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Kinetics of *Escherichia coli* inactivation employing hydrogen peroxide at varying temperatures, pH and concentrations

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ABSTRACT

The effectiveness of hydrogen peroxide on the destruction of planktonic cells of *Escherichia coli* at different temperatures, pH and sanitizer concentrations was studied. Inactivation kinetics of *E. coli* exhibited a clear dependence on hydrogen peroxide concentration, pH and temperature. A Weibullian mathematical model successfully described the inactivation curves. Quantitative kinetic results obtained allowed to identify various combinations H_2O_2 concentration–pH–temperature for 5-log cycles reduction of *E. coli*. Flow cytometry analysis revealed induced H_2O_2 cytoplasm membrane damage. TEM observations indicated that H_2O_2 treatment resulted in rupture of outer and cytoplasm membranes and a more uniform granularity.

1. Introduction

Fruit and vegetable surfaces are exposed to natural contaminants (soil, insects, animals and/or humans) during growing or harvesting and in the processing plant. Contaminations of fresh and minimally processed fruit and vegetable and foodborne illnesses have been linked to *Escherichia coli* O157:H7, *Salmonella* spp. *Shigella* and *Listeria monocytogenes*, among others (Beuchat, 1996; FDA, 2001). Chlorination of wash water up to 200 ppm is routinely applied to reduce microbial contamination in processing lines. However, the use of chlorine is of concern due to the potential formation of harmful byproducts and the reduced antibacterial effect (less than 2–3 log reductions of native microflora) (Beuchat, 2000; Sapers, Miller, Pilizota, & Mattrazzo, 2001). Thus, there is much interest in developing safer and more effective sanitizers (Zhang & Farber, 1996; Ukuku, Pilizota, & Sapers, 2004).

The use of hydrogen peroxide (H_2O_2) has been proposed as an alternative for decontaminating fruit and vegetables due to its low toxicity and safe decomposition products. It is effective against a wide spectrum of bacteria, yeast, molds, viruses and spore-forming organisms (Cords, Burnett, Hilgren, Finley, & Magnuson, 2005). H_2O_2 rapidly degrades into oxygen and water (nontoxic products) upon contacting organic material, thus having no long term residual activity. H_2O_2 is classified as Generally Regarded as Safe (GRAS) for use in food products as a bleaching agent, oxidizing and reducing agent and antimicrobial agent (Sapers & Simmons, 1998).

H_2O_2 has been shown to damage bacterial proteins, DNA and cellular membranes of microbial cells and to remove protein from the coat of the bacterial spore (Juven & Pierson, 1996). Its toxicity appears to be not due to its oxidative properties in its molecular state but to the formation of other more reactive oxidant species (hydroxyl radical, superoxide and singlet oxygen) in oxygen and H_2O_2 reduction.

Various factors have been reported to greatly influence the antimicrobial activity of H_2O_2, such as sanitizer concentration, pH and temperature, contact time and produce surface (Beuchat, 2000; Cords et al., 2005; Gil, Selma, López-Gálvez, & Allende, 2009). However, systematic studies and quantitative kinetic results that could help understanding the effect of these factors on the effectiveness of the disinfection process are very scarce. Recently, we published a work concerning the inactivation kinetics of *E. coli* ATCC 35218 in hydrogen peroxide solutions at different concentrations (0–3.00% w/v) and pH values (3.0–7.2) working at a constant temperature of 25 °C (Raffellini, Guerrero, & Alzamora,

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2008). Effectiveness of H_2O_2 solutions against *E. coli* ATCC 35218 appeared to improve at both higher H_2O_2 concentrations and more acidic pH values, ranging the times necessary to obtain 5-log reductions between 1 min and 9 min approximately. Inactivation kinetics was successful modeled by using a Weibullian type distribution of resistances. Other authors (Labas, Zalazar, Brandi, & Cassano, 2008) modeled the inactivation reaction of *E. coli* ATCC 8739 at pH 7, 20 °C but they used very low concentrations (15–300 ppm H_2O_2) and large exposure times, reaching very low inactivation levels.

FDA (2001) recommends the use of indicator microorganisms for validating effectiveness of microbial control measures. *E. coli* is usually employed as indicator of fecal contamination in raw fruit and vegetables because of its presence in raw vegetables has been linked to the presence of pathogens such as *Salmonella* spp. (Sagoo, Little, Ward, Gillespie, & Mitchell, 2003).

The purpose of this study was to examine and to model the effectiveness of hydrogen peroxide on the destruction of planktonic cells of *E. coli* at different temperatures, pH and sanitizer concentrations. This research's significance was lying in the quantitative assessment of the effect *per se* of temperature, pH and hydrogen peroxide concentration during free floating cell inactivation. Several additional variables influencing sanitization process, such as surface attachment of microorganisms, surface characteristics and presence of organic material other than the bacteria as well as studies with other pathogens, would be explored in a subsequent work to optimize hydrogen peroxide treatment.

2. Materials and methods

2.1. Organisms, culture conditions, media and inoculum

E. coli ATCC 35218 was used in experiments with a single strain. This microorganism had yet been employed in previous studies to compare the toxicity of hydrogen peroxide, hydroxyl radicals and other oxidant agents (Watts, Washington, Howsawkeng, Loge, & Teel, 2003), as well as to evaluate the effect of pH on the inactivation produced by H_2O_2 (Raffellini et al., 2008). In experiments with mixed-strain cultures, *E. coli* ATCC 8739, *E. coli* ATCC 11229 and *E. coli* ATCC 25922 were used in addition. These strains are commonly used as indicators in internationally standardized methodologies for the evaluation of antimicrobial activity (Block, 2001).

The stock cultures were maintained at −20 °C in Tryptic Soy Broth (TSB; Difco Laboratories, Detroit, MI, USA) supplemented with 10% v/v glycerol (Merck KGaA, Darmstadt, Germany). Preceding use of each strain as inoculum for inactivation experiments, a loopful from the corresponding frozen stock culture was transferred in TSB and statically incubated 18 h at 37 °C (±1 °C). After a second transfer under similar conditions, this culture was used to inoculate (0.5% v/v inoculum) fresh TSB. Cells were incubated at 37 °C to stationary-phase (6 h), harvested by centrifugation at 3000*g* in a Rolco centrifuge (Rolco S.A., Buenos Aires, Argentina), washed twice with sterile 0.85% w/w physiological saline solution and concentrated 10-fold. Final microorganism concentration of this suspension ($\approx 5 \times 10^9$ CFU/ml) was determined in duplicate by pour plating onto Plate Count Agar (PCA, Biokar Diagnostics, Beauvais, France) followed by 48 h of incubation at 37 °C before colonies were counted.

The test culture was either the individual *E. coli* ATCC 35218 or a pool of equal volumes of the cultures of the four test organisms in the sterile physiological saline solution.

2.2. Inactivation experiments

Inactivation experiments of *E. coli* ATCC 35218 were carried out at 12.5 °C, 25.0 °C, 37.5 °C and 50.0 °C in 250 mL Erlenmeyer flasks with 99 mL of sterile citric acid–Na_2HPO_4 buffer solutions (pH 3.0; 5.8 and 7.2) containing different H_2O_2 concentrations (0–6.00% w/v). The solutions were prepared no more than 30 min before use and placed in a water bath (Thermomix 1460 Braun, Germany) at 12.5 °C, 25.0 °C, 37.5 °C or 50.0 °C ± 0.5 °C to reach the desired temperature before inoculation. At pH 7.2, tests were conducted only at 25 °C and 50 °C, due to the reduced effectiveness of hydrogen peroxide treatment at this pH reported in a previous work (Raffellini et al., 2008). *E. coli* was challenged with the sanitizing solutions at designated concentrations, pH and temperatures by adding 1 mL of the inoculum prepared as indicated in the first section to 99 mL sanitizing solution (final cell concentrations were between 3×10^7 and 5×10^7 CFU/mL). After inoculation, 1000-μL aliquots were periodically collected, immediately neutralized with sterile buffered (pH 7.0) 4% w/v $Na_2S_2O_3$ $5H_2O$ (Merck KGaA, Darmstadt, Germany) solution to stop the inactivation reaction, serially diluted in 0.1% peptone water (Difco Laboratories, Detroit, MI USA) and pour plated in duplicate using PCA. The plates were counted following incubation at 37 °C for 48 h. Survival curves were represented as log surviving fraction versus time. Three independent experiments were carried out and three replicate samples were used per each treatment time.

Inactivation experiments of mixed-strain suspensions were performed at pH 5.8 with 1.00% w/v H_2O_2 at 25.0 °C and 0.50% w/v H_2O_2 at 50.0 °C according to the protocol previously described for the single strain test.

The antimicrobial agent solutions were prepared from 30% peroxide hydrogen solution (Merck KGaA, Darmstadt, Germany). The buffer solutions were prepared from 0.1 M citric acid monohydrate (J.T. Baker, Phillipsburg, USA) and 0.2 M Na_2HPO_4 (M&B, Dagenham, England). Neutralizing solutions were obtained from $Na_2S_2O_3$ $5H_2O$ diluted in 0.25 M KH_2PO_4 buffer adjusted at pH 7.0 with 1 N NaOH. NaOH and KH_2PO_4 were obtained from Merck Química Argentina (Buenos Aires, Argentina).

H_2O_2 levels were monitored by titration with 0.1 N potassium permanganate (Mallinckrodt, Phillipsburg, USA) immediately before and after each treatment. The pH was measured with a pH meter METTLER TOLEDO (model MP 220, Schwerzenbach, Switzerland).

All buffer solutions were prepared with deionized water obtained from a Milli-Q system (Model OM-140, Millipore, Billerica, USA).

2.3. Evaluation of toxicity and efficacy of neutralizing solution

The evaluation of toxicity of neutralizing solution on the *E. coli* strains, the neutralizer efficacy and the qualitative estimation of residual peroxide after neutralization were performed according to the protocol proposed by Raffellini et al. (2008). Briefly, to assess the neutralizer toxicity 1 mL of dilution of each *E. coli* strain ($\approx 10^7$ CFU/ml) was inoculated into 9.0 mL of sterile water or 9.0 mL of sterile buffered (pH 7.0) 4% w/v $Na_2S_2O_3$ $5H_2O$ solution and incubated at 25 °C for 30 min. Then decimal dilutions of each treatment were pour plated in duplicate PCA plates and incubated at 37 °C for 48 h. To evaluate the neutralizer efficacy 1 mL of each hydrogen peroxide solution (different concentrations and pH) was mixed with 9 mL of each sterile buffered neutralizing solution, challenged with each *E. coli* strain, and incubated at 25 °C for 15 min. Then decimal dilutions of each test were pour plated in duplicate PCA plates.

2.4. Mathematical modeling

2.4.1. Primary model of inactivation pattern

The Weibullian distribution was used as a model to describe the spectrum of resistances of the population to experimental factors (Peleg & Cole, 1998; Aragao, Corradini, Normand, & Peleg, 2007).

If each individual of a microbial population is inactivated at a specific time (t_{ci}) and considering t_{ci} as having a continuous distribution, the survival ratio $S(t)$ can be written as:

$$S(t) = \int_0^1 f[t, t_c(\phi)]d\phi \quad (1)$$

where t_c is the time at which the microorganism dies or loses its viability; $S(t)$ is the survival fraction, N/No (No is the initial number of the cells and N the number of the cells at time t) and f [t, $t_c(\varphi)$] is a function of the exposure time t and the fraction of organisms φ which share any given t_c.

Assuming t_c has a Weibullian type distribution, i.e.

$$\frac{d\phi}{dt_c} = bnt_c^{n-1}\exp(-bt_c^n) \quad (2)$$

where b and n are constants, the survival curves can be assumed to be the cumulative form of a temporal distribution of lethal events. Operating, the cumulative form of Weibullian distribution, after their logarithmic transformation, results:

$$\log S(t) = -bt^n \quad (3)$$

The Weibullian model has two parameters: the scale or nonlinear rate parameter b (with dimensions $time^{-n}$) and the dimensionless shape parameter n, which indicate the overall steepness and the shape of the survival curves respectively. The survival curves were fitted using Eq. (3) and Weibullian parameters (n, b) were obtained for each experimental condition. These values were then used to calculate the frequency distribution of resistances or sensitivities using Eq. (2), and the distribution's mode, t_{cm}; the mean, $\bar{t}_c$; the variance, σ^2_{tc}; and the coefficient of skewness, v_1

$$t_{cm} = [(n-1)/nb]^{1/n} \quad (4)$$

$$\bar{t}_c = \{\Gamma[(n+1)/n]\}/b^{1/n} \quad (5)$$

$$\sigma^2_{tc} = \left\{\Gamma[(n+2)/n] - (\Gamma[(n+1)/n])^2\right\}\Big/ b^{2/n} \quad (6)$$

and

$$v_1 = \mu_3/\mu_2^{3/2} \quad (7)$$

where:

Γ = gamma function

$$\mu_3 = \Gamma(1+3/n)/b^{3/n} \quad (8)$$

$$\mu_2 = \Gamma(1+2/n)/b^{2/n} \quad (9)$$

The distribution mode, t_{cm}, represents the treatment time at which the majority of population dies or inactivates. The mean, $\bar{t}_c$, corresponds to the inactivation time on average with its variance, σ^2_{tc}. The coefficient of skewness, v_1, represents the skew of the distribution.

Non linear regressions were carried out using the STATGRAPHICS Plus for Windows 3.0® Package (StatPoint Inc., Hendon, VA, USA). Internal validation of the model was carried out through the comparison between observed and predicted values and the evaluation of the adjusted coefficient of determination (R^2_{adj}).

The significance of the differences of survival curves of single and mixed strains of *E. coli* was determined by means of ANOVA and the *F*-test between the overall regression and the sum of the individual regressions.

2.5. Time to reach 5-log reductions in the microbial population

Response surface methodology was employed to model the time of exposure needed to reach 5-log reductions in *E. coli* ATCC 35218 population (single strain test, pH 5.8). An incomplete three level two factor design was adopted (Gacula & Singh, 1984). It was assumed that a mathematical function, φ, exists for the response variable, Y (time to 5-log reductions, TR5L), in terms of two independent process variables X_1 (temperature) and X_2 (% w/v H_2O_2):

$$Y = \varphi(X_1, X_2) \quad (10)$$

The two independent variable levels were coded as −1 (lowest level), 0 (middle level) and +1 (highest level). Their actual values (X_i), and the corresponding coded values (x_i) are given in Table 1. The complete design consisted of 13 experimental trials which included five replications of the center point ($x_1 = x_2 = 0$). For each experimental condition, the time required to reduce by 5 log cycles the initial population of microorganisms was estimated from the kinetic parameters of primary Weibullian type model.

To approximate the function φ, a second order polynomial on two variables was used:

$$Y = \beta_o + \sum_{\mu=1}^{2}\beta_\mu x_\mu + \sum_{\mu=1}^{2}\beta_{\mu\mu}x_\mu^2 + \sum_{\mu=1}^{2}\sum_{j=\mu+1}^{2}\beta_{\mu j}x_\mu x_j \quad (11)$$

where Y is the response (time to 5-log reductions), $x_{\mu j}$ are the coded independent variables, linearly related to X_μ, and β_0, β_μ, $\beta_{\mu\mu}$ and $\beta_{\mu j}$ are the regression coefficients.

In order to select the best equation, analysis of variance, test of lack of fit, partial *F*-test for individual parameters and analysis of residuals were performed following the backward selection procedure (Gacula & Singh, 1984). Computer generated contour plots were obtained using the best regression equations. The Design Expert 6.0.6 Package (Stat-Ease, Inc, Minneapolis, USA) was employed for these calculations.

2.6. Flow cytometry analysis

Multiparametric flow cytometry analysis was used to evaluate the effect of H_2O_2 on different structural and physiological aspects of *E. coli* cells, following the protocol proposed by Schenk, Raffellini, Guerrero, Blanco, and Alzamora (2011).

Application of double staining with fluorescein diacetate (FDA) (Calbiochem, Darmstadt, Germany) and propidium iodide (PI) (Sigma–Aldrich St Louis, MO, USA) was used for flow cytometry analysis. FDA is used for the evaluation of enzymatic activity and membrane integrity. In the intracellular compartment of metabolically active cells, FDA undergoes hydrolysis by unspecific esterases into fluorescein (F) giving green fluorescence. PI is a nucleotide-binding probe used for the assessment of membrane integrity. PI can not penetrate cells with intact membranes due to

Table 1
Coding of levels of independent variables used in developing experimental data at pH 5.8.

Independent variable	Symbol		Level	
	Uncoded	Coded	Uncoded	Coded
Temperature (°C)	X_1	x_1	12.5	−1
			25.0	0
			37.5	1
H_2O_2 (% w/v)	X_2	x_2	0.5	−1
			1.5	0
			2.5	1

its positive charge. Following loss of membrane integrity, PI diffuses into and intercalates to double stranded DNA or RNA giving red fluorescence (Ananta, Heinz, & Knorr, 2004).

Experiments were made with *E. coli* ATCC 11229 exposed at 3.00% H_2O_2 (w/v), pH 3.0 and 25.0 °C during 5 min, following protocol described above. Non-treated cells were used as control of H_2O_2 treatment. Non-treated stained cells and cells treated at 85 °C for 15 min and subsequently stained with PI were used as controls of PI negative and positive histogram regions, respectively. Control, heat treated (85 °C, 15 min) or H_2O_2 treated cells were initially incubated with 20 μL FDA (5 mg/mL acetone) at 37 °C for 30 min. After this labeling, the cells were centrifuged (7880*g*, 5 min, 10 °C) and resuspended in 1 mL phosphate-buffered saline (PBS, pH 7.0) (Emeve Medios, C.A.B.A., Argentina). This step was followed by addition of 2 μL PI (1 mg/mL sterile water) and 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 15 min).

Analysis of microorganisms was performed on a flow cytometer Partec PAS III (Germany) equipped with an air-cooled 488 nm argon laser. Scatter or 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 (585 ± 21 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. Determinations were made by triplicate. The software WinMDI 2.8 was used to analyze flow cytometric data.

2.7. Transmission electronic microscopic (TEM) observations

To evaluate the effect of H_2O_2 on ultrastructural aspects of *E. coli*, the same conditions as for cytometry analysis were used.

These cells were subjected to three cycles of centrifugation and washing with Millonig buffer and were fixed in 2% osmium tetroxide (Sigma–Aldrich, St. Louis, USA) during 2 h (Dykstra, 1993). Fixed cells were packed in agar, subjected to stepwise dehydration in ethanol-acetone with increasing concentrations and embedded in Spurr's resin (Sigma–Aldrich, St. Louis, USA) (Sorrivas & Morales, 1983). Thin sections were cut with a Sorvall MT2

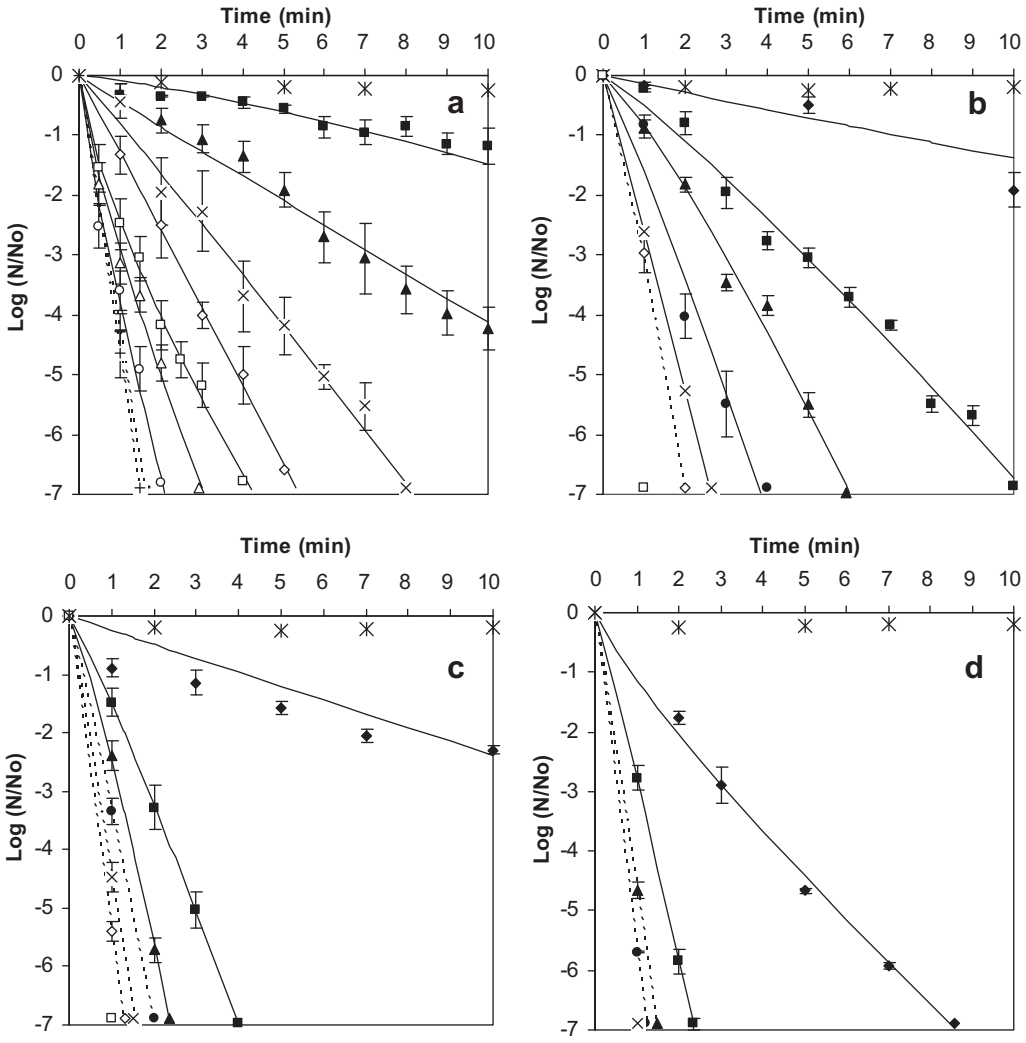

Fig. 1. Effect of H_2O_2 concentration and temperature on semilogarithmic survival curves of *E. coli* at pH 5.8. Experimental (points) and fitted values derived from the Weibullian model (lines). Control (∗); 0.10% w/v H_2O_2 (◆); 0.50% w/v H_2O_2 (■); 1.00% w/v H_2O_2 (▲); 1.50% w/v H_2O_2 (•); 2.00% w/v H_2O_2 (×); 2.50% w/v H_2O_2 (◇); 3.00% w/v H_2O_2 (□); 3.50% w/v H_2O_2 (△); 4.00% w/v H_2O_2 (○); 5.00% w/v H_2O_2 (−);6.00% w/v H_2O_2 (+). a) 12.5 °C; b) 25.0 °C; c) 37.5 °C; d) 50 °C. Dashed lines correspond to fitted inactivation curves with limited experimental data due to the rapid reduction in *E. coli* population.

ultramicrotome (Dupont Instruments, Newtown, USA), stained with Reynold's solution and examined by TEM using a JEOL transmission electronic microscope model JEM-1200 EX II (Tokio, Japan).

3. Results and discussion

Investigations on inactivation of microorganisms by chemical agents require preliminary experiments conducted to evaluate the efficacy and toxicity of the neutralizer to be used in trials. A neutralizer must effectively inhibit the action of the biocidal and must not itself be toxic to the microorganisms (Sutton, Proud, Rachui, & Brannan, 2002). The neutralizing solution assayed in this work (4.0% w/v sodium thiosulfate (w/v) in phosphate-buffered water pH 7.0) was not toxic to the *E. coli* strains and neutralized completely the highest peroxide hydrogen concentration tested (6%), since no decline was observed in the viable cell numbers in both tests .

In Figs. 1–3 the semilogarithmic experimental survival curves of *E. coli* ATCC 35218 for various H_2O_2, pH and temperature levels are presented. The three variables greatly influenced the hydrogen peroxide ability to inactivate the microorganism. In general, more *E. coli* ATCC 35218 cells were inactivated as the exposure temperature and agent concentration increased and the pH decreased.

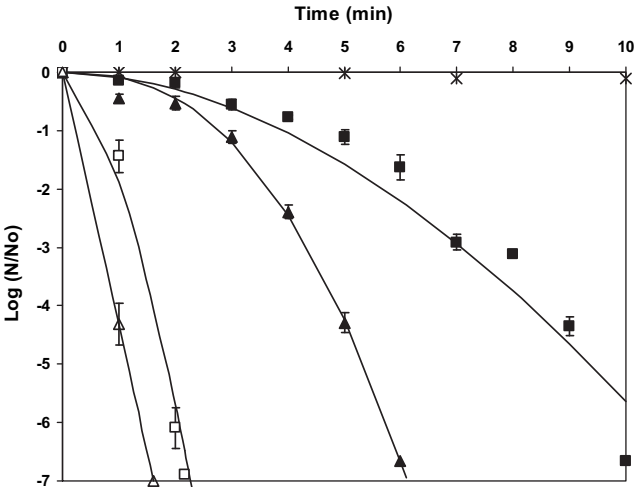

Fig. 3. Effect of H_2O_2 concentration and temperature on semilogarithmic survival curves of *E. coli* at pH 7.2. Experimental (points) and fitted values derived from the Weibullian model (lines). Control (∗); 0.50% w/v H_2O_2 at 25.0 °C (■); 1.00% w/v H_2O_2 at 25 °C (▲); 0.50% w/v H_2O_2 at 50.0 °C (□); 1.00% w/v H_2O_2 at 50.0 °C (△).

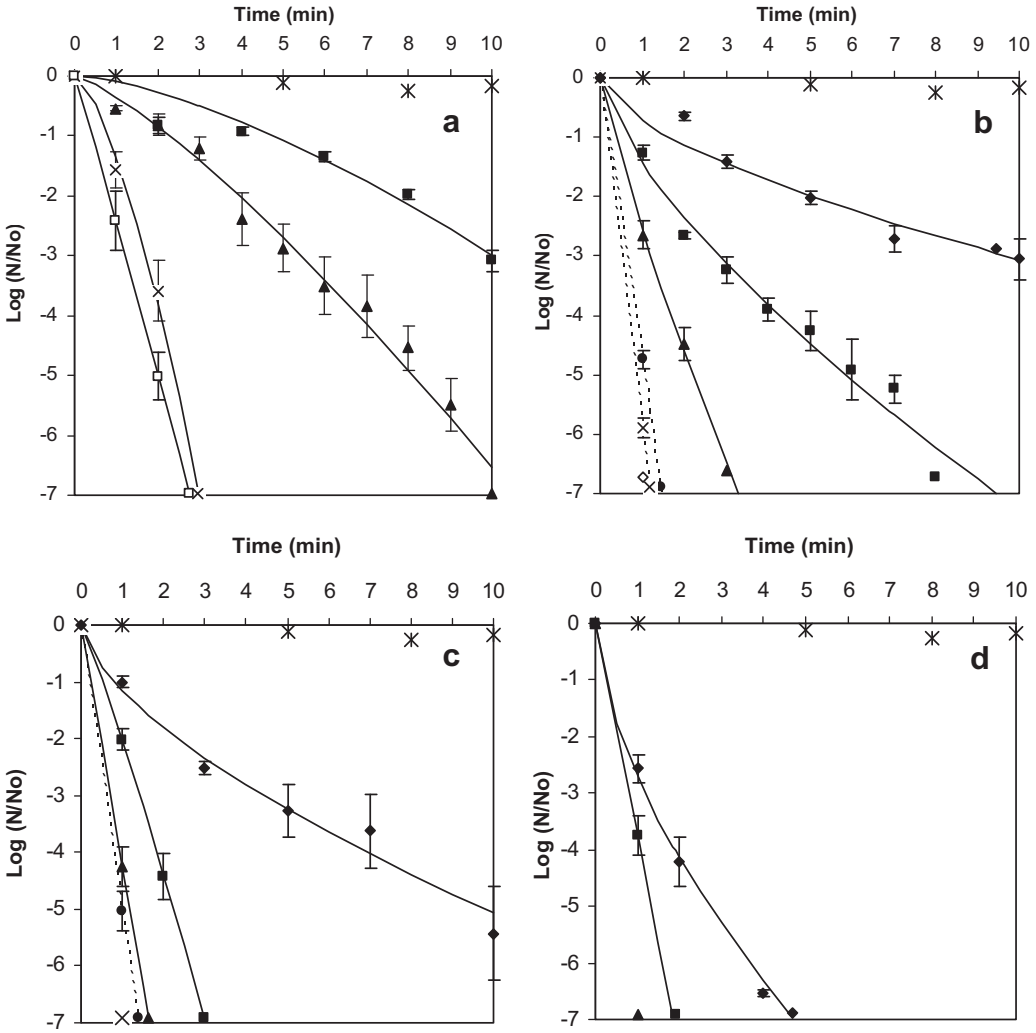

Fig. 2. Effect of H_2O_2 concentration and temperature on semilogarithmic survival curves of *E. coli* at pH 3.0. Experimental (points) and fitted values derived from the Weibullian model (lines). Control (∗); 0.10% w/v H_2O_2 (◆); 0.50% w/v H_2O_2 (■); 1.00% w/v H_2O_2 (▲); 1.50% w/v H_2O_2 (•); 2.00% w/v H_2O_2 (×); 2.50% w/v H_2O_2 (◇); 3.00% w/v H_2O_2 (□). a) 12.5 °C; b) 25.0 °C; c) 37.5 °C; d) 50 °C. Dashed lines correspond to fitted inactivation curves with limited experimental data due to the rapid reduction in *E. coli* population.

At a given pH value, as temperature decreased, the efficacy of hydrogen peroxide noticeably declined as its concentration decreased. For instance, at 0.50% w/v H_2O_2 and pH 7.2, 2.2 min and 10 min exposure time at 50.0 °C or 25.0 °C were respectively required to reduce *E. coli* ATCC 35218 viable counts to undetectable values (approximately 7.0-log reductions) (Fig. 3). But when hydrogen peroxide concentration increased to 1.00% w/v, equivalent times to reach comparable levels of inactivation were 1.6 and 5 min at those temperatures. Doubling H_2O_2 concentration reduced in only 27% the time to reach the desired inactivation at the highest temperature while at 25.0 °C the time was decreased in 50%.

In a similar fashion, at a given H_2O_2 concentration, the influence of pH on bacterial destruction was more notorious as temperature decreased. This observation can be illustrated considering treatments with 2.00% w/v H_2O_2 at pH 5.8 and 3.0 (Figs. 1 and 2). The decrease in pH from 5.8 to 3.0 reduced in 33% the exposure time needed to destroy 7 log units of *E. coli* ATCC 35218 at 37.5 °C, but at 12.5 °C this reduction achieved 62%.

Semilogarithmic survival curve patterns were linear or non linear depending on H_2O_2 concentration, exposure temperature and pH. The different survival patterns could be well described in terms of Weibullian distribution, considering that the individual microorganisms in a population do not have identical resistance and that microbial sensitivity to lethal agents is distributed (i.e. biological variability). Downward concavities (shoulders) and upward concavities (tailing) are due to underlying physiological reactions of the cells/spores to lethal conditions. The Weibullian distribution function takes biological variation into account and is used to describe the spectrum of resistances of the population to a lethal agent under different conditions (van Boekel, 2002; Peleg, 2000, 2006; Peleg & Cole, 1998). Survival patterns are explained without assuming the validity of any kinetic model. There will be a distribution of inactivation times and the survival curve should not be treated in kinetic terms but should be considered the cumulative form of the temporal distribution of lethal events. So, the shapes of the survival curves are reflections of treatment resistance distributions having different mode, mean, variance and skewness. Figs. 1–3 also show the experimental inactivation data fitted using the cumulative Weibullian distribution function described by Eq. (3). Table 2 shows the underlying b and n regression parameters as well as the corresponding mode, mean, variance and skewness values (Eqs. (3)–(6)). Experimental curves were highly correlated to predicted data, obtaining very significant adjusted determination coefficients R^2_{adj} (0.945–0.998). For high sanitizer concentrations, high temperature and low pH, the number of experimental points was too small to ascribe specific inactivation behaviour, because of the rapid reduction in *E. coli* population. For those inactivation curves, model parameters were calculated only with comparison purpose and appeared in Table 2 in cursive numbers, while the fitting of Eq. (3) in Figs. 1 and 2 are shown with dashed lines. The parameter n or shape parameter, as expected, was >1 for concave downward survival curves (all curves obtained at pH 7.2 and some curves obtained at pH 5.8 and pH 3.0), indicating that the remaining cells became increasingly susceptible to the treatment (van Boekel, 2002). Inactivation pattern at pH 5.8 mainly exhibited n values nearly 1, meaning that the probability of dying did not depend on treatment time (i.e. there was no biological variation). On the contrary, at pH 3.0, the shape parameter was <1 for most of the inactivation curves, showing that sensitive members of the population were destroyed while the remaining cells had less probability of dying and were the sturdy ones. The occurrence of tailing has been attributed to: 1) phenotypic or genotypic differences within a population which provide enhanced survival for a minority of cells, and/or 2) the protection resulting from the contents of dead cells which shield the remaining survivors (Shadbolt, Ross, & McMeekin, 1999). On the other hand, Lambert, Johnston, and Simons (1999) and Lambert and Johnston (2000) developed a mechanistic model of disinfection (the Intrinsic Quenching Model) derived from the Chick–Watson model but assuming that the disinfection concentration changes during the course of the test. They demonstrated that if biocide concentration can be reduced during the disinfection treatment by some means, then tailing will be the natural consequence. However, it is to be noticed that in the present research the bulk concentration of hydrogen peroxide, as well as the pH, maintained constant along the period of time of the experiments.

Overall at a given pH and temperature, the parameter b or scale parameter gradually increased as H_2O_2 level increased. The higher the temperature, the higher was the relative increase in b parameter.

The continuous probability density function for $\bar{t}_c$, generated according to Eq. (2), is shown in Fig. 4 for 0.50%, 1.00% and 2.00% w/v H_2O_2 concentrations. Frequency distribution profiles markedly differed with exposure temperature and pH and H_2O_2 level. In general, the greater the H_2O_2 concentration and the temperature and the lower the pH, the narrower were the distribution, and the lower were the mean, the mode and the variance values. At 37.5 °C and 50.0 °C, for all H_2O_2 levels assayed at pH 3.0 and 5.8, the frequency distributions showed an extreme skewness to the right. They did not appear to have a peak (i.e. without mode) or had a very small mode (Fig. 4). That is, the majority of the population was destroyed within a very short time after being exposed to the sanitizer. On contrast, at 50.0 °C and pH 7.2, the distributions did exhibit mode. In fact, this behaviour denoted the incidence of pH on inactivation rate, even at high temperatures. When treatment was performed at 12.5 °C and low H_2O_2 concentrations (0.10% w/v and 0.50% w/v), there was a small shift to the right in the distribution's mode and an important increase in its overall spread, with a pronounced tailing, meaning that a substantial fraction of the microbial population survived long after the majority was inactivated. For a given pH and sanitizer concentration, as temperature increased, *E. coli* became not only more sensitive to the sanitizer but it had a very narrow distribution of resistances (the response of the population was more uniform) as evidenced by very low variance values.

A secondary polynomial model was obtained to compare the effectiveness of the different combinations lethal agent concentration–temperature. The time of exposure needed to reach 5-log reductions in *E. coli* ATCC 35218 populations (estimated by applying the Weibullian distribution) was modeled as a function of the independent variables H_2O_2 concentration (0.50–2.50% w/v) and temperature (12.5–37.5 °C) at pH 5.8 using a Box–Behnken incomplete factorial design. Inactivation response was ln-transformed to homogenise the variance. The second order polynomial equation in terms of actual variables is presented in Eq. (12):

$$\ln \mathrm{TR5L} = 5.80 - 0.16\,\mathrm{T} - 1.73\%\,\mathrm{H_2O_2} + 0.0013\,\mathrm{T}^2 + 0.19\%\,\mathrm{H_2O_2^2} + 0.015\,T\%\,\mathrm{H_2O_2} \quad (12)$$

The equation was tested for adequacy and fitness by analysis of variance and residuals (Gacula & Singh, 1984). The analysis of variance for the linear, quadratic and cross effects of the independent variables is shown in Table 3. The statistical analysis indicates that the proposed model was adequate possessing no significant lack of fit and with very satisfactory values of the R^2_{adj} statistic. The correlation equations account for more than 99% of the variation in the response variable ($R^2_{adj} = 0.998$). All linear, quadratic and interaction terms appeared to be significant although both independent variables were the most relevant factors according to F partial values (Table 3). To visualize the direct and interactive

Table 2
Weibullian distribution parameters of single *E. coli* ATCC 35218 strain or mixed-strain cultures (*E. coli* ATCC 35218, *E. coli* ATCC 8739, *E. coli* ATCC 11229 and *E. coli* ATCC 25922) under treatments with different H_2O_2 concentrations and temperatures at pH a) 3.0; b) 5.8; c) 7.2.

a) pH 3.0

T (°C)	H_2O_2 concentration (% w/v)	b (min^{-n})	SE	n	SE	R^2_{adj}	t_{cm} (min)	$\bar{t}_c$ (min^2)	ν_1 (−)	σ^2_{tc} (min^2)
12.5	0.50	0.10	0.03	1.49	0.15	0.984	2.3	4.3	1.5	8.77
	1.00	0.35	0.06	1.28	0.08	0.984	0.7	2.1	1.7	2.83
	2.00	1.34	0.17	1.51	0.12	0.994	0.4	0.7	1.5	0.25
	3.00	2.42	0.01	1.04	0.01	0.999	0.02	0.4	2.0	0.16
25.0	0.10	0.73	0.08	0.62	0.03	0.987	–	2.4	3.9	16.06
	0.50	1.44	0.16	0.70	0.06	0.978	–	0.8	3.3	1.19
	1.00	2.57	0.09	0.84	0.04	0.998	–	0.4	2.6	0.18
	1.50	*4.73*		*1.00*			–	*0.2*	*2.1*	*0.04*
	2.00	*5.89*		*0.99*			–	*0.2*	*2.1*	*0.03*
37.5	0.10	1.16	0.10	0.64	0.03	0.992	–	1.1	3.8	3.20
	0.50	2.03	0.01	1.11	0.01	0.999	0.1	0.5	1.9	0.21
	1.00	*4.25*		*1.00*			*0.0*	*0.2*	*2.1*	*0.06*
	1.50	*5.03*		*1.00*			*0.0*	*0.2*	*2.1*	*0.04*
50.0	0.10	2.71	0.14	0.61	0.04	0.995	–	0.29	4.1	0.24
	0.50	*3.75*		*1.00*			0.0	0.27	2.1	0.07

b) pH 5.8

T (°C)	H_2O_2 concentration (% w/v)	b (min^{-n})	SE	n	SE	R^2_{adj}	t_{cm} (min)	$\bar{t}_c$ (min)	ν_1 (−)	σ^2_{tc} (min^2)
12.5	0.50	0.08	0.01	1.26	0.05	0.986	2.1	6.8	1.7	30.12
	1.00	0.43	0.04	0.98	0.04	0.991	_	2.4	2.2	5.86
	2.00	0.80	0.11	1.03	0.08	0.984	0.04	1.2	2.1	1.42
	2.50	1.27	0.07	1.01	0.04	0.997	0.01	0.8	2.1	0.60
	3.00	2.42	0.08	0.73	0.03	0.995	_	0.4	3.1	0.25
	3.50	2.88	0.14	0.79	0.06	0.990	_	0.3	2.8	0.15
	4.00	3.80	0.23	0.82	0.11	0.979	_	0.2	2.7	0.07
	5.00	*4.29*		*1.00*			_	*0.2*	*2.1*	*0.05*
	6.00	*4.65*		*1.00*			_	*0.2*	*2.1*	*0.05*
25.0	0.10	0.14	0.05	0.98	0.09	0.966	_	7.3	2.2	55.42
	0.50	0.50	0.07	1.13	0.07	0.987	0.3	1.8	1.9	2.48
	1.00	0.82	0.12	1.20	0.09	0.987	0.3	1.1	1.8	0.88
	1.00[a]	1.39	0.21	0.82	0.09	0.971	_	0.7	2.7	0.83
	1.50	1.60	0.39	1.09	0.20	0.959	0.1	0.6	1.9	0.33
	2.00	*2.61*	*0.01*	*1.01*	*0.01*	*0.990*	*0.1*	*0.4*	*2.1*	*0.14*
	2.50	*3.10*	*0.04*	*1.17*	*0.02*		*0.1*	*0.4*	*1.8*	*0.10*
37.5	0.10	0.25	0.09	0.98	0.12	0.945	–	4.2	2.2	18.02
	0.50	1.50	0.02	1.11	0.01	0.999	0.1	0.7	1.9	0.36
	1.00	2.46	0.08	1.19	0.03	0.999	0.1	0.4	1.8	0.14
	1.50	*3.34*		*1.08*			*0.03*	*0.3*	*2.0*	*0.09*
	2.00	*4.47*		*1.00*			_	*0.2*	*2.1*	*0.05*
	2.50	*5.40*		*1.00*			_	*0.2*	*2.1*	*0.03*
50.0	0.10	1.15	0.10	0.84	0.04	0.995	–	0.9	2.6	1.25
	0.50	2.79	0.02	1.06	0.08	0.999	0.03	0.4	2.0	0.12
	0.50[a]	3.28	0.34	0.66	0.12	0.982	–	0.2	3.6	0.12
	1.00	*4.66*		*1.00*			–	*0.2*	*2.1*	*0.04*
	1.50	*5.69*		*1.00*			–	*0.2*	*2.1*	*0.03*

c) pH 7.2

T (°C)	H_2O_2 concentration (% w/v)	b (min^{-n})	SE	n	SE	R^2_{adj}	t_{cm} (min)	$\bar{t}_c$ (min)	ν_1 (−)	σ^2_{tc} (min^2)
25.0	0.10	0.07	0.07	1.12	0.18	0.950	1.4	10.1	1.9	81.52
	0.50	0.08	0.04	1.84	0.25	0.946	2.6	3.5	1.4	3.82
	1.00	0.08	0.02	2.47	0.13	0.995	2.3	2.5	1.2	1.14
	1.50	0.53	0.17	1.57	0.21	0.970	0.8	1.3	1.5	0.76
	2.00	1.21	0.10	1.59	0.08	0.998	0.5	0.8	1.5	0.26
	2.50	1.72	0.33	1.67	0.19	0.990	0.4	0.6	1.4	0.16
	3.00	*3.18*	*0.03*	*1.11*	*0.02*		*0.1*	*0.3*	*1.9*	*0.09*
50.0	0.10	1.88	0.30	1.60	0.15	0.992	0.4	0.6	1.5	0.15
	0.50	*4.32*		*1.00*			*0.0*	*0.2*	*2.1*	*0.05*

T = temperature; b, n = constants of Weibullian model; SE = standard error; R^2_{adj} = statistic; t_{cm} = distribution's mode; $\bar{t}_c$ = distribution's mean; σ^2_{tc} = variance and ν_1 = coefficient of skewness; (−) distribution function with no peak.
Cursive numbers correspond to model parameters of fitted inactivation curves with limited experimental data due to the rapid reduction in *E. coli* population.
[a] Data corresponding to mixed-strain cultures.

effects of the independent variables, response surfaces and contour plots for ln TR5L as function of temperature and sanitizer concentration are presented in Fig. 5. As it can be seen, a very smooth curvature of surfaces occurred due to the significance of both pure quadratic terms (x_{12}, x_{22}) as was shown through the analysis of variance (Table 3).

A match between observed time to 5-log reduction values recorded in independent experiments and predicted values

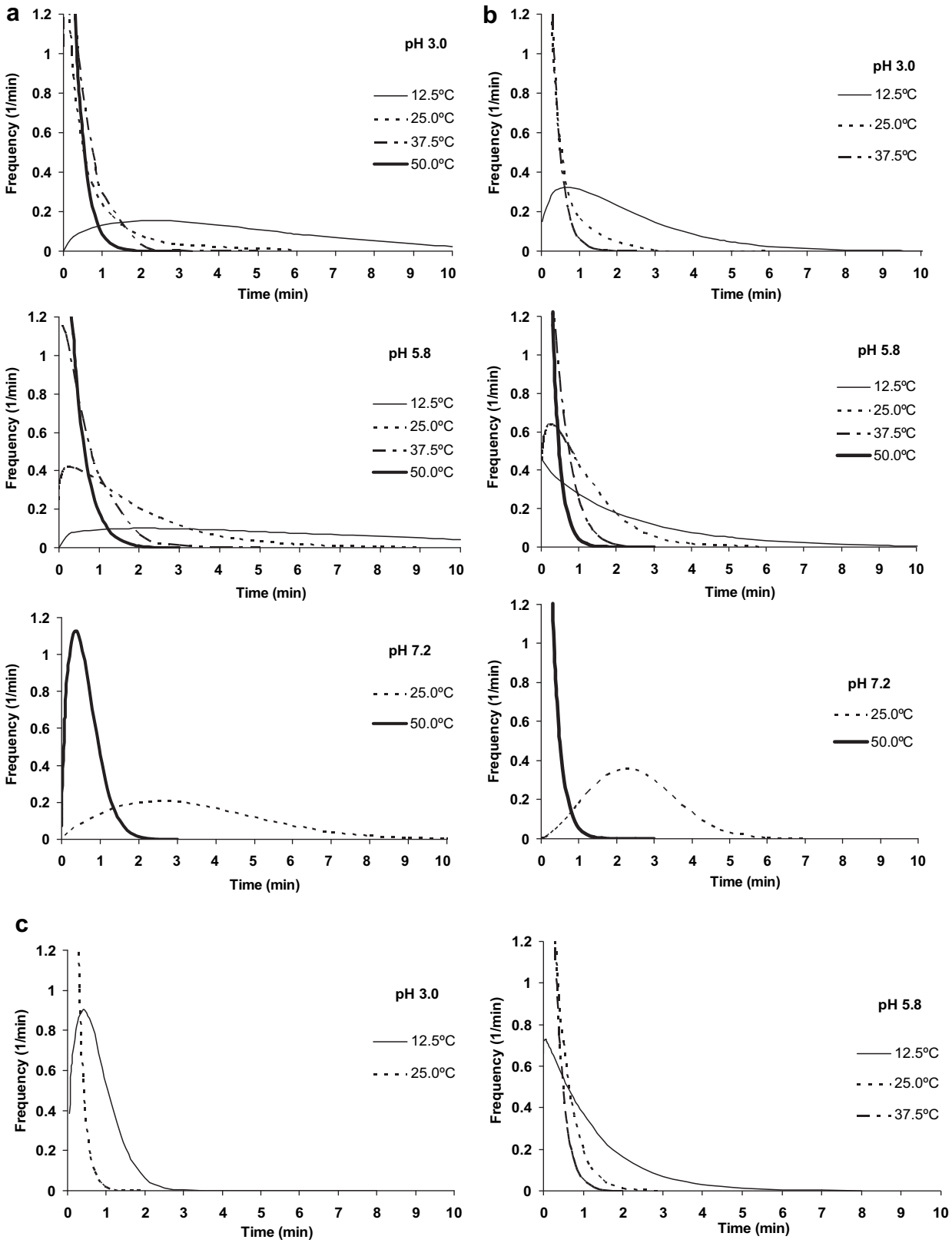

Fig. 4. Frequency distributions of resistances of *E. coli* obtained by applying Eq. (2) at different pH, temperatures and H_2O_2 concentrations: (–) 12.5 °C% w/v; (- -) 25.0 °C; (— — —) 37.5 °C; (▬) 50.0 °C. a) 0.50% w/v; b) 1.00% w/v; c) 2.00% w/v.

Table 3
Analysis of variance showing the effect of treatment variables as linear, quadratic and cross-product terms on time to 5-log reductions.

Source	DF	Mean square (MS)	F[a]
Model	5	1.80	1121****
Linear			
x_1[b]	1	4.81	2989****
x_2[c]	1	3.73	2318****
Quadratic			
x_1[b]	1	0.11	67.8****
x_2[c]	1	0.10	59.5****
Cross			
x_1x_2	1	0.15	91.1****
Residual	7	0.002	
Lack of fit	3	0.002	1.62 (NS)
Pure error	4	0.001	
Total	12	0.752	
Variability explained (R^2_{adj})		0.998	

[a] Significant at ****0.01% level, NS: no significant
[b] x_1: temperature
[c] x_2: % w/v hydrogen peroxide

validated the model. Predicted and experimental TR5L values are presented in Table 4. Errors associated to the prediction with Eq. (12) ranged between 0 and 16%, excepting for the combination 37.5 °C–1.00% w/v H_2O_2, where a 33% error was registered. However, this treatment condition resulted in very low TR5L value and the difference with the prediction was of only 0.5 min. This comparison evidenced the ability of the quadratic polynomial model to correctly predict the treatment times necessary for 5-log reduction of the microorganism at pH 5.8 in the ranges of temperatures and sanitizer concentrations used to develop the model.

A mixed inoculum of four reference strains (*E. coli* ATCC 35218, *E. coli* ATCC 8739, *E. coli* ATCC 11229 and *E. coli* ATCC 25922) was used to assess differences in susceptibility to H_2O_2 treatment. It is to be noted that one of these strains, *E. coli* ATCC 25922, had been employed as surrogate of *Salmonella* (Eblen, Annous, & Sapers, 2005) and *E. coli* O157:H7 (Kim & Harrison, 2009; Sapers & Sites, 2003) in the evaluation of the efficacy of different food processes, including fresh produce decontamination with hydrogen peroxide (Sapers & Sites, 2003). Shown in Fig. 6 are inactivation curves (experimental and fitted by the Weibullian model) for the mixed-strain cultures and the single *E. coli* ATCC 35218 strain using 1.00% w/v H_2O_2 at 25.0 °C and 0.50% w/v H_2O_2 at 50.0 °C at pH 5.8. Small differences, although not significant (p values: 0.126 and 0.690) were observed between them. Downward concavity (1.00% w/v H_2O_2 at 25.0 °C) or near linear (0.50% w/v H_2O_2 at 50.0 °C) curves were obtained for the single inoculum whereas mixed-strain inoculum presented upward concavity curves in both treatments. For short treatment times, mixed strains showed some greater sensibility to the sanitizer but this tendency was inverted at greater exposure times. The small difference in inactivation behaviour was manifested in the parameters of the Weibullian model: the survival curves of mixed strains exhibited lower shape parameter and media and greater scale parameter and skewness coefficient than survival curves of the single strain (Table 2). Experimental TR5L values were similar for single and mixed strains. This behaviour was expected since inactivation curves for both inoculums crossed over when 4–5 log cycle reductions were reached. In spite the Eq. (12) had been obtained for the single strain, it predicted a TR5L value of 4.4 min at 1.00% w/v H_2O_2 and 25.0 °C while the experimental value in the same conditions was 5.0 min. The greater resistance of *E. coli* ATCC

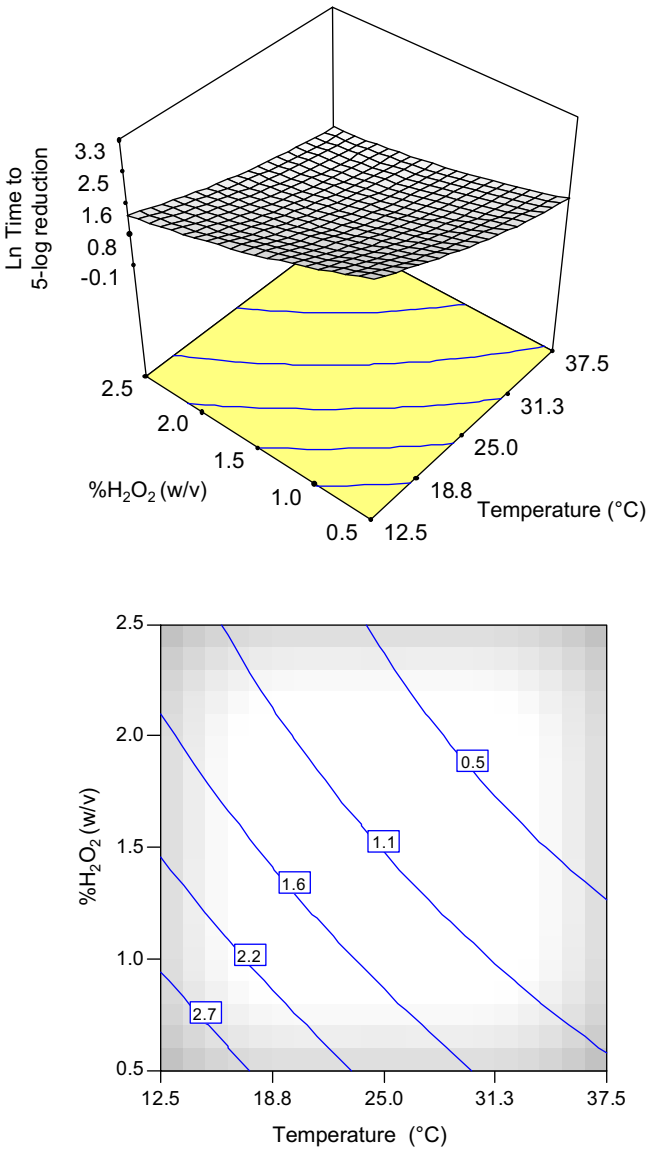

Fig. 5. Response surface (a) and contour plots (b) for the effect of H_2O_2 concentration (%HP w/v) and temperature on Ln time to 5-log reductions of *E. coli* ATCC 35218 at pH 5.8.

35218 to hydrogen peroxide during short exposure times as compared with the one of other strains of the same specie indicates that this strain would be adequate to study the efficacy of inactivation processes by oxidant agents.

Table 4
Predicted values by the polynomial model (Eq. (12)) and observed experimental time to 5-log reductions (T5LR) of *E. coli* ATCC 35218 population exposed to different H_2O_2-temperature treatments.

Treatment		T5LR	
Temperature (°C)	H_2O_2 (% w/v)	Predicted value (min)	Experimental datum (min)
12.5	1.00	14.4	12.4
	2.00	5.4	6.0
25.0	0.75	5.7	5.9
	1.00	4.4	4.7
	2.00	2.0	1.9
37.5	1.00	2.0	1.5
	2.00	1.1	1.1

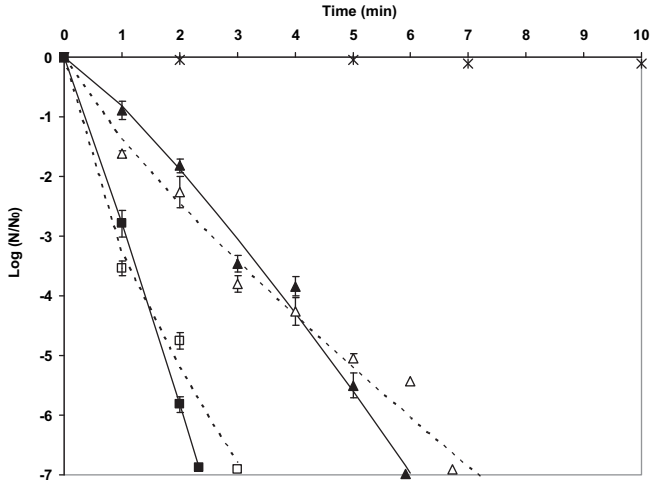

Fig. 6. Effect of H_2O_2 concentration and temperatures on semilogarithmic survival curves of E. coli ATCC 35218 (single strain) and on semilogarithmic survival curves of a mixed-strain cultures (mixed strains) of four reference strains (*E. coli* ATCC 35218, *E. coli* ATCC 8739, *E. coli* ATCC 11229 and *E. coli* ATCC 25922), at pH 5.8. Experimental (points) and fitted values derived from the Weibullian model (lines). Control (∗); 1.00% w/v H_2O_2 at 25.0 °C single strain (▲−); 1.00% w/v H_2O_2 at 25.0 °C mixed strains (△ - - -); 0.50% w/v H_2O_2 at 50.0 °C single strain (■−); 0.50% w/v H_2O_2 at 50.0 °C mixed strains (□ - - -).

Changes in pH may appear to affect the activity of a compound by inducing changes either in the cell wall or in the structure of the compound (Bean, 1967). However it has been reported that H_2O_2 action is less affected by pH that are many other disinfectants and that little activity change occurred between pH 2 and 10 (Block, 2001). These findings are not in agreement with results reported by other authors. Cords et al. (2005) reviewed the effects of pH on bactericidal and sporicidal action of hydrogen peroxide and concluded that it appears to be a greater H_2O_2 activity in the acidic range while its overall effectiveness decreases at alkaline pH. In a previous study made at constant temperature, Raffellini, Guerrero and Alzamora (2008) found that effectiveness of H_2O_2 solutions against *E. coli* ATCC 35218 improved at both higher concentrations and more acidic pH values. These authors reported that, as $\%H_2O_2$ increased, the differences in time to 5-log reductions for the different pHs tended to decrease especially in the range 3.0–5.8. In the present study, a similar behaviour was confirmed at constant sanitizer level and varying temperatures: as exposure temperature increased, T5LR values were less influenced by pH.

For a given temperature and pH, inactivation of *E. coli* ATCC 35218 increased between 0.10% and 2.00–3.00% w/v H_2O_2. Baatout, De Boever, and Mergeay (2006) analyzed physiological changes induced in *E. coli* following oxidative stress using flow cytometry. Working with similar sanitizer levels as in this work, they found that PI fluorescence intensity increased in function of H_2O_2 concentration. PI, a nucleic-acid stain, is a small hydrophilic molecule and cells that have exclusively lost their selective membrane permeability take up the dye and emit fluorescence. This gradual increase in PI fluorescence would mean that an increase in sanitizer concentration would increase membrane damage. A change in intracellular pH at high H_2O_2 concentration and loss of membrane potential were also reported.

A temperature rise almost invariably increased the rate of the bactericidal reaction and increased the efficacy of H_2O_2 treatment. The dependence of the scale parameter b with temperature is complex. For a given pH and sanitizer level, *b* parameter increased as temperature increased. For those combinations of H_2O_2 concentration–temperature–pH in which b values could be obtained at four temperatures (pH 5.8 and 3.0; 0.50% w/v H_2O_2 level), it clearly appeared that *b* parameter was more influenced by temperature at low exposure temperature values (12.5–25.0 °C) than at moderate and high temperature values (37.5–50.0 °C) studied. This behaviour could be explained as follows. In addition of acting on the membrane lipids by a peroxidation reaction affecting membrane integrity, hydrogen peroxide as a small molecule is able to cross cytoplasmic membrane and, once inside, to generate oxygen derivable radicals that impact on almost all components of the cell (including DNA and proteins) producing a range of significant physiological alterations induced by oxidative stress (Baatout et al., 2006; Labas et al., 2008). It could be assumed that a diffusive step is in series with chemical reactions. The diffusion process is less temperature dependent than chemical reaction. At high temperatures, reactive oxygen species (mainly hydroxyl radicals) would be able to react with extremely high rates with almost every type of molecule found in living cells (Halliwell & Gutteridge, 1984) and so, diffusion transport of hydrogen peroxide through membranes would be controlling the destruction reaction. At low temperatures, chemical attack would be the controlling step and consequently b parameter would show a greater dependence on temperature.

Multiparameter flow cytometry studies about the hydrogen peroxide (3.00% w/v H_2O_2; pH 3.0; 25.0 °C; 1 and 5 min exposure) induced damage on *E. coli* ATCC 11229 cells supported some of the previous comments. The effect of H_2O_2 treatment at different times was evaluated by the ability to accumulate and retain F as an indicator of membrane integrity and enzyme activity, and the uptake of PI to assess membrane damage. The dual-parameter density plot of green fluorescence (*x*-axis) and red fluorescence (*y*-axis) was represented in Fig. 7a–c, where each dot constituting the cell cloud represents one single cell, which is plotted as a co-ordinate of its green and red fluorescence value. The percentage of microbial populations which falls in each quadrant can be also found in Fig. 7a–c. Untreated *E. coli* stained with FDA showed a heterogeneous behaviour in their fluorescence labeling properties (Fig. 7a). Not all cells yielded high green fluorescence and appeared in quadrant 4. This could possibly due to: a) the presence of the outer membrane of lipopolysaccharides (LPS) (Hewitt, Boon, McFarlane, & Nienow, 1998), which does not allow FDA to freely diffuse across cytoplasmic membrane or b) the active expulsion of F outside the cell by bacteria pumps and consequently the lack of green fluorescence despite the existence of metabolic activity (Ananta & Knorr, 2009). A minor sub-population (16%) was detected in quadrant 1 demonstrating compromise of membrane allowing PI diffusion into the cells.

A changing pattern of fluorescence with H_2O_2 dose was observed. Exposure of *E. coli* during 1 min resulted in a shift of cells from quadrant 4 to quadrants 3 and 1 (Fig. 7b). Clearly, sanitizer destroyed membrane integrity allowing PI to penetrate cells. When treatment increased to 5 min, the proportion of cells with a permeabilized membrane (quadrant 1) and therefore non-viable, continued increasing (from 33% to 54%) at expenses of cells in quadrants 3 and 4 (Fig. 7c). The fluorochrome PI continued accumulating in the inner of cell, binding to DNA and causing higher concentration of red fluorescence. A minor proportion of cells was distinguished in quadrant 2 (~4–6%) both in control and treated systems (cells with esterase activity and damage membrane). Flow cytometry results tended to overestimate the viability of studied microorganisms. It is to be noticed that, after 1 min treatment, *E. coli* plate counts were reduced in approximately 7 log cycles (data not shown) while only 33% of the cells were stained with PI and 6% were PI^+/F^+. Therefore, the penetration of PI reflects the state of the integrity of the membrane in the cell, but cannot be viewed as the sole indicator of cell viability. Light scattering data for *E. coli* before

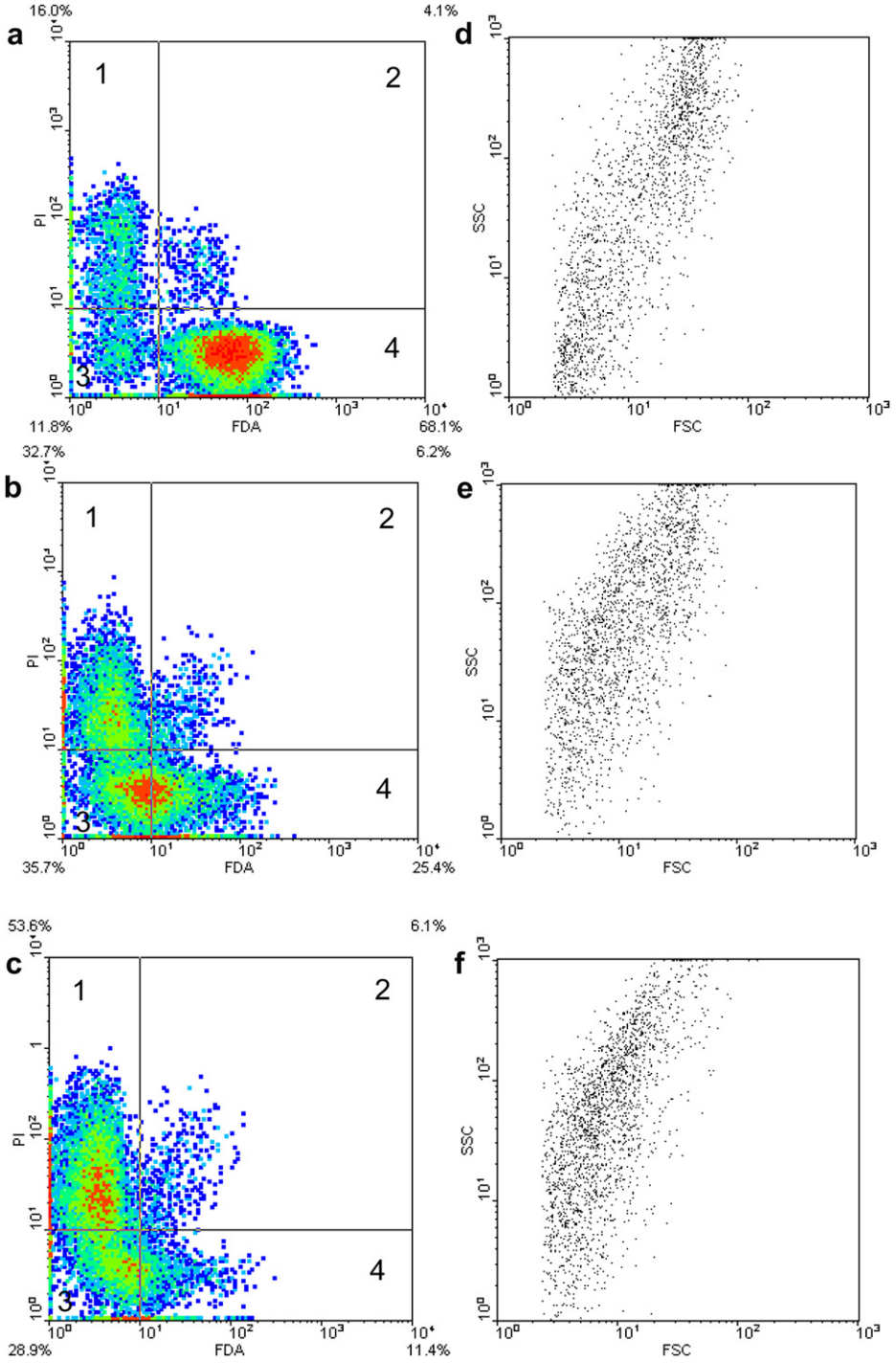

Fig. 7. Fluorescence density plots (a–c) and dot plots representing forward scatter light (FSC) vs. side scatter light (SSC) (d–f) of *E. coli* ATCC 11229 cells treated or not with H_2O_2 (3.00% w/v, pH 3.0). a,d) Untreated cells; b,e) cells treated with H_2O_2 during 1 min; c,f) cells treated with H_2O_2 during 5 min. Fluorescence density plot quadrants: 1: F– PI+, Esterase activity not detectable, compromised membrane; 2: F+ PI+, Active esterase, minimally damaged membrane; 3: F– PI–, Esterase activity not detectable (or F extruded out of the cells), intact membrane; 4: F+ PI–, Active esterase, intact membrane.

and after H_2O_2 exposure during 1 and 5 min is shown in Fig. 7d–f. Untreated *E. coli* generated a unimodal distribution with respect to FSC but a bimodal distribution with respect to SSC. That resulted in a diffuse cluster shape that is characteristic for rod shaped micro-organisms and can be attributed to the different orientation of the elongated cells as it travels through the laser beam (Fig. 7d) (Hewitt & Nebe-von Caron, 2001). After treatment, a unimodal distribution with respect to both FSC and SSC could be observed as well as a concentration of events in the medium size and granularity values (Fig. 7e,f).

Further evidence for multiple targets of H_2O_2 comes from TEM observations of *E. coli* ATCC 11229 cells treated during 5 min with 3.00% w/v H_2O_2 (pH 3.0, 25.0 °C) (Fig. 8). Structure and composition studies of Gram-negative show that cells are surrounded by a complex multilayered cell wall, composed of a thin inner layer of peptidoglycan and an outer membrane. The outer membrane

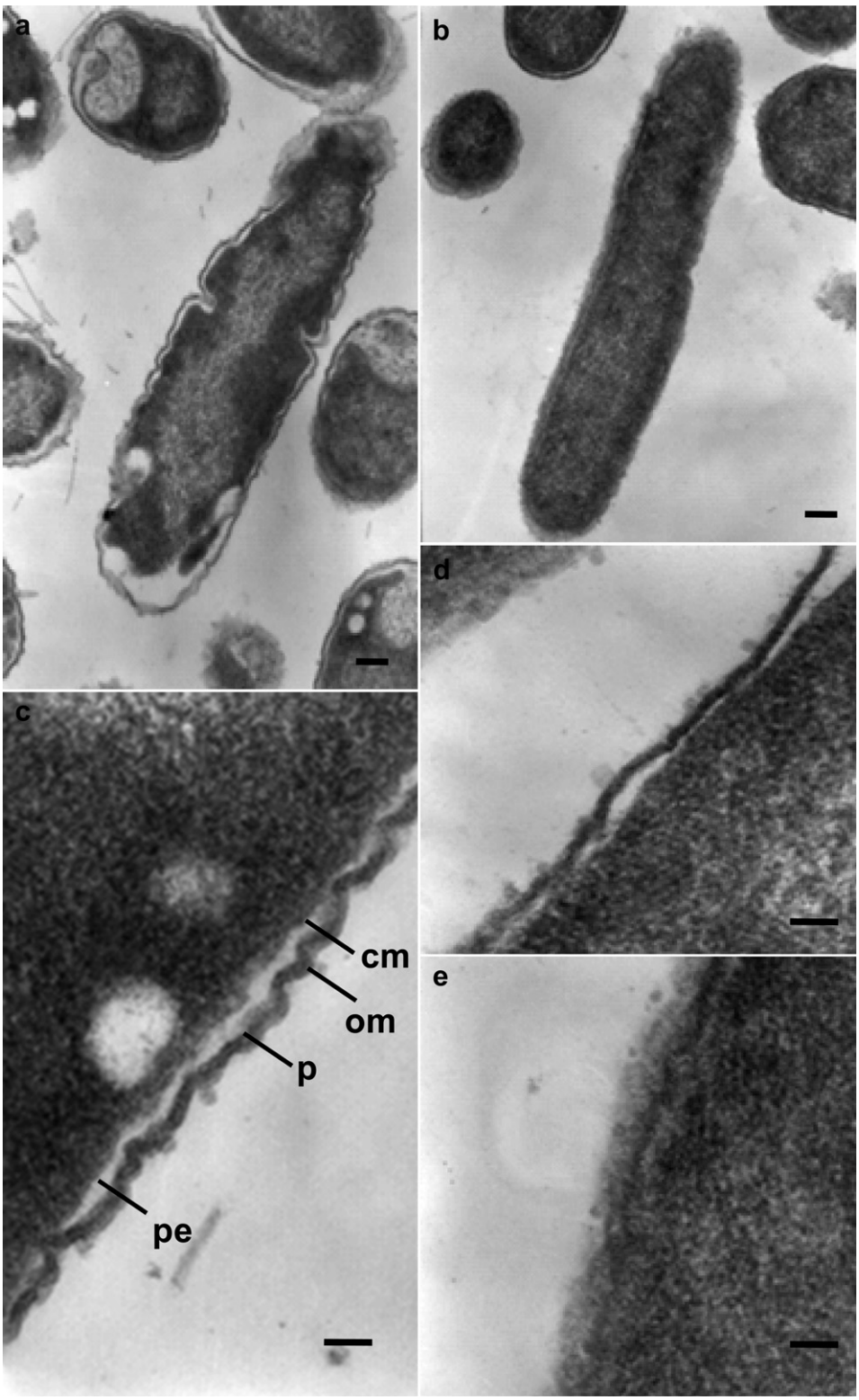

Fig. 8. TEM photomicrographs of *E. coli* ATCC 11229 cells after 5 min exposure to 3.0% p/v H_2O_2 (pH 3.0, 25 °C). a, c: control. b, d, e: treated cells. om: outer membrane; p: peptidoglycans; cm: cytoplasmic membrane, pe: periplasm. Scale: a, b 200 nm; c, d, e 100 nm.

appears as a lipid bilayer and is composed of phospholipids, lipoproteins, lipopolysaccharides (LPS), pore-forming proteins and surface proteins (Schnaiman, 1970; Eltsov & Zuber, 2006). The periplasm is a gelatinous material between the outer membrane, the peptidoglycan and the cytoplasmic membrane. Porins in the outer membrane allow diffusion of small molecules into the periplasmic space; the peptidoglycan layer provides the cell with its shape and prevents osmotic lysis and the periplasm contains enzymes for nutrient breakdown and binding proteins to facilitate the transfer of nutrients across cytoplasmic membrane (Leptihn et al., 2009). As seen in Fig. 8a,c, the bilayer outer and cytoplasmic membranes and the periplasm of untreated cells were well

distinguishable. The nucleoids appeared as electrolucent cavities partially filled with coarse aggregates of chromatin in the ribosome free area (Fig. 8a,c). The application of H_2O_2 produced many structure changes (Fig. 8b–e). The periplasmic space was not limpid and the outer and cytoplasmic membranes appeared broken in many points, with vesicle formation. Cell walls were not as undulated as in the control. The nucleoid region was not very clear (the contrast between DNA-plasm and ribosome area was less marked). In agreement with flow cytometry results, the granularity of cells was more uniform.

4. Conclusions

The inactivation rate of *E. coli* exhibited a clear dependence on hydrogen peroxide concentration, pH and temperature. The Weibullian mathematical model described the inactivation rate of *E. coli* by H_2O_2 at different exposure times and temperatures and sanitizer levels with a good correlation. Quantitative kinetic results obtained allowed identifying various combinations H_2O_2 concentration–pH–temperature for 5-log cycles reduction of *E. coli*. Flow cytometry analysis revealed induced H_2O_2 cytoplasmic membrane damage. TEM observations indicated that H_2O_2 treatment resulted in rupture of outer and cytoplasmic membranes and a more uniform granularity.

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