced unimodal clustering with respect to SSC and FSC, extended practically from small to high sizes and granulometries, although more concentrated in the range from smaller to intermediate values. Cells showed great heterogeneity with respect to their size and granularity. This could be attributed, at least partially, to the different orientation of the elongated cells as they travel through the laser beam generating different granularity and size (Hewitt and Nebe-Von Caron 2001). After PL treatment (Fig. 2 b1, b2, and b3), more uniform distribution with respect to FSC and SSC signals was obtained, and the major population shifted to slightly lower forward and sideward scatter values, indicating that cells became more symmetric in shape, with smaller size and granularity. This light scattering pattern suggests that cells deform and/or shrink after 60 s of PL exposure and that the refractive index of the individual cells decreased possibly by leakage. As it will be shown in this work, this assumption had been partially justified by TEM observations, which demonstrated more rounded cells and cells that lost partially or totally (i.e., "ghost" cells) their content.

Figure 3 shows dual parameter density plots of the green fluorescence (*y*-axis) and the red fluorescence (*x*-axis), monitoring the ability of *S. cerevisiae* cells in peptone water systems or apple juice to accumulate and retain F as an indicator of membrane integrity and enzyme activity and uptake of PI to assess membrane damage.

Untreated *S. cerevisiae* cells suspended in peptone water systems or in apple juice and stained with FDA (Fig. 3a–c; 0 s) showed a similar pattern in their fluorescence labeling properties, although not all cells yielded green fluorescence and appeared in quadrant 1. This was mainly observed in peptone water with pH 5.6 (56.6 % cells) and in apple juice (57.6 % cells), possibly due to the active extrusion of F outside the cell mediated by an ATP-driven transport system and consequently the lack of green fluorescence. This probe efflux is an additional indicator of the metabolic performance of the cell, a vitality marker (Bunthof et al. 2000). Exposure of *S. cerevisiae* cells to PL at increasing doses resulted in a gradual shift of cells from quadrant 1 (cells with esterase activity and intact membrane) to quadrant 4 (cells with permeabilized membranes). PL treatment destroyed membrane integrity allowing PI to penetrate cells. The PI probe continued accumulating in the inner of the cell, binding to DNA and causing higher concentration of red fluorescence. Finally, upon 60 s of PL application, PI-stained cells (quadrant 4) predominated in a range between 87.0 % in apple juice and more than 98 % in both peptone water systems, while nonsignificant fractions were detected in the remaining quadrants.

This behavior was well correlated with the inactivation curve determined by the plate count method shown in Fig. 1. According to the survival curves in peptone water systems, after 5–10 s (6.0–11.9 J/cm^2) of PL treatment, *S. cerevisiae* plate counts were reduced in approximately 5.5 log cycles (Fig. 1), being in accordance with the great decline in a subpopulation with green fluorescence (Fig. 3a, b; quadrant 1), and viable yeast cells could not be detected at 20 s (47.8 J/cm^2) of PL treatment. At this treatment time, a large subpopulation of yeast cells (87.0–99.3 %) could be distinguished in the density plots (quadrant 4) corresponding to dead cells (uptake of PI) and practically none of the yeast cells (0.0–2.0 %) retained F (quadrant 1), except for apple juice where 10.8 % of cells was distinguished in gate 2 (cells double stained). In apple juice, 60 s of PL treatment only reduced the yeast count by 3.8 log cycles (Fig. 1), leaving a resistant subpopulation evidenced for a less proportion of damaged cells (87.0 %) compared with peptone water systems (Fig. 3a–c; 71.6 J/cm^2).

The shift from quadrant 1 to quadrant 4 was faster in peptone water with pH 3.5. For example, for 5–10 s of PL treatment, the percentage of cells stained with PI (Fig. 3a, quadrant 4) ranged between 74.8 and 98.9 %. In peptone water with pH 5.6, the change was more gradual. It is possible that the incorporation of an additional stress factor like the decrease of pH was able to accelerate this shift. Paparella et al. (2008) reported that *L. monocytogenes* viable cells (quadrant 1) treated with antimicrobials (oregano, thyme, and cinnamon essential oils) were reduced faster by the addition of a complementary stress factor like NaCl (2–15 % *w*/*v*).

After 5 s of PL treatment (3.5 log reduction of viable cells), only 43.6 % of cells were PI-stained and a significant proportion (32.9 %) of double stained cells were found (Fig. 3b, quadrant 2). Red fluorescence increased in the subpopulation gated in quadrant 2, indicating that cytoplasmic membrane integrity was being affected while

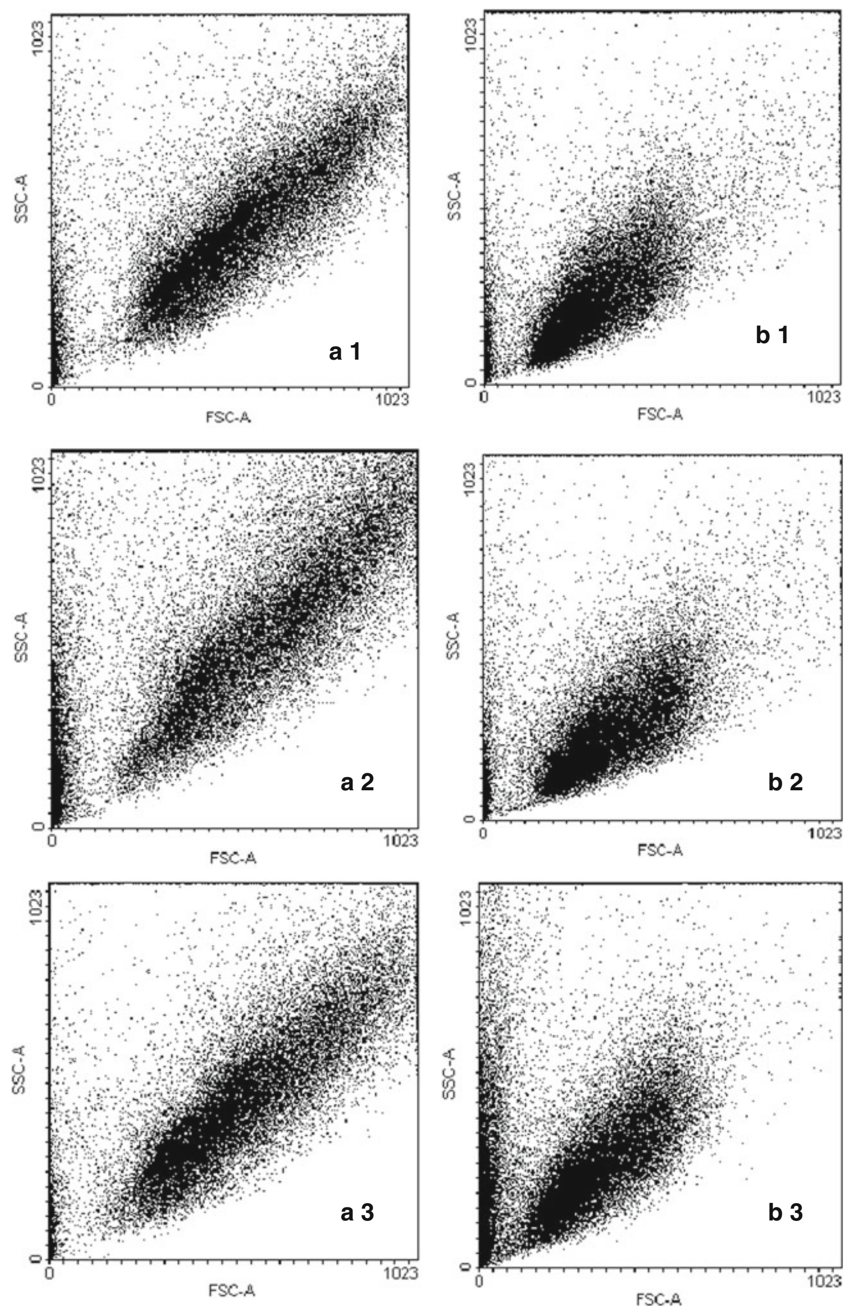

Fig. 2 Dot plot representing forward scatter light (FSC) versus side scatter light (SSC) of *S. cerevisiae* KE162 cells in peptone water or apple juice treated or not with PL. *a* Untreated cells; *b* PL-treated cells (60 s); *1* peptone water, pH 3.5; *2* peptone water, pH 5.6; *3* apple juice

accumulating PI, but simultaneously, cells retained esterase activity, hydrolyzing FDA fluorochrome. This stressed or injured subpopulation has been denoted as "viable but not cultivable cells" because they have metabolic activity but they cannot be detected by conventional cultivation assays (Ueckert et al. 1995; Schenk et al. 2011). Cell sorting of these fractions showed that cells could not be able to recover in peptone water with pH 3.5 (5 and 10 s PL), at least in the time frame of the experiment, while there was a slight recovery in peptone water with pH 5.6 (1 s (1.2 J/cm^2) PL, 1.6×10^3 CFU/mL) and apple juice (1 and 5 s (1.2; 6.0 J/cm^2) PL, 1.5×10^3 CFU/mL; 10 s (11.9 J/cm^2) PL, 4×10^2 CFU/mL). The presence of this sublethally injured population could seriously affect shelf life in juices treated by low PL dose (Zhao et al. 2011). *S. cerevisiae* cells with double staining (PI$^+$F$^+$, quadrant 2) in apple juice (Fig. 3c) reached a maximum value (67.3 %) at 5 s (6 J/cm^2) of PL treatment, while in peptone water with pH 3.5 and 5.6, the greater proportion (23.7 and 43.8 %, respectively) of double stained cells was reached at 1 s (1.2 J/cm^2) of PL treatment.

By culturing in selective and nonselective agar, Pataro et al. (2011) also reported the presence of sublethally injured cells with compromised membrane of *E. coli* and *L. innocua* inoculated in PL-treated apple and orange juices. In agreement with our findings, the proportion of this subpopulation depended on the energy dose delivered and the type of

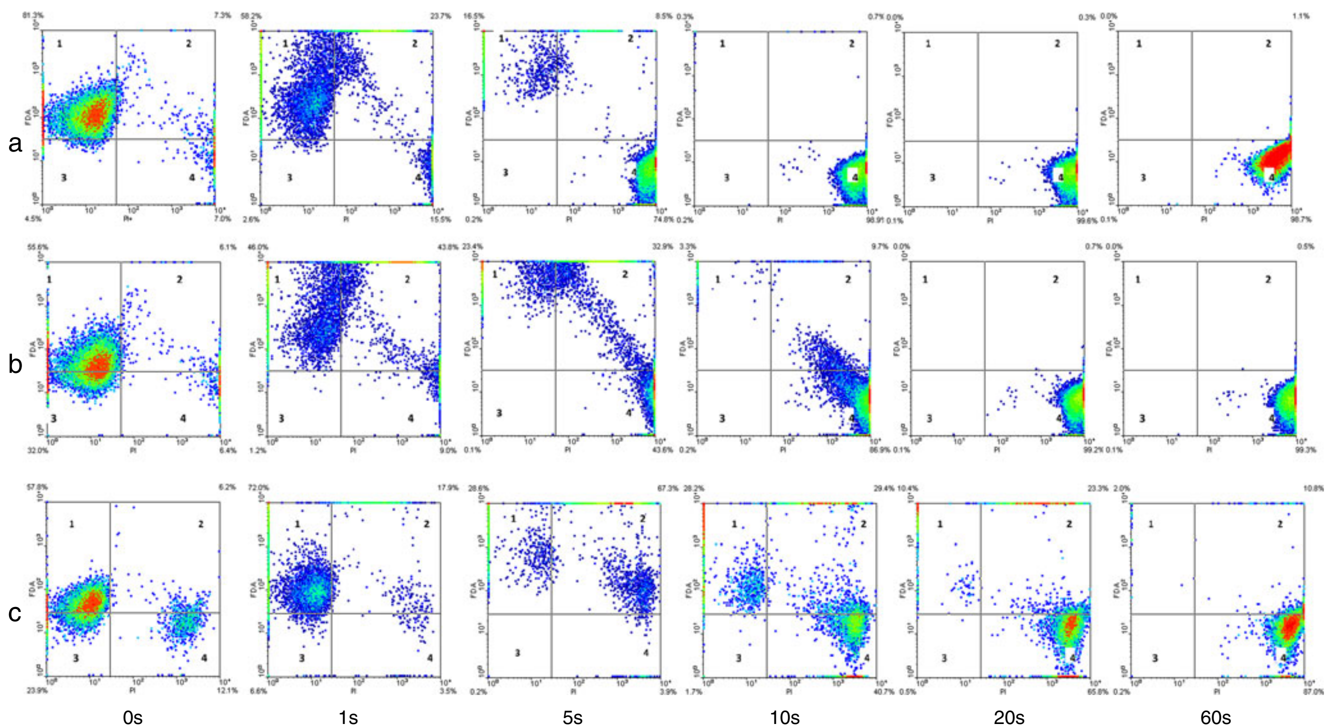

Fig. 3 Fluorescence density plots of *S. cerevisiae* in response to staining with FDA and PI after PL treatment at different exposure times (1.2, 6.0, 11.9, 47.8, and 71.6 J/cm^2 or 1, 5, 10, 20, and 60 s of PL exposure): **a** peptone water, pH 3.5; **b** peptone water, pH 5.6; and **c** apple juice. The percentage of microbial populations which falls in each quadrant can be found in the four edges of each plot

matrix, the orange juice having more absorptivity where more recovery was found.

The increase in the number of *S. cerevisiae* cells in gate 4 (PI-stained population), expressed as log reductions as a function of treatment time, is shown in Fig. 4a. Residual esterase activities following PL treatments calculated using Eqs. (1) and (2) are shown in Fig. 4b. In peptone water systems, the number of cells that took PI increased and the residual esterase activity decreased faster up to 20 s of PL treatment (23.9 J/cm^2) remaining almost constant until the maximum dose assayed (60 s; 71.6 J/cm^2). In those systems, a similar number of permeabilized cells and percentage of esterase activity gated in quadrant #1 or in quadrants #1 + #2 were achieved for PL exposures beyond 23.9 J/cm^2 (Fig. 4a, b).

On the contrary, *S. cerevisiae* suspended in apple juice showed scarce membrane permeabilization throughout the entire range of PL doses assayed compared with peptone water systems. This fact demonstrated that membrane permeabilization was dependent on the type of medium. This fact was in accordance with a less pronounced decline in the esterase activity level and the results from the culturing method shown in Fig. 1.

After PL treatment, scarce differences were observed among esterase activities at all evaluated doses in peptone water systems, but in apple juice, relevant differences were found between esterase activity gated in #1 and overall esterase activity gated in #1 + #2 (Fig. 4b). These observations confirmed that in apple juice, there were a greater proportion of post-PL-treated cells with minimal damage, capable of growing in a culture media, than in peptone water systems, correlating well with a greater proportion of survivors observed in the inactivation curve (Fig. 1).

The evolution of permeabilized cells (nonviable cells) and the percentage of overall esterase activity (viable cells) were plotted against the increase of nonviable cells by the plate count method to analyze the relationship between viable and nonviable cells determined by both FCM and plate count methods (Fig. 4c, d). While in apple juice only 4 log of nonviable cells was reached, for peptone water systems, a rapid increase of nonviable cells was encountered, indicating a biphasic type behavior (Fig. 4c). It is worthy to note that more cells were determined as nonviable as cells with compromised membrane increased. For example, a 71.6-J/cm^2 PL exposure in apple juice resulted in 4 log reductions of *S. cerevisiae* cells, but only a 0.83 log increase of permeabilized cells was observed. Regarding peptone water systems, although there was a change in the relationship slope, when observing, for example, a 6 log increase in nonviable cells by plate count method, only a 2 log increase of permeabilized cells was detected (Fig. 4c). These results are in agreement with those reported by Aronsson et al. (2005), who also found a higher number of inactivated cells in relation to the permeabilized cells observed after

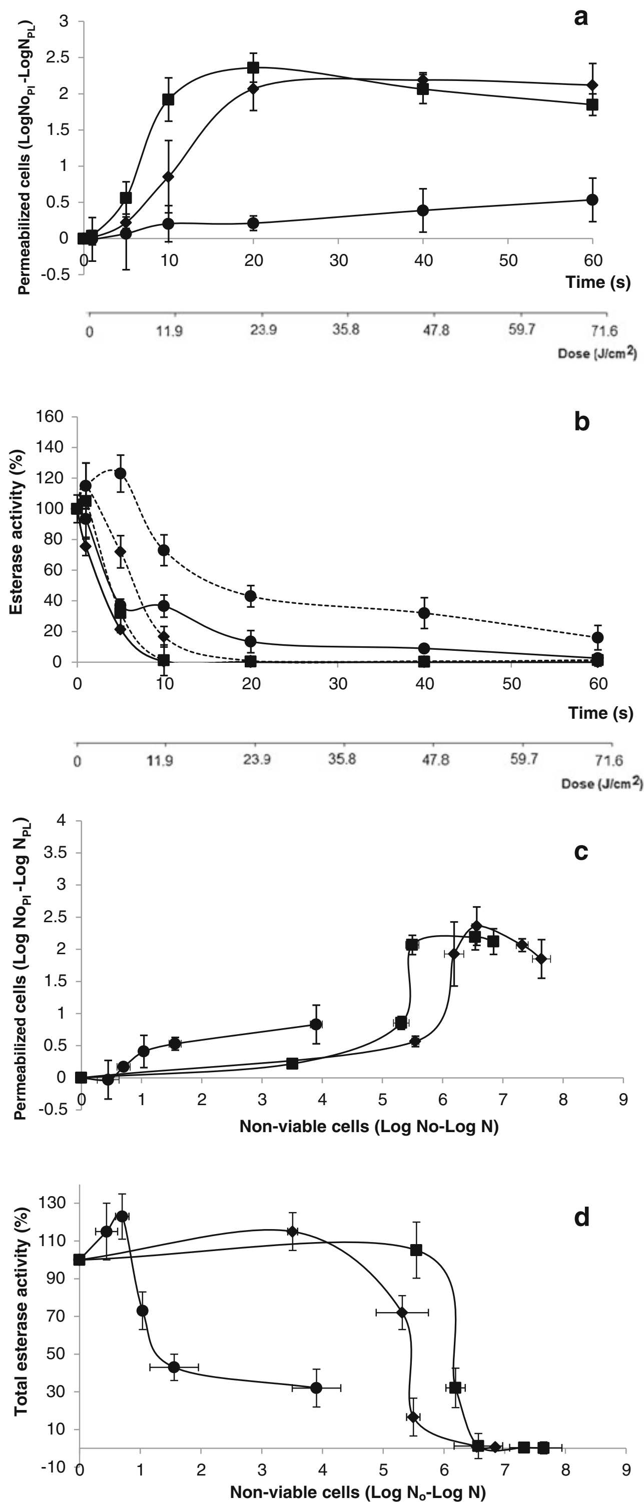

Fig. 4 **a** Permeabilized *S. cerevisiae* cells determined by PI uptake as a function of PL treatment time. **b** Relative changes of overall esterase activity, %$EA_{overall}$ (*dotted line*) or esterase activity in cells with intact membrane, %EA (*solid line*) as affected by time/dose of PL treatment (seconds). **c** Permeabilized cells determined by PI uptake as a function of nonviable cells determined by the plate count method. **d** Relative changes of overall esterase activity as a function of nonviable cells determined by the plate count method. Peptone water pH 3.5 (*square*) or pH 5.6 (*diamond*) and apple juice (*circle*). *Whiskers* Mean ± standard deviation of three replicates

treatment with pulsed electric fields. They attributed this phenomenon to a possible reversible permeabilization, which would not be revealed by the PI uptake. However, the damage would be severe enough to cause molecular transport across the membrane. In contrast, changes in overall esterase activity seem to correlate well with the increase in nonviable cells by the plate count method. This parameter was completely lost at high doses for both peptone water systems, while only a 30 % of the enzyme activity remained in apple juice (Fig. 4d). At low doses (10 s, 11.9 J/cm^2), *S. cerevisiae* cells in apple juice maintained almost the total metabolic activity, while in peptone water pH 5.6, 70 % of its activity was detected, and in peptone water pH 3.5, only 30 % (Figs. 1 and 4d). These results suggest that the loss of metabolic activity as a result of PL exposure would not be a reversible phenomenon as the membrane permeabilization.

Transmission electronic micrographs of untreated and PL-treated yeast cells in peptone water with pH 3.5 or in apple juice for 11.9, 47.8, and 71.6 J/cm^2 of PL exposure are exhibited in Fig. 5. As shown in Fig. 5a–c, healthy cells showed a characteristic ellipsoidal shape. Cell structures, such as walls, organelles, and membranes, appeared intact, both in cells suspended in peptone water and in apple juice. All untreated cells exhibited high electronic density and a continuous and smooth cell wall, and buds, bud scars, and birth scars were clearly observed. After PL exposure, many distinct structural changes in the yeast cells were visualized, evidencing various PL targets other than membranes (Fig. 5d–l). Overall, these changes increased in severity as the PL dose increased. Similar structure modifications appeared at lower doses when cells were suspended in peptone water compared with apple juice. Many cells presented raised and expanded vacuoles (Fig. 5h, j, l). The cytoplasm of cells exposed to PL during 10 s (11.9 J/cm^2) appeared to be less differentiated (Fig. 5e). Cells looked deformed (Fig. 5k) or with more rounded unnatural shape (Fig. 5g). A disrupted cytoplasm appeared slightly separated from the wall to the inner in some cells (Fig. 5f). Efflux of intracellular material can be visualized to originate from puncture sites (Fig. 5i, j) at the cell surface and from discontinuity of plasma membrane with leakage of content (Fig. 5k). PL treatment resulted in occasionally swollen walls (Fig. 5g). Many cells lost the inner content showing themselves empty (Fig. 5i), while others seemed to lose their cell wall (Fig. 5j). In general, PL-treated cells appeared with less electronic density than those without treatment. As PL treatment time increased, the inner content of the cell looked more coagulated, vacuolated, and coarse, with disruption of organelles and a generalized rupture of membranes, without any possibility of distinguishing any cell structure (Fig. 5g, h, i).

Takeshita et al. (2003) studied the damage of *S. cerevisiae* IFO2347 in model media by pulsed light and

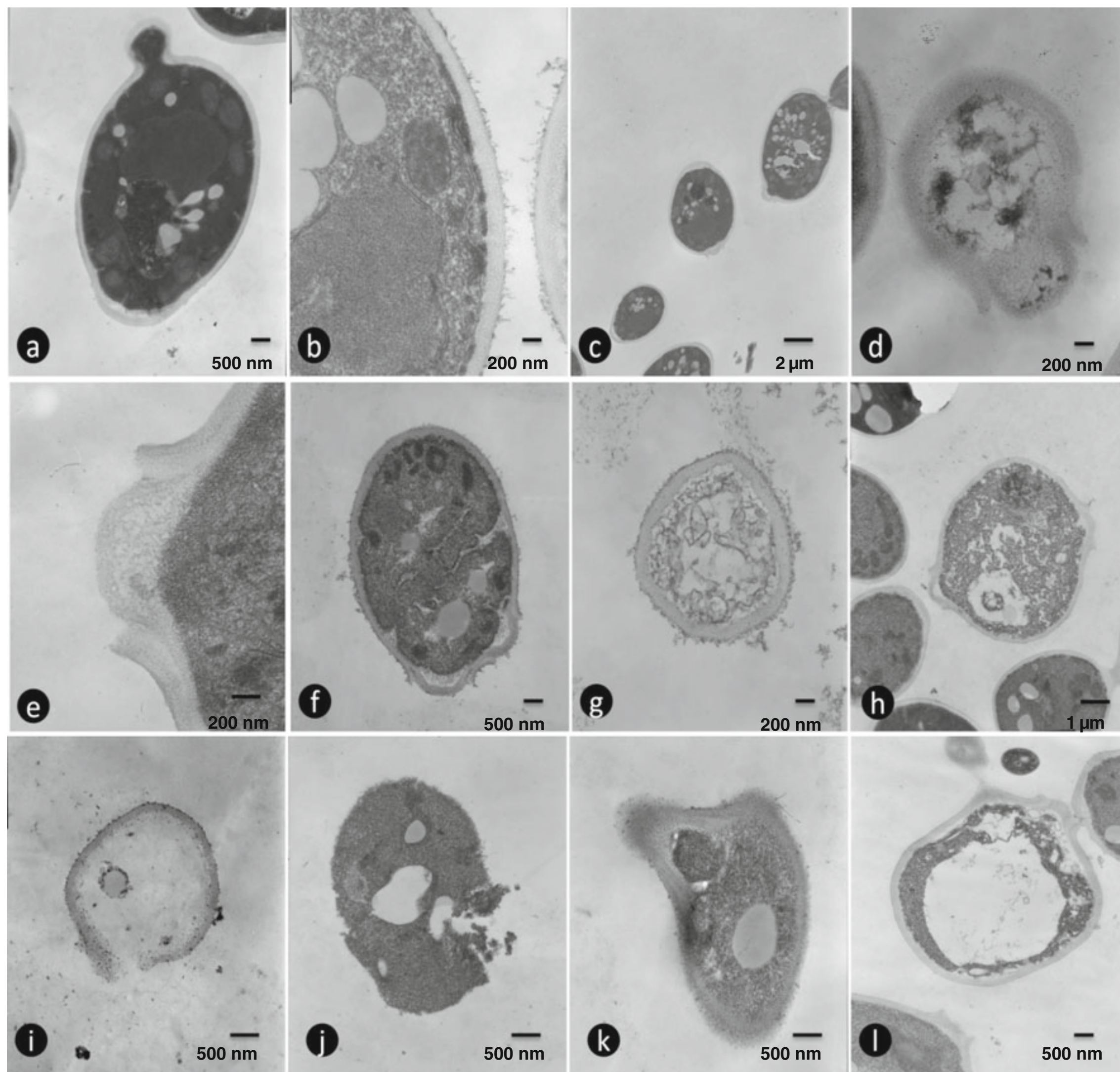

Fig. 5 TEM images of *S. cerevisiae*. **a**–**c** Untreated cells; **d**–**f** 10 s of PL treatment of cells; **g**, **h** 40 s of PL treatment of cells; **i**–**l** 60 s of PL treatment of cells. **a** General aspect of intact daughter and mother yeast cells showing well-defined organelles and membranes. **b** Detail of an electronically dense cell wall and continuous plasma membrane. **c** Group of intact cells with similar general aspect. **d** Broken cell wall and plasmalemma and inner material radiating from the cell. **e** Single puncture site at the cell surface with efflux of inner material. **f** Deformed cell with electronic dense walls; cytoplasm material separated from the cell wall. **g** Discomposed inner structure but cell wall and cytoplasmic membrane not broken. **h** Deformed cell with disrupted inner structure rounded by cells without ultrastructural modifications. **i** Remains of cell wall with scarce inner material. **j** Cell content without wall. **k** Puncture sites and distortion of cell shape. **l** Deformed cell with important degradation, efflux of inner material and vacuolization. Media: **a**, **c**, **d**, **e**, **g**, **i**, **j**, **k**—peptone water with pH 3.5; **b**, **f**, **h**, **l**—apple juice. Scale: **a**, **f**, **i**, **j**, **k**, **l**—500 nm; **b**, **d**, **e**, **g**—200 nm; **c**—2 μm; **h**—1 μm

continuous UV light using TEM among other techniques. Although the authors reported scarce number of images, in accordance with the results presented in this study, they observed alteration of cell shape (yeasts turned more rounded than control cells), plasma membrane distortion, and an increase in vacuole size. Otherwise, Krishnamurthy et al. (2008b) also reported cell wall damage and content leakage in *S. aureus* cells treated with PL. They remarked the importance of the photothermal effect which could cause a localized heating of the cell content and its consequent vaporization, even when the bulk temperature would not increase. In this work, this effect could be responsible for the damage observed in cells whose inner content was coarse (Fig. 5d, g, h).

The greater proportion of nonviable cells compared to those with compromised membrane is in agreement with the results observed by TEM. A great proportion of cells appeared with apparently intact wall and membrane but with their inner content disrupted. The structural changes observed in the TEM micrographs, such as expanded vacuoles,

distortion of cell shape, and disruption of inner material while preserving cell wall and membrane, strongly suggested that they contributed to the damage caused by PL irradiation and, consequently, to a significant reduction of viable population. On the other hand, permeabilized cells detected by FCM could be associated to cells exhibiting loss of wall or inner content and puncture sites with efflux of material (p.e. Fig. 5d, i, j).

Aronsson et al. (2005) observed the same behavior pattern when *E. coli* ATCC 26, *L. innocua* ATCC 33090, and *S. cerevisiae* CBS 7764 membrane permeabilization (by uptake of PI) caused by pulsed electric field (2 μs; 30 kV/cm) were studied by FCM. They registered 2.8 log reductions in viability, but only 1.9 log reductions in membrane integrity. A similar behavior was observed in the case of *L. innocua*, but the relationship in the case of *S. cerevisiae* was different; the number of cells with a permeated membrane was relatively high (1.9 log reduction) at low levels of inactivation (0.5 log reduction).

Conclusions

FCM and TEM studies contributed to a better understanding of the inactivation mechanism of PL. The inactivation rate of *S. cerevisiae* by PL treatment exhibited a clear dependence on the type of media and pH. PL was markedly more effective in peptone water (pH 5.6 or 3.5) systems than in apple juice (pH 3.5) probably due to an increased absorbance of apple juice which reduces the effective contact between light photons and microorganisms. In peptone water, a reduction of pH from 5.6 to 3.5 provoked a significant increase in the observed yeast inactivation in the first seconds (up to 8 s) of PL treatment. However, absorption of light limited the inactivation reached in apple juice with the same pH value (pH 3.5). TEM observations revealed many PL dose-dependent changes in cell structure, indicating that the loss in viability was not only due to wall and plasma membrane damage, but also PL provoked a significant disorder mainly in the inner cells exposed to this agent. According to the results, the determination of viability from different physiological and metabolic parameters is important to evaluate process effects on microorganisms in apple juice. PL treatment induced sublethal injury of *S. cerevisiae* cells at low doses (up to 12 J/cm^2, 10 s), showing that the recovery of cells is dependent on the medium. Additionally, it was demonstrated that loss of capacity of forming colonies on agar as a consequence of PL treatment correlated with the absence of metabolic activity, but much more log reductions of viable cells by the CFU method were obtained in comparison with the log increase in permeabilized cells for a given condition of PL treatment.

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