Reaction of *B. cereus* Bacteria and Peroxidase Enzymes under Pressures >400 MPa

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It is known that *B. cereus* (Gram-positive bacteria) and peroxidase enzymes are resistant to pressures of ∼400 MPa in fruit and vegetable products among others. The aim of the present work is to have knowledge about their behavior when using pressures >400 MPa without other combined treatments. The results showed that *B. cereus* was not inactivated at pressures of 1000 MPa for 15 min at 20 °C. In peroxidase enzymes the results remained similar and the pressure of 1000 MPa for 30 min was not enough to inactivate them. The apple cell structure at these high-pressure levels revealed that it changed and the cells were less cemented. The treated apple presented a translucent aspect, and some fluids migrated from the inside to the outside of the cell.

Keywords: *High pressure; microorganisms; Bacillus cereus; peroxidase; apple; horseradish; cell structure*

INTRODUCTION

In Japan, pressure-processed foods, such as jams and jellies, first became available to the public in 1990 (*1*). Later, in 1991 more varieties of hihg-pressure (HP) processed products such as fruit yogurts, dressings, and sauces were also introduced to the market.

The knowledge acquired from the HP field has enabled the discovery of further new applications in food processing. In Spain, for instance, a commercial company (Esteban-Espuña S.A., Olot, Girona, Spain) processes HP-pasteurized ham, which is commercially available in the food market. As the food industry is interested in the use of high pressure, more new products and applications will be discovered in view of the growing interest with regard to this.

As a result of hydrostatic pressure, microbial inactivation has been extensively described (*2*−*9*) and particular attention has been paid to this, as a nonthermal method of food preservation. However, the information is still insufficient to fully understand the behavior of microorganisms in HP treatment.

It is generally admitted that high pressure induces a number of changes to microorganisms' morphology, biochemical reactions, genetic mechanisms, and cell wall membrane. Although most Gram-negative bacteria, molds, and yeast are inactivated by pressure of 400 MPa, Gram-positive bacteria are very resistant to HP treatment. This type of bacteria (Gram-positive) might respond to pressure by increasing its tolerance to pressure, triggering a physiological response to producing and synthesizing high-pressure resistant proteins, just as heat produces heat shock response (*10*).

Color is one of the attributes that changes in some fruits and vegetables after HP treatment (*9*) and probably the most significant one that must be controlled in order for the product to be acceptable to consumers. In addition to this, changes in color occur in some pressurized fruit due to HP treatment, giving the product a slight brown color (*8*, *9*, *11*, *12*). As a result, the HP treatment switches on some enzymatic reactions due to the oxidative stress developed, which are responsible for the browning (*11*, *13*−*16*).

Contrary to what is usually found with the application of HP to microorganisms, some enzymes may display activation or enhance, in addition, possible inactivation.

The involvement of polyphenol oxidase (EC 1.14.18.1; PPO) (*17*, *18*) and peroxidase (EC 1.11.1.7; POD) in enzymatic browning has been assumed by numerous authors (*11*, *15*, *17*−*19*).

Unexpectedly, some fruits, such as melon (*20*), have been reported to be more suitable for preservation with HP. However, apples are very sensitive to enzymatic browning due to their high concentration of phenolic compounds, polyphenol oxidase (*18*, *21*), and peroxidase enzymes (*15*). Taking into account that phenolic compounds are in all fruits as secondary metabolites, they play an important role in the processes of oxidation as either antioxidant or substrates (*22*).

Concerning the peroxidase activity, Préstamo and Arroyo (*15*) reported that in apples, HP of 400 MPa for 30 min at 5 °C was not enough to inactivate the activity of peroxidase. Less information is available for pressures >400 MPa.

The combination of scanning electron microscopy (SEM) and freeze−fracture is an excellent technique to visualize the interior of a cell, enclosed by the membrane, and the three-dimensional organization with exceptional clarity. Moreover, these techniques avoid any risk of artifacts caused by fixation. Therefore, they are very useful for revealing changes that the cell structure undergoes after pressurization. The changes in the permeability of the cell, due to the HP treatment, enable the movement of water and metabolites from the

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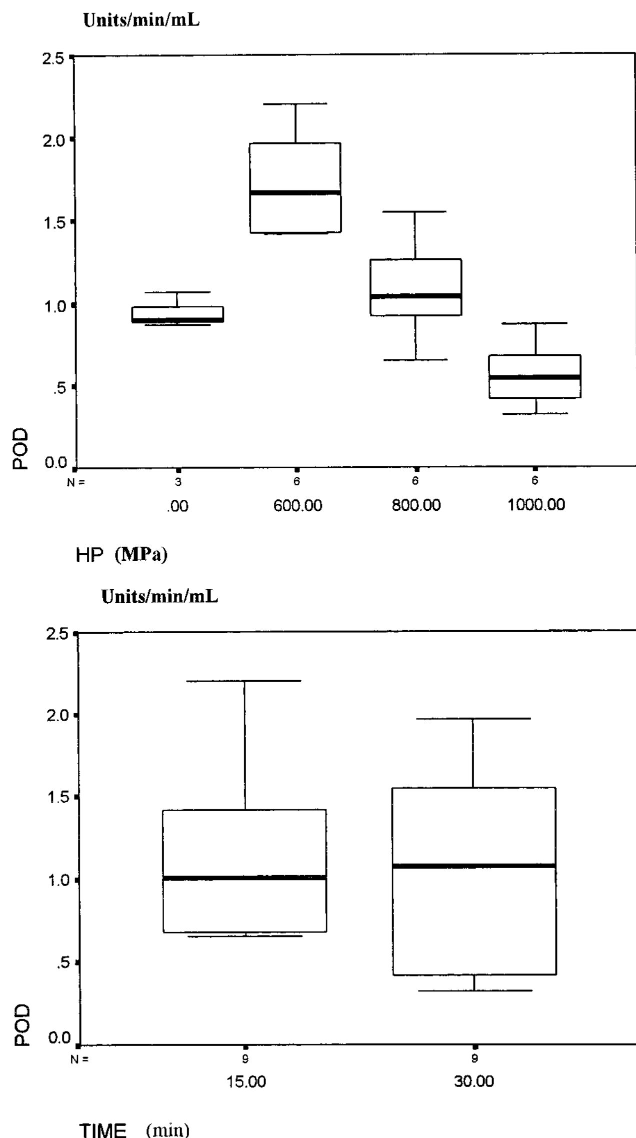

Figure 1. Apple POD units/min/mL in control samples and samples treated with HP of 600, 800, and 1000 MPa for 15 and 30 min at 20 °C.

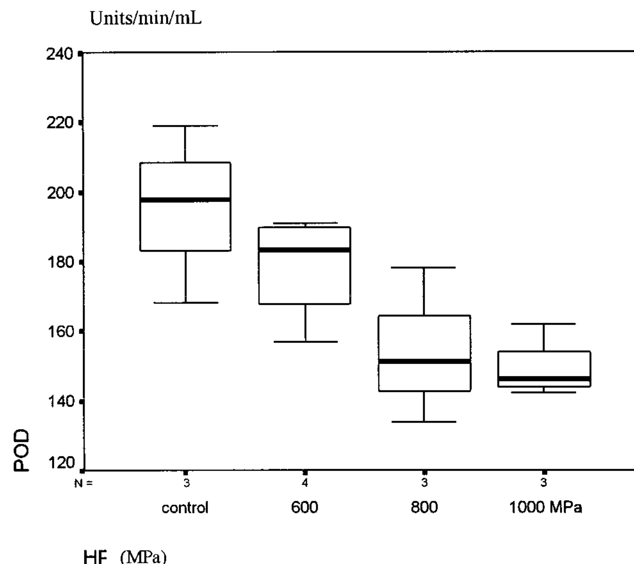

Figure 2. Horseradish POD units/min/mL in control samples and samples treated with HP of 600, 800, and 1000 MPa for 15 and 30 min at 20 °C.

inside to the outside of the cell. As a consequence of the breaking of the membranes in the compartments of the cell, an effect of transparency occurs (*12*).

The aim of this work is to know the effect of high pressure on peroxidase enzymes and *Bacillus cereus* bacteria at a pressure >400 MPa, without any other complementary treatment.

MATERIALS AND METHODS

Samples. Apples (Reineta variety) were purchased from the local market, peeled, and cut into pieces of ∼7−8 mm wide and 30 mm long. They were introduced afterward in freezing vials of 2 mL (Sigma) and filled with sterile distilled water. Pure POD enzyme (Sigma, horseradish) was prepared at 10 mg/50 mL concentration in sterile distilled water and kept in freezing vials of 2 mL to be subjected to HP treatment.

The interest in using apple and pure POD is to compare their behaviors and to prove that the increase of peroxidase after HP treatment is due to a major extraction of the enzyme from the product. When using pure enzyme, the activity has to remain stable or decrease but never increase.

Microbial Analysis. *Bacillus cereus* bacteria strain (Spanish type culture collection, Lab 56) was cultured on tryptone soy agar (TSA) at 30 °C for 24−72 h. This culture was then used to prepare suspensions on tryptone soy broth (TSB), which were incubated at 30 °C for 24 h. Freezing vials of 2 mL were filled with the grown culture to be subjected to HP treatment.

The number of microorganisms was determined by plate counting before and after HP treatment by plating 0.1 mL of culture of serial dilutions on the control samples and without dilutions in the treated samples. The microbial results were expressed as percent of viable bacteria in three replicate samples.

Peroxidase Activity. The preference of using POD enzyme is due to its high level of activity in relation to the PPO enzyme, although the inactivation of POD leads to the inactivation of PPO enzymes.

POD activity was determined following the method described by Préstamo and Manzano (*23*) using *o*-dianisidine as a chromogenic indicator. The peroxidase released from the product to the solution was used to measure the activity of POD. The total reaction volume was 1.5 mL, containing 1.200 mL of 50 mM sodium acetate, pH 6, buffer; 0.1 mL of 0.5% hydrogen peroxide; 0.1 mL of 0.25% *o*-dianisidine (w/v); and 0.1 mL of enzyme (0.005 mL in 0.095 mL of water for pure enzyme and 0.1 mL of released enzyme from apples). The reaction was measured at room temperature by spectrophotometry at 460 nm. POD activity was expressed in units of POD per milliliter. A unit of POD was defined to be an increase of 0.001 unit of absorbance per minute. The assay was made in three replicate samples.

High Hydrostatic Pressure. The samples were subjected to high pressure of 600, 800, and 1000 MPa for 15 and 30 min at 20 °C. These conditions have been chosen to complement previous work in which we observed that 400 MPa for 30 min was not enough to inactivate POD enzyme (*12, 15*) and *B. cereus* (*9*). The experiments were carried out in a piston−cylinder type high-pressure vessel with a maximum pressure of 1300 MPa, developed in the High-Pressure Research Center (Warszawa, Poland), using ethanol as a pressure-transmitting medium. The samples were pressurized in 2 mL freezing vials. After the pressure was released, the pressurized samples were kept at 5 °C for 2 days and then analyzed at room temperature.

The samples (*B. cereus*) subjected to pressures of 200, 300, 350, and 400 MPa for 30 min at 20 °C and at 400 MPa for 15, 30, and 60 min were carried out in a high-pressure apparatus (ACB GEC Alsthom, Nantes, France). The pump used in this case was a hydrostatic pump, and water was used as a fluid of low compressibility. The vessel capacity was 2.35 L in volume (steel container 100 mm in diameter and 300 mm in height).

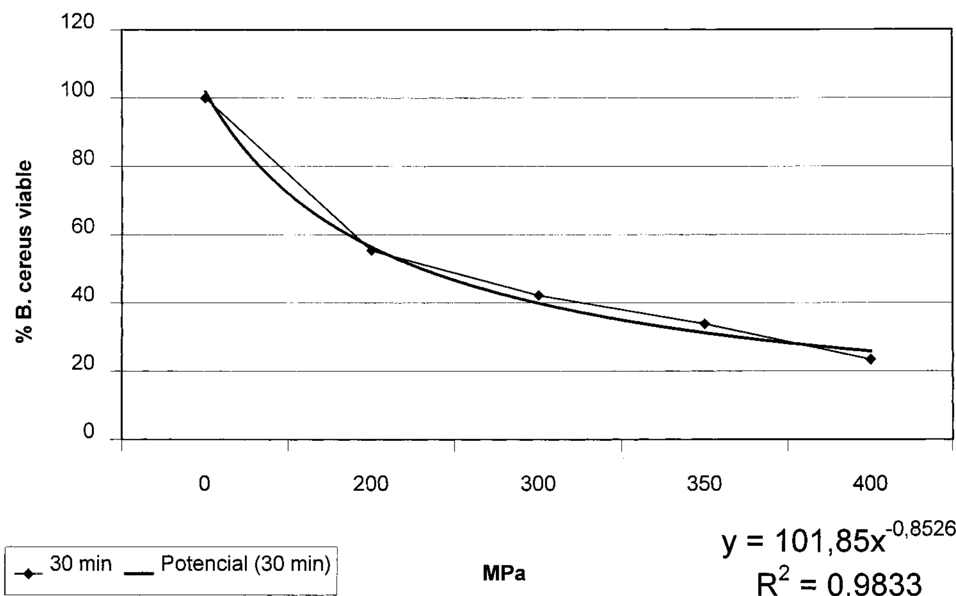

Figure 3. Percent *B. cereus* viable on control and samples treated with HP of 200, 300, 350, and 400 MPa for 30 min.

Low-Temperature Scanning Electron Microscopy. The combination of SEM (Zeis) and freeze−fracture is an excellent technique to study the structure of cells. The internal structure of apple cells was observed using the CT 1500 cryo-trans for SEM, whereby the samples can be examined for an extended period in a vacuum without loss of water by rapidly cooling and maintaining them below −150 °C. Preservation is an alternative and more satisfactory approach to determining the real structure than the fixation. The internal structure of cooled samples can be revealed by freeze−fracture provided care has been taken to minimize the growth of ice crystal.

The samples were adhered with O.C.T. compound (Gurr, a kind of glue) and mechanically fixed on a sample holder. Rapid freezing of the specimen in the cryostat was achieved by plunging the specimens into nitrogen slush, and the specimens were then transferred to the preparation unit via an airlock transfer device. The samples were fractured in the preparation unit and transferred directly via a second airlock to the microscope cold platform, where the samples were heated. Greater detail could often be revealed by carefully raising the temperature to a point at which water began to sublimate at a controlled rate (−90 to 100 °C for 2 min). This etching process was halted by rapidly lowering the temperature. Finally, it was usually necessary to coat the specimen with a film of conducting metal (gold) at low temperature to prevent charging during examination and photography. After coating, the specimen was transferred again to the Zeis SEM cold platform and scanned at a cold platform temperature of −135 °C using an accelerating voltage of 15 kV.

Statistical Analysis. All of the data were analyzed with the SPSS program following ANOVA one-way lineal model (time) and ANOVA two-way lineal model (time, pressure) using as Post-Hoc the Bonferroni test.

RESULTS AND DISCUSSION

This experiment was carried out to check some enzymatic response to pressures >400 MPa such as peroxidase and also as in some Gram-positive bacteria, such as *B. cereus*.

For a start, when the apple samples were subjected to 600, 800, and 1000 MPa for 15 and 30 min, respectively, the activity of POD increased at 600 MPa when compared with control, as shown in Figure 1, whereas at 800 and 1000 MPa, the activity decreased when compared with 600 MPa. In addition to this, the values of POD activity at 800 MPa were similar to the control value. Taking into consideration the statistical analysis, significant differences ($P \leq 0.05$) were found between the treated samples and the control, with the exception of pressure at 800 MPa, as shown in Figure 1.

On the contrary, the activity of the POD enzyme presented no significant differences between 15 and 30 min in all of the pressures assayed. Everything seems to indicate that the decrease of activity is not a factor of time but a factor of pressure.

When the POD was determined on pure enzyme (horseradish), the activity decreased at pressures of 600, 800, and 1000 MPa for 30 min, as shown in Figure 2, although there were no significant differences ($P \leq 0.05$) between the treated samples and the control in the Bonferroni test. However, in the DMS test, there are differences between the samples treated at 800 and 1000 MPa and the control. In short, the results of POD activity on pure enzyme and apple sample showed that pressures of 1000 MPa for 30 min were not enough to inactivate the POD enzyme. With regard to the browning effect developed on apples after the HP treatments, we found that the color was less dark at 1000 MPa than at a lower pressure, due to the diminution of the activity. As a result of these data, there is no doubt that the POD enzyme is an HP-resistant enzyme.

As for the microorganisms, whereas most Gram-negative bacteria are inactivated by pressures at 400 MPa, Gram-positive bacteria are quite resistant to HP (*8, 9*). Having this in mind, we chose *B. cereus* as Gram-positive bacteria to observe its behavior under HP.

Regarding *B. cereus*, it followed a decreasing potential curve when pressures of 200, 300, 350, and 400 MPa were applied for 30 min, as depicted in Figure 3. In other words, the data showed that the microbial population decreased when the HP increased, yet the pressure of 400 MPa for 30 min was not enough to inactivate *B. cereus*. Moreover, when pressures of 600, 800, and 1000 MPa were applied for 15 min to the *B. cereus* culture, the microbial population continued to decrease, as depicted in Figure 4. However, the pressure of 1000 MPa for 15 min was not enough to inactivate *B. cereus*. Besides this, the statistical analysis revealed that there were significant differences ($P \leq 0.05$) from the control in comparison with each of the samples treated at 600,

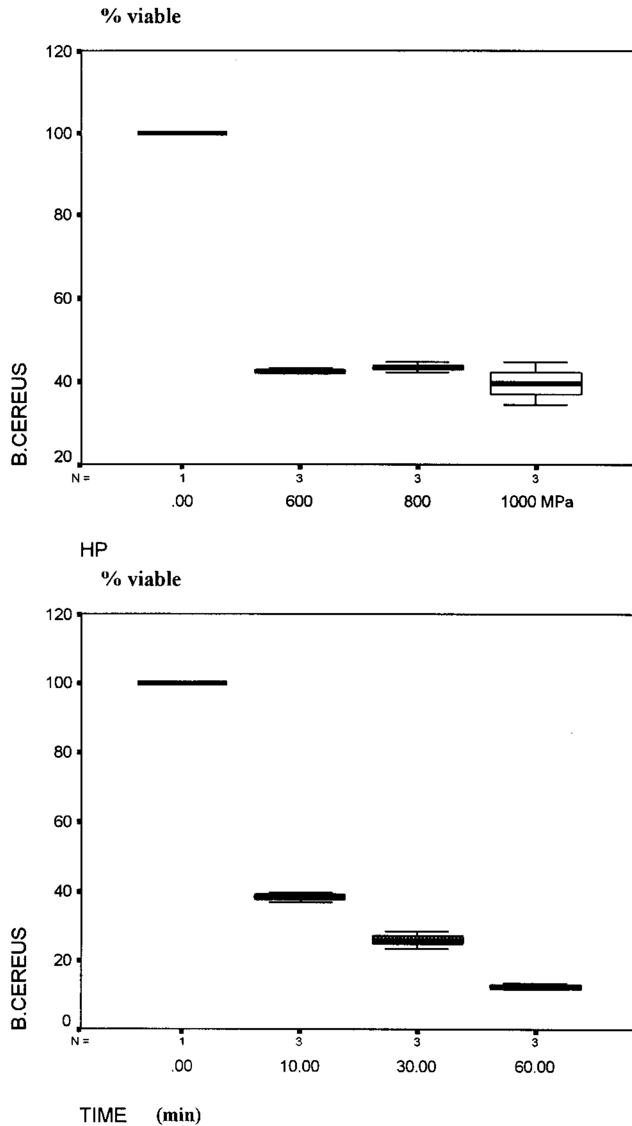

Figure 4. Percent *B. cereus* viable on control and samples treated with HP of 600, 800, and 1000 MPa for 15 min and percent *B. cereus* viable on control samples and samples treated with HP of 400 MPa for 15, 30, and 60 min.

800, and 1000 MPa for 15 min at 20 °C. Nonetheless, no significant differences were found between the treated samples.

Analyzing the behavior of *B. cereus* with regard to HP treatment and time, we have observed that the microbial population decreased and significant differences were found on the control in comparison with the samples treated at 400 MPa for 10, 30, and 60 min, respectively. However, no significant differences were found between the treated samples (Figure 4). At 400 MPa for 15 min, half of the population was inactivated. On the other hand, the same pressure for 60 min was not enough to inactivate *B. cereus*.

From the results, it is obvious that pressures of 400 and 1000 MPa for 60 and 15 min, respectively, at 20 °C were not enough to inactivate *B. cereus.* Consequently, *B. cereus* could be described as a pressure-resistant Gram-positive microorganism. All of this naturally leads to the conclusion that a treatment complementary to the HP treatment has to be done to inactivate Gram-positive bacteria, such as *B. cereus*.

The cryofracture observed in the SEM revealed a compact and turgid ultrastructure in apple cells for the control, as shown in Figure 5. Moreover, the cell had a large vacuole, which almost filled the whole cell. However, when the samples were treated at 1000 MPa for 30 min at 20 °C, we observed that the cell structure changed. As a consequence, the cells were less cemented with a translucent aspect and some fluids migrated from the inside to outside of the cell (Figure 6). The migrated fluids contain water and other constituents, such as salts, vitamins, and enzymes as well as substrates that also migrate. As a result of the HP treatment, the permeability of the cell changes, pushing the water and metabolites from the inside to the outside. Because of this, more metabolites are released into the solution (water). Préstamo et al. (*15*) reported that by measuring the electric conductivity they could determine the increase in the released metabolites. It is obvious that water is the main component of the cell and that it plays

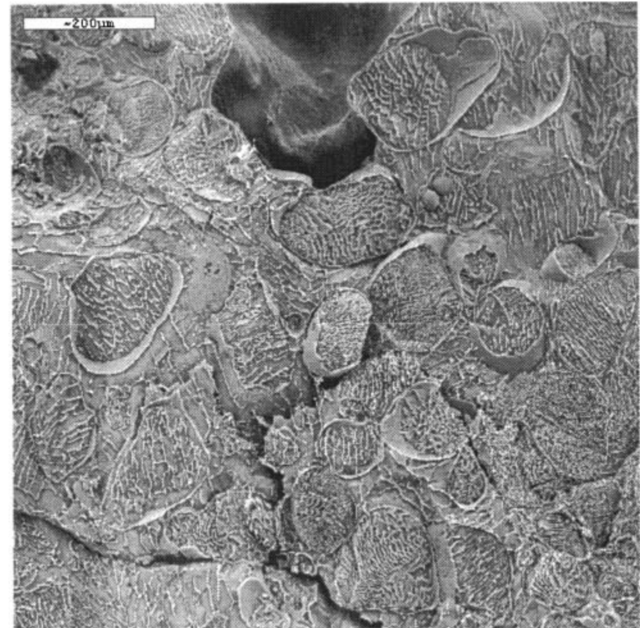

Figure 5. Apple freeze–fracture SEM on control.

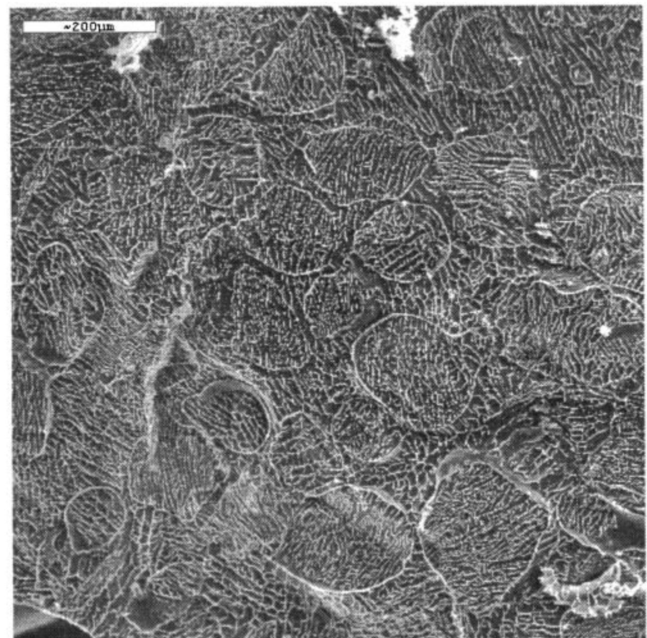

Figure 6. Apple freeze–fracture SEM on treated samples at 1000 MPa for 30 min at 20 °C.

an important role in all of the changes produced as a result of the HP treatment.

Fruits and vegetables are very sensitive to HP and suffer morphological alteration, tissue and cellular disruptions, slurries, and enhancement of enzymatic reactions (*13*). All of these naturally lead the POD enzyme to react with substrates producing the enzymatic browning. Besides that, it is well-known that some products such as apple subjected to HP produce a response to the browning (*11*−*13*) as a result of oxidative stress (*16*). We observed an increase of POD activity in apples when HP was applied, due to the higher extractability of the enzyme from the product. However, on pure enzyme, this increase was not detected. In this case, the activity of POD decreased gradually as HP increased, confirming that the increase of the POD mentioned above occurred due to the HP effect upon the product.

Little information on the behavior of POD under HP is available at pressures >400 MPa. At the start, the response of the product to HP tends to increase the activity of the POD because of the higher extractability, caused by the loss of permeability. Time seems to be a parameter with no significant difference on POD activity, as we observed in Figure 1 for 15 and 30 min of HP treatment. However, pressure plays an important role because when HP increases up to 800 MPa, the activity of POD decreased when compared with 600 MPa. This remarkable decrease is also observed in the changes of color that occur in the apples treated at 1000 MPa for 30 min, which presented a color close to that of the control, yet the apple treated at 600 MPa for 30 min had a dark brown color.

In the case of *B. cereus*, the response to pressures >400 MPa is that there are some remaining bacteria which are resistant to the HP treatment and about half of the *B. cereus* amount were inactivated. However, when the pressure was increased to 1000 MPa, some were still viable. Apart from that, similar values were found at 1000 MPa for 15 min and at 400 MPa for 60 min. In other words, when the time was increased to 60 min at 400 MPa, the *B. cereus* population decreased but not enough to be inactivated.

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