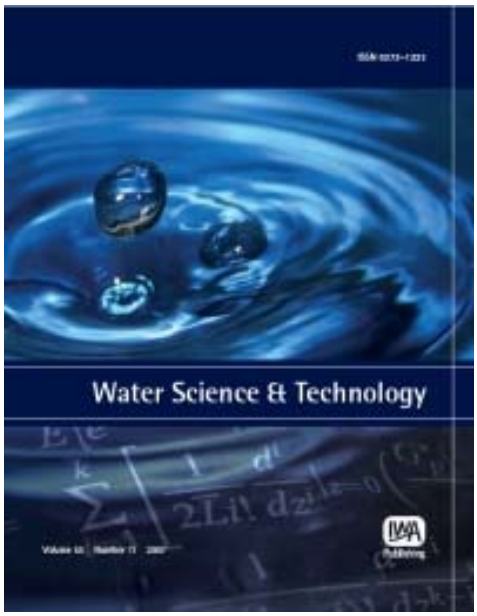

A novel approach to explain the inactivation mechanism of *Escherichia coli* employing a commercially available peracetic acid

Marina J. Flores, Maia R. Lescano, Rodolfo J. Brandi, Alberto E. Cassano and Marisol D. Labas

ABSTRACT

The chemical inactivation of *Escherichia coli* employing a commercial mixture of peracetic acid (PAA) was studied. For this purpose, experiments were carried out using dilutions of the unmodified mixture, and also the same mixture but altered with hydrogen peroxide (HP) previously inhibited. Also, these results were compared to those obtained before employing HP alone. It was found that the mixture is much more efficient than HP and PAA acting separately. Furthermore, it was found that PAA without HP is much more efficient than HP alone. A plausible explanation is presented. The homolysis of PAA would give rise to a chain reaction that generates a significant number of highly oxidizing radicals. An attacking scheme to bacteria in two stages is proposed, where the initial step, mainly caused by PAA, is very fast and eliminates some specific components of the bacteria that would otherwise inhibit the parallel action of HP. Thereafter, the emergence of a potentiating synergetic action of the second oxidant seems to be immediately unveiled.

Key words | commercial peracetic acid, *Escherichia coli*, hydrogen peroxide, synergism, water disinfection

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INTRODUCTION

Water disinfection is carried out to prevent the spread of human pathogens that may be present in wastewater effluents. The efficient inactivation of pathogenic bacteria, viruses and protozoan parasites from water and wastewaters is critical, since sewage discharges may increase the risks of waterborne infections. Studies have pointed out that untreated wastewater is the first contributor of bacteria to the aquatic ecosystem. Chlorine is the most commonly used disinfectant but can also have an important drawback such as disinfection by-products (Nieuwenhuijsen *et al.* 2000).

Peracetic acid (PAA) is a strong oxidant. Its oxidation potential is larger than the one of chlorine or chlorine dioxide (Kitis 2004; Rossi *et al.* 2007) and it is a much more potent antimicrobial agent than hydrogen peroxide (HP), being rapidly active at low concentrations. The equilibrium state of commercial PAA is a mixture of peracetic and acetic acid, as well as water and HP. Although HP also contributes to the inactivation power of the mixture and to the formation of hydroxyl radicals (Caretti *et al.* 2002; Caretti & Lubello 2003), PAA is a stronger biocide for a wide spectrum of microorganisms (Baldry 1983; Baldry & French 1989), while HP requires much larger doses for the same level of inactivation (Wagner *et al.* 2002). Some of the desirable attributes of PAA are the easiness of treatment implementation, its broad spectrum of activity even in the presence of heterogeneous organic matter, and a minor dependence on the pH.

Regarding the specific mechanism of the PAA attack against microorganisms, one may speculate that PAA functions in a similar way to other peroxides and oxidizing agents; thus, possibly PAA disrupts sulfhydryl (–SH) and disulphide (S–S) bonds in proteins and enzymes, and then breaks important components in the membranes and inside the cell by oxidative disruption (Malchesky 1993). An important advantage of PAA is that it inactivates catalase, an enzyme that is known to act by inhibiting highly oxidant hydroxyl radicals (Block 1991). Additionally, intracellular PAA action may oxidize essential enzymes,

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impairing vital biochemical pathways, active transport across membranes and intracellular solute concentrations (Kitis 2004).

Different ways have been proposed to explain the chemical inactivation process. It can be thought that the oxidizing action takes place on the cellular wall or that, after regular or facilitated diffusion, the oxidant acts on the components of the interior of the bacteria or that in fact it operates with a combination of both processes. However, for optimization purposes, it is also very relevant to explain why PAA behaves in a manner so different than the one observed applying other disinfectants, for example, HP.

Research studies that show the synergistic effect between the PAA and HP are virtually non-existent, with the exception of the work of Alasri *et al.* (1992). In this work an experimental study adding different amounts of HP to a PAA solution was performed in order to observe those synergistic effects.

Therefore, for practical purposes, it is important to study the inactivation results produced by the mixture and identify the mechanism of the observed oxidation activity. Due to this fact, the use of commercial PAA as an alternative disinfectant was studied in this report. Its efficiency was tested employing a microbial indicator of water contamination, *Escherichia coli*, commonly used in this process.

MATERIALS AND METHODS

In all experiments, a well-stirred batch annular reactor having a total reaction volume of 2 L was employed. Stirring was achieved with an external orbital shaking device. A cooling jacket connected to a thermostatic bath surrounds the reactor to keep the reacting system at a constant temperature of 20 °C. The top of the reactor has provisions for sampling, pH and temperature measurements. For the experimental runs, a PAA commercial mixture (Química Agroindustrial Neo: PAA 15% v/v; HP 20%; acetic acid 25% and water 40%) was used. It is important to study separately the effect of the two oxidizing components of the mixture. Therefore, the reactant was also investigated free from HP. Inhibition of HP was achieved using catalase (from *Aspergillus niger*, Biochemika), allowing in this way the study of the efficiency of PAA alone.

Escherichia coli strain ATCC 8739 was used throughout this work. The culture was grown in a complex medium: a nutrient broth. The complete broth composition was: tryptone: 10 g L^{-1}; beef extract: 5 g L^{-1}; and NaCl: 5 g L^{-1}.

The bacterial inoculums remained in the stove for 24 hours at a constant temperature of 37 °C. The solution used for the experimental runs was prepared from a culture that had reached the beginning of the stationary phase of growth and afterwards was brought to a 1/1000 dilution with physiological saline. This dilution ensured that there was no bacteria growth during the inactivation run because the growing culture concentration was sufficiently diluted. The prepared culture was mixed with the desired concentration of PAA in the reactor.

The initial concentrations of bacteria a $t = 0$ were always around 10^5 CFU (colony forming units) mL^{-1}. Afterwards, samples were withdrawn at different intervals. To quench the PAA and HP action during the time interval between sampling and spread plating, a known volume of the sample was mixed with the required amount of sodium thiosulfate (200 μL) and catalase (500 μL) solutions respectively. These experiments were very effective in achieving their goals, which were twofold. Different concentrations of catalase and thiosulfate were tested until the obtained combination of the concentration of both compounds showed that (i) the desired inhibition was obtained and (ii) this combination did not affect in any way the existing population of bacteria. The plates were incubated for 24 h at 37 °C in an EMB (Eosin Methylene Blue) plate. Runs were duplicated and samples were subjected to triplicate determinations.

RESULTS AND DISCUSSION

PAA inactivation effects

Figure 1 shows the average results obtained during a series of inactivation tests that employed concentrations from 1 to 15 mg L^{-1} using the commercial PAA mixture. It is well documented that a plot of log of survivors versus time may give a straight line (type I), or curves with different shoulders (type II), or curves with shoulders and tails (type III). Thus, when the disinfectant concentration is changed, each family of curves represents more markedly the phenomenon that prevails in the different circumstances of the process. Curves named 'Type I' show clearly a rapid inactivation, with a small shoulder and an important portion of their trajectory having the characteristics of a straight line. Those of 'Type II' do not allow distinguishing with precision if they are the result of a very slow inactivation or a shoulder that extends for a very long time. Those of 'Type III' show a marked shoulder and tail, and therefore these cannot be represented by a straight line.

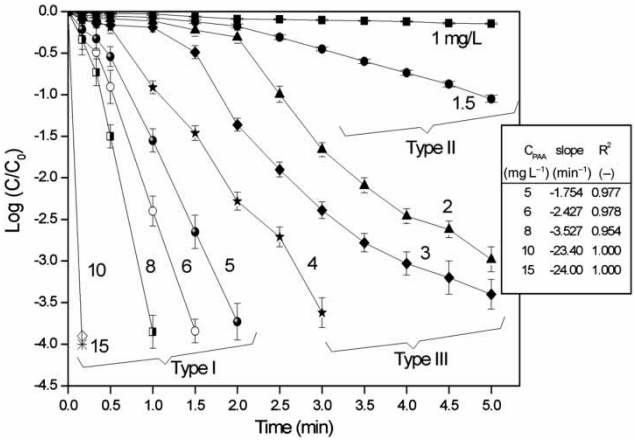

Figure 1 | Decrease in CFU as a function of time employing the commercial mixture of PAA. (Slope and R^2 for those plots that show features corresponding to a straight line in a significant portion of their trajectory.)

Notice, however, that for concentrations larger than 5 mg L^{-1}, the plots are straight lines and 99.99% inactivation is obtained in less than 2.1 minutes. It may be interesting to note that approximately 6 mg L^{-1} is coincident with the concentration usually applied in water disinfection processes when PAA is employed (Lefevre *et al.* 1992; Colgan & Gehr 2001). In the inactivation curves of Figure 1, two typical deviations from a straight line can be observed: shoulders and tails. There are several reasons for the shoulder: if clumps of microorganisms exist in the suspension, all cells in the clump needed to be inactivated before the colony-forming ability of the cluster is fully inactivated. Another possible explanation is that the bacteria populations exposed to low concentrations of disinfectant required a successive accumulation of injuries to reach their threshold limit.

The presence of tailing in an inactivation curve may have a different plausible interpretation: if some of the existing microorganisms are intrinsically more resistant than others, they can survive under the studied conditions and display a reduction in the inactivation rate and, consequently, the disappearance of the CFU will be appreciably slowed down. Additionally, the competition for the subsequent oxidation of the lysate (products resulting from the dead bacteria lysis) with the active bacteria for the existing oxidizing agents can contribute also to the tailings appearance.

Organic peroxides as PAA contain peroxide groups that are an indisputable source of high oxidation potential. In any event, these results should be compared with those obtained employing HP alone. They are summarized in Table 1. Values on the left were extracted from results published by Labas *et al.* (2008, 2009) for a total reaction time of

Table 1 | Inactivation results employing HP and commercial PAA

HP alone[a] (t = 150 min)		Commercial PAA (t = 5 min)	
H_2O_2 (mg L^{-1})	Inactivation (%)	PAA (mg L^{-1})	Inactivation (%)
15	80	1	28.4
45	92	2	99.9
160	99.9	5	> 99.99
185	99.99	15	> 99.99

[a]From Labas *et al.* (2009).

150 minutes, employing the same experimental procedure. From Table 1, in the experiments with the commercial PAA (2 mg L^{-1}) a much greater inactivation was obtained (99.9%) in just 5 minutes. To reach the same level of inactivation, in runs that lasted 2.5 h, using HP, a concentration of 160 mg L^{-1} was required.

Synergetic effect of the mixture

The next step is to elucidate if, when employing commercial PAA in the absence of HP, the experimental results show significant differences. Two sets of experiments were performed inhibiting HP activity. For PAA concentrations of 5 and 8 mg L^{-1} the results are shown in Figure 2. It can be seen that, to get the same level of inactivation (99.9%) achieved by the mixture of PAA without inhibition of the HP, it is necessary to increase the contact time by approximately three times.

This outcome implies a larger increase in the inactivation rate operating with the mixture. This is explained by the synergistic effect generated by the presence of HP.

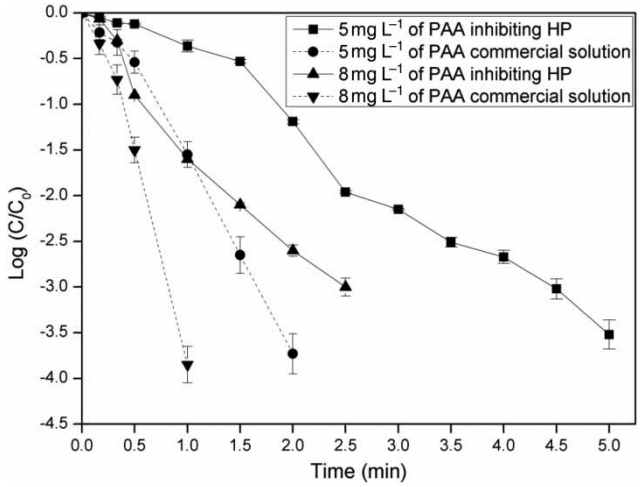

Figure 2 | Decrease in CFU as a function of time employing PAA without the presence of H_2O_2 and using the commercial PAA.

Interpretation of the obtained results

The following explanation of the results is based on the hypothesis that chemical inactivation is just a particular case of a rather unusual oxidation reaction mechanism. Table 2 shows an interesting comparison between the inactivating activity using HP intervening solely, PAA acting alone and the commercial mixture of HP and PAA.

From Table 2, it can be seen that to achieve 99.9% inactivation, the dose required is 24,000 and 22.5 mg min L^{-1} for HP and PAA respectively (the HP is 1,067 times slower than the PAA alone), and the inactivation process with the mixture requires a dose of 8.16 mg min L^{-1} and inactivation is 2.76 times faster than with PAA alone. Furthermore it can be seen that the effect of the mixture is greater than the sum of the individual effects of the two isolated disinfectants. Clearly there is a potentiating synergistic effect between HP and PAA.

The commercial mixture shows a unique result, which raises the thought of the existence of a very distinct mechanism of action. The generation of strong oxidative radicals from HP results from a well-known mechanism. The action of the HP is based primarily on the oxidation caused by hydroxyl radicals almost exclusively. On the other hand, one can venture to say that, in the case of the PAA, something substantially different takes place.

PAA oxidation mechanism

There may be more than one possible explanation to interpret the results of inactivation of *Escherichia coli* with the PAA. In this work, a tentative interpretation of the data is proposed, which advances a new approach to explain chemical inactivation processes for microorganisms. It is the result of adapting chemical oxidation reactions produced by the presence of hydroperoxide groups on organic substances.

The explanation proposed for the fast oxidation rate of PAA considers the homolytic PAA reaction proposed by Bach *et al.* (1996) and studied and confirmed in details by Rokhina *et al.* (2010). These authors have shown that a chain reaction occurs with a pathway described as follows:

$$CH_3C(=O)OOH \rightarrow CH_3C(=O)O^{\bullet} + HO^{\bullet} \quad (1)$$

$$CH_3C(=O)OOH + HO^{\bullet} \rightarrow CH_3C(=O)^{\bullet} + O_2 + H_2O \quad (2)$$

$$CH_3C(=O)OOH + HO^{\bullet} \rightarrow CH_3C(=O)OO^{\bullet} + H_2O \quad (3)$$

$$CH_3C(=O)O^{\bullet} \rightarrow H_3C^{\bullet} + CO_2 \quad (4)$$

$$2\,CH_3C(=O)O^{\bullet} \rightleftarrows 2\,H_3C^{\bullet} + 2\,CO + O_2 \quad (4a)$$

$$H_3C^{\bullet} + O_2 \rightarrow OOCH_3^{\bullet} \quad (5)$$

$$CH_3C(=O)O^{\bullet} + HO^{\bullet} \rightarrow CH_3C(=O)OOH \quad (6)$$

Reaction (1), which represents the initiation step, is very important because it forms the radical $HO^{\bullet}$ and it was found to be the rate controlling step. The authors claim that all the generated radical species are active contributors to any oxidation mechanism but $HO^{\bullet}$, and to some extent the $H_3C^{\bullet}$ radicals, are the most significant ones. The reaction requires the presence of an eligible catalyst that should be of the types usually encountered in Fenton or Fenton-like reactions (Bianchini *et al.* 2002). It has been shown that the existing intra- or extra-cellular Fe^{2+} is able to produce this type of reaction (Imlay & Linn 1988). It is important to note that only traces of some transition metal compounds are needed to induce the reactions mentioned above (Li *et al.* 1997; Nieto-Juarez *et al.* 2010; Jung *et al.* 2012).

Free radicals such as peroxy radicals, the superoxide anion, and the hydroxyl radical are responsible for many of the possible damaging reactions (McDonell & Russell 1999; Denyer & Maillard 2002). The chain reactions represented by Equations (1)–(6) may provide an adequate explanation for the rapid kinetics of inactivation by PAA.

Table 2 | Comparison of efficiencies of different processes of *Escherichia coli* inactivation (Temperature: 20 °C)

Disinfectant	Percent inactivation	Concentration (mg L^{-1})	Reaction time (min)	Dose; $D_{99.9}$ (mg min L^{-1})	Reference
HP	99.9%	160	150	24.000	Labas *et al.* (2009)[a]
PAA alone	99.9%	5	4.5	22.5	This work
PAA commercial mix	99.9%	5	1.6	8.16	This work

[a]From Labas *et al.* (2009).

Potentiated, synergetic effect of HP

From the dose results presented in Table 2, it can be concluded that the efficiency of HP is much lower than that of PAA acting alone. Moreover, a potentiating synergistic effect when working with the commercial mixture, having both PAA and HP, can be surely inferred. From the results presented in Figure 2, it should be noted that this enhancement happens only after the PAA has initiated the attack against the cell, indicating that the protecting systems that existed before have been removed and only then can HP participate actively in the rapid inactivation reaction.

Considering the above reasoning, as a first approximation to the inactivation reaction modeling with the PAA commercial mixture, the following scheme incorporating the bacteria attack in two stages can be proposed:

$$B_{Act} \xrightarrow[HP(very\ slow)]{} B_{Inj} \xrightarrow[HP(very\ fast)]{} B_{De} \quad (7)$$

$$B_{Act} \xrightarrow[PAA(very\ fast)]{} B_{Inj} \xrightarrow[PAA(fast)]{} B_{De} \quad (8)$$

Here, B_{Act} represents an active bacteria and B_{Inj} and B_{De} portrait injured bacteria and dead bacteria respectively. In this process, injured bacteria have suffered a certain level of damage, but they are not lysate. So, active bacteria plus injured bacteria are considered as viable bacteria.

When HP is used alone (reaction (1)), the inactivation rate is slow, since the first stage (from active bacteria to damaged bacteria) is the controlling step. In the case of the mixture of PAA having HP inhibited (reaction (8)), the reaction is much faster and the controlling step is the second (from bacteria damaged to dead bacteria). This can be explained because it is known that some microorganisms may be protected against HP by their catalase enzymatic activity. This enzyme does not act against PAA; in fact, this compound can also inactivate or inhibit catalase activity (Malchesky 1993; Wagner *et al.* 2002; Galvan *et al.* 2010).

When working with the commercial mixture of PAA (having HP), the contribution of HP to the process becomes important, but only after a fast PAA attack has occurred, producing damage in vital parts of the cell metabolism, particularly inactivating catalase. Therefore, PAA rapidly attacks the bacteria in the first stage, facilitating the subsequent attack of the damaged bacteria by HP. In a second stage, both PAA and HP, acting together, produce the very fast death of the bacteria, with a notable increase in the rate of inactivation as compared with the one observed when PAA acts alone (potentiating synergism).

CONCLUSIONS

Water disinfection employing a commercial mixture of PAA was studied. Experiments have demonstrated that there is a much greater inactivation efficiency of PAA (after inhibition of HP existing in the mixture) than that of HP alone.

The inactivation process with the commercial mixture of PAA (5 to 8 mg L^{-1}) is 2.76 times faster than with PAA alone. It can be seen that the effect of the mixture is greater than the sum of the individual effects of the two isolated disinfectants. A potentiating synergetic effect of the existing HP in the commercial mixture was found.

A tentative interpretation for the formation of strong oxidant species, based on a chain reaction and a scheme of attack on bacteria in two stages, has been proposed to explain the observed results.

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