ESTIMATION OF THE THERMOCHEMICAL NONISOTHERMAL INACTIVATION BEHAVIOR OF *BACILLUS COAGULANS* SPORES IN NUTRIENT BROTH WITH OREGANO ESSENTIAL OIL

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ABSTRACT

The aim of this work was to validate the prediction of the nonisothermal thermochemical inactivation of *Bacillus coagulans* spores in nutrient broth, adjusted to 4 °Brix and pH 4.2, with 400 ppm of oregano essential oil (EO), using the Weibull dynamic model proposed by Peleg. First, the spores were heat treated at four constant temperatures. Afterwards, empirical secondary models were employed to describe the influence of temperature on the primary parameters. Finally, primary and secondary models were used to simulate microorganism inactivation under two nonisothermal temperatures, ranging from 90 to 95C every 1 min; and from 90 to 95C for 5 min at each temperature. Even if a limited experimental data was employed for the nonisothermal analysis, it appears that the proposed model can be used to estimate the nonisothermal inactivation of *B. coagulans* spores in nutrient broth with 400 ppm of oregano EO.

PRATICAL APLICATIONS

Oregano essential oil (EO) demonstrates an important antibacterial action against food spoilage microorganisms, as *Bacillus coagulans* spores. This EO is capable of decreasing the time of the spores' inactivation and, then, the food thermal processing time can be reduced. The industrial heat treatment of foods includes nonisothermal temperatures, as temperature fluctuations during the process, and it is important to understand the behavior of spoilage microorganism all through this temperatures oscillations.

INTRODUCTION

Heat treatment is the most common method for food preservation. The main goal of this treatment in the food industry, in order to project a microbiologically safe process, is to promote microbial inactivation. Most of the industrial processes include nonisothermal heating-up phases and temperature fluctuations during the process (Peleg 2006). Microbial responses under isothermal conditions may differ from the ones observed under dynamic conditions, thus compromising the process safety (Miller *et al.* 2010). Even though, the majority of studies related to microbial inactivation were carried out under isothermal conditions, in recent years, the literature has reported works on dynamic models, i.e., models that allow predicting microorganism death under variable temperature conditions (Conesa *et al.* 2003; Periago *et al.* 2006; Valdramidis *et al.* 2006).

Traditionally, to develop a nonisothermal predictive model, for either inactivation or growth, two steps are required (Valdramidis *et al.* 2008). First, kinetic parameters of a model describing the inactivation of microorganisms according to time, the so-called primary model, are estimated for at least four isothermal temperatures. Then, the influence of temperature on the inactivation primary

parameters is described by secondary models. Finally, primary and secondary models are used to simulate microorganism inactivation under dynamic temperature conditions. To validate the dynamic model obtained, experimental data are compared with model prediction. If predicted and measured results are close or similar to each other, it can be concluded that the parameters derived from isothermal inactivation are useful to predict the inactivation under nonisothermal conditions (Dolan 2003; Dolan *et al.* 2007; Valdramidis *et al.* 2008).

Bacillus coagulans is an important food spoilage microorganism. This thermotolerant spore-forming bacteria is able to germinate at pH values as low as 4, so it is often isolated from acid-canned vegetables (De Clerck *et al.* 2004; Lucas *et al.* 2006). Likewise, *B. coagulans* is responsible for the flat sour spoilage in canned products, which is a drastic acidification of media because of the production of lactic acid without gas formation (Lucas *et al.* 2006).

Oregano essential oil (EO) and other EOs derived from spices and herbs have had their antimicrobial activities tested against a wide range of food spoilage microorganisms. Oregano EO has been tested (1) in ground beef against *Salmonella* (Juneja *et al.* 2010); (2) in meat stored under aerobic, vacuum and modified atmosphere conditions against *Listeria monocytogenes* (Tsigarida *et al.* 2000); (3) in chitosan films against *B. cereus, Salmonella enteritidis*, *Staphylococcus aureus* and *Escherichia coli* (Pelissari *et al.* 2009); (4) in an active food packaging against *E. coli* and *St. aureus* (Becerril *et al.* 2007); (5) in carrot broth against *B. cereus* (Valero and Salmeron 2003); among many other studies. No study describing the antimicrobial action of oregano EO against *B. coagulans*, as the addition of this EO in canned foods, was found in the consulted literature. The relation between the antimicrobial and organoleptic effect of EOs in foods should be also taken into account (Haberbeck *et al.* 2012). Sensory evaluation in Mediterranean octopus resulted in a limit of acceptability for odor of 6 days in samples without oregano EO, and of 17 and 23 days in samples added with 2,000 and 4,000 ppm of oregano EO, respectively (Atrea *et al.* 2009).

The objective of this study was to validate the prediction of the thermochemical inactivation of *B. coagulans* spores in nutrient broth (NB) with 400 ppm of oregano EO under variable temperature, using the dynamic model proposed by Peleg (2006). The obtained model was compared with experimental data under two temperature profiles. To achieve this goal, the inactivation curves of *B. coagulans* spores in NB with 400 ppm of oregano EO under four different temperatures (90, 95, 97 and 100C) were fitted with the Weibull model and a secondary model was used to represent the temperature dependence of inactivation parameters.

MATERIALS AND METHODS

Strain and Preparation of Inocula

B. coagulans ATCC7050 was pre-cultivated in NB (HiMedia, Mumbai, India) at 37C for 24 h. The microorganism sporulation was performed in petri dishes containing nutrient agar (Biolife, Milan, Italy) supplemented with 5 ppm of manganese sulfate (Vetec Quimica Fina Ltd., Rio de Janeiro, Brazil) (Pacheco and Massaguer 2004). Then, plates were incubated over 10 days at 37C; previous studies, carried out in our laboratory, showed that these conditions resulted in the most resistant *B. coagulans* spores. After incubation, spores were collected by flooding the dish surfaces with sterile distilled water, followed by scratching them with sterile rubber rod. The collected spores were sedimented by centrifugation (2,000 × g, 15 min) and washed with sterile distilled water. The centrifugation and washing steps were accomplished five times. The final spore suspension was stored under refrigeration until used. The population density was determined by serial dilutions in 0.1 % peptone water, then dilutions were pour plated in tryptone dextrose agar ([TDA] Biolife). The plates were incubated at 37C for 48 h to determine the initial number of bacterial spores expressed in CFU/mL.

Oregano EO

The EO was provided by Givaudan Brazil Ltda. (Sao Paulo, Brazil). Oregano EO was emulsified in order to improve its solubility. Soy lecithin (Alfa Aesar, Ward Hill, MA) was used as surfactant. Initially, the organic phase (EO + soy lecithin) was stirred magnetically for 50 min, at a ratio of 4% (m/m) of soy lecithin/OE. The aqueous phase (NB + distilled water) was added to the organic phase, at a rate of 4 g of aqueous phase per g of organic phase. Then, they were agitated for 20 min in a magnetic stirrer. After that, the solution underwent sonification using an ultrasound (Sonic Dismembrator Model 500, 400 W, Fisher Scientific, Pittsburg, CA) for 4 min with 70% amplitude. The emulsion was stored under refrigeration until used.

Heat Medium

NB was prepared with distilled water, and adjusted to 4 °Brix by adding glucose (Nuclear, Sao Paulo, Brazil), standardization was performed with the help of a digital refractometer (AR200, Reichert, Depew, NY). The medium pH was standardized at 4.2 by adding citric acid solution at 18% (v/v) and measured through a pH meter (AN2000, Analion, Sao Paulo, Brazil). Soluble solid concentration and pH values were chosen aiming at simulating tomato pulp, the product in which the oregano EO can be easily

employed. The heat medium was autoclaved at 121C for 15 min. There was no change in soluble solids and pH after this treatment.

Thermochemical Inactivation under Isothermal and Nonisothermal Conditions

Inactivation tests were performed by using sealed thermal-death-time (TDT) tubes (glass tubes of 8×120 mm with wall thickness of 1 mm) (Stumbo 1978). Contact time between *B. coagulans* and oregano EO before heat treatment was standardized at 15 min. The heating medium, containing appropriate concentrations of homogenized EO emulsion, was inoculated with spores of *B. coagulans*, and the contact time started being recorded immediately. The oregano EO concentration was chosen as 400 ppm for all test, according to previous research (Haberbeck *et al.* 2012).

Initial concentration of bacterial spores was, approximately, 10^6 CFU/mL. Over the contact time, TDT tubes were filled with 2.0 mL of the solution (NB + EO + spore suspension), and then they were sealed by gas flame (LPG/O$_2$). After the contact time, TDT tubes were submerged into a thermostatic water bath. The come-up-time for the temperature in the TDT tubes has been determined experimentally with thermocoples, and the average of 2 min has been assumed for all temperatures. Next, TDT tubes were individually removed in predetermined times and immediately cooled in an ice bath.

For the nonisothermal inactivation, two water baths were used; each bath at a temperature of the inactivation condition. The tubes were placed in an iron grid and displaced between the baths according to predetermined times. A thermocouple was placed inside a sealed TDT, and its recovery temperature was used to determine the nonisothermal temperature profile.

TDT tubes were aseptically opened with the aid of a diamond glass cutter. Population density was determined by serial dilutions in 0.1 % peptone water, and dilutions were pour plated in TDA. The plates were incubated at 37C for 48 h to determine the number of bacterial spores expressed in CFU/mL.

The isothermal inactivation, with EO concentration fixed at 400 ppm, were performed at 90, 95, 97 and 100C. For the nonisothermal inactivation with 400 ppm of EO, two different temperature profiles were studied. In profile 1, the temperature ranged from 90 to 95C every 1 min for 13.5 min, and in profile 2, from 90 to 95C for 5 min at each temperature in a total of 10 min. During the experiments, temperatures were recorded every 5 s by thermocouples attached to a data acquisition system (Agilent System Acquisition 34970a, Santa Clara, CA).

Nonisothermal Mathematical Model

For primary modeling, the Weibull distribution function (Eq. 1) was adjusted to the experimental data.

$$\log S(t) = -b(T) t^{\alpha(T)} \tag{1}$$

where $S(t)$, the momentary survival ratio, is equal to $N(t)/N_0$; N_0 (CFU/mL) is the initial number of spores in the heat-treated population, and $N(t)$ (CFU/mL) is the number of spores after time t (min); b is a parameter of the model, α is known as the shape factor, and T is the temperature (C).

The influence of the temperature or EO concentration on parameter b was described by an exponential equation (Eq. 2).

$$b(T) = a \cdot \exp(c \cdot T) \tag{2}$$

where a and c are empirical parameters of the equation; T is the temperature (C).

Peleg (2006) has proposed a nonisothermal model for microorganism inactivation that had a good fit with the Weibull primary model. The derivate of the isothermal Weibull model (Eq. 1), with respect to time, is shown in Eq. (3).

$$\frac{d \log S(t)}{dt} = -b(T) \cdot \alpha \cdot t^{\alpha - 1} \tag{3}$$

The time that corresponds to the survival ratio at any given moment, determined as t*, is the inverse of Eq. (1) and is shown in Eq. (4):

$$t^\star = \left(\frac{-\log S(t)}{b(T)} \right)^{\frac{1}{\alpha}} \tag{4}$$

The empirical $b(T)$ model was combined with the temperature profile models of the nonisothermal treatments to produce the corresponding $b[T(t)]$. Replacing the time t in Eq. (3) by $t^\star$ (Eq. 4) and the constant temperature T by the treatment's temperature history $T(t)$, provides the nonisothermal inactivation rate equation (Eq. 5).

$$\frac{d \log S(t)}{dt} = -b[T(t)] \alpha \left\{ \frac{-\log S(t)}{b[T(t)]} \right\}^{\frac{\alpha - 1}{\alpha}} \tag{5}$$

Eq. (1) was fitted to experimental isothermal inactivation curves at 90, 95, 97 and 100C through the program Matlab (The MathWorks Inc., Natick, MA). And Eq. (2) was adjusted to the experimental data of parameter $b(T)$ values related to the temperature through the program Excel (Microsoft Corporation, Redmond, WA).

The temperature profiles during nonisothermal inactivation, obtained with the data acquisition system, were

described by a sinusoidal equation, as given by Eq. (6), through the program Matlab (The MathWorks Inc.).

$$T(t) = a_1 \cdot \sin(b_1 \cdot t + c_1) + a_2 \cdot \sin(b_2 \cdot t + c_2) + \ldots + \\ a_8 \cdot \sin(b_8 \cdot t + c_8) \qquad (6)$$

where $T(t)$ is the temperature profile (C) at time t, and a_i, b_i and c_i are the model parameters.

The expressions $b(T)$ and $T(t)$, then calculated, were combined to produce the $b[T(t)]$ term for each nonisothermal profile. The resulting $b[T(t)]$ was then inserted into Eq. (5). The program Matlab (The MathWorks Inc.) was used to solve the dynamic Weibull-type model (Eq. 5) by means of an ode15s solver. The equation was solved numerically and compared with the experimental inactivation curves.

Statistical Analyses

In order to check the quality of the fits, the following statistical parameters were calculated: correlation coefficient (R^2) and mean square error (MSE). The correlation coefficient (R^2) measures the fraction of variation over the mean that is explained by a model. The higher the value ($0 < R^2 < 1$), the better the prediction by the model is (Jin *et al.* 2009). The MSE (Eq. 7) shows the modeling error for data, i.e., how close the predicted values are to observed values (Zimmermann *et al.* 2011).

$$MSE = \frac{\sum (v_{observed} - v_{predicted})^2}{n - p} \qquad (7)$$

The value of experimental data is given by $v_{observed}$; the value estimated by the model is given by $v_{predicted}$; while n is the number of experimental observations and p is the number of parameters in the model.

RESULTS

First, in order to test the efficiency of EO emulsion, a thermochemical resistance with 500 ppm of oregano EO, concentration chosen randomly, was performed with non-emulsified EO and emulsified with soy lecithin (data no showed). The time needed to reduce a 6 log level of spores with pure EO was 2.8 min, whereas with the EO emulsion it was 1.4 min. Thus, all the following analyses were accomplished with emulsified EO.

Figure 1 shows the isothermal inactivation curves of *B. coagulans* at 90, 95, 97 and 100C with 400 ppm of oregano EO, fitted by the Weibull model with a fixed parameter α, equal to 2.65 (Haberbeck *et al.* 2012). As already shown in the literature, the value of α does not vary with temperature and it can be considered constant (Van Boekel 2002; Corradini *et al.* 2008). Previous work from this research group, showed that the parameter α, from the Weibull model with a varying α, does not change significantly with temperature and it can be considered constant for the temperature range of 90–100C in the heat medium added with 400 ppm of oregano EO (Haberbeck *et al.* 2012). The value of 2.65 is the mean value of α for the Weibull model with varying α, adjusted to this experimental
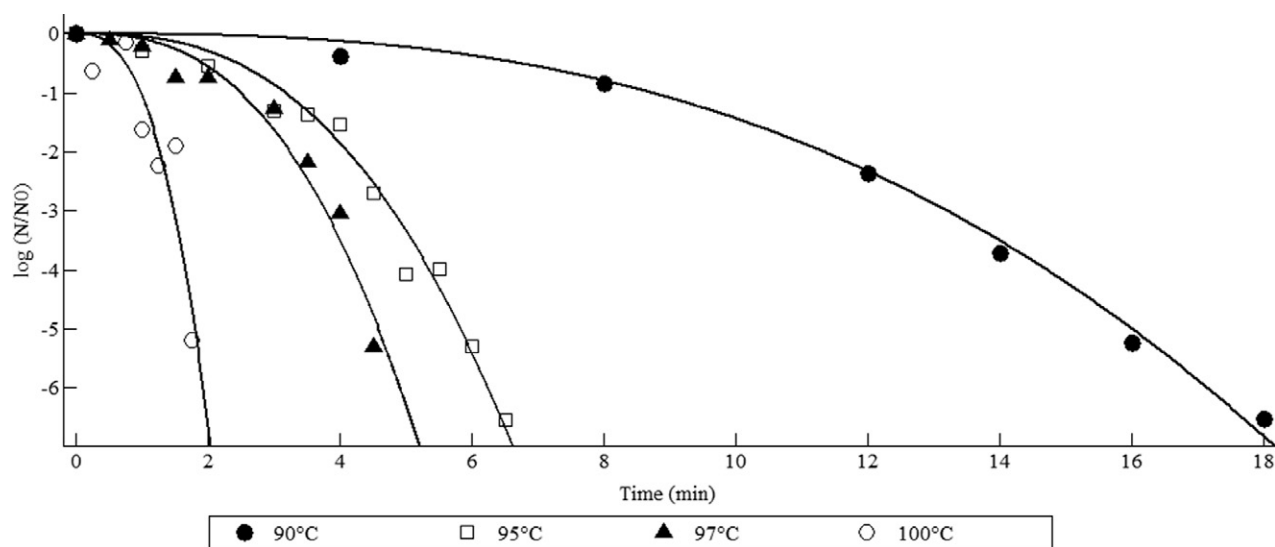

FIG. 1. EXPERIMENTAL INACTIVATION ISOTHERMAL CURVES OF *BACILLUS COAGULANS* IN NUTRIENT BROTH (4 °BRIX, PH OF 4.2) WITH 400 PPM OF OREGANO ESSENTIAL OIL THE CONTINUOUS LINE REPRESENTS THE FIT OF THE WEIBULL MODEL WITH A FIXED PARAMETER α TO THE EXPERIMENTAL DATA

TABLE 1. MEAN VALUE AND SD OF THE PARAMETER *b* FOR INACTIVATION OF *BACILLUS COAGULANS* SPORES WITH 400 PPM OF OREGANO ESSENTIAL OIL

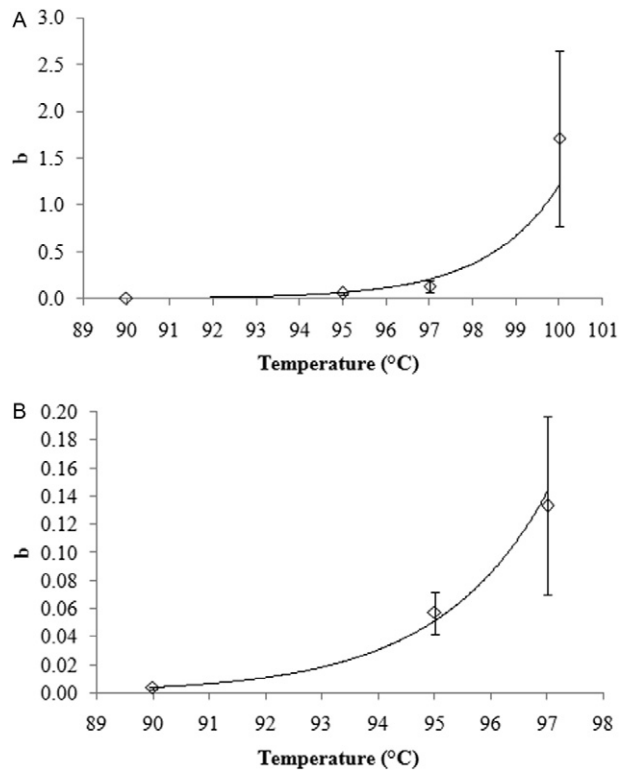| Temperature (C)	$b \pm SD$
90	0.004 ± 0.001
95	0.057 ± 0.015
97	0.133 ± 0.063
100	1.716 ± 0.939

SD, standard deviation.

isothermal inactivation data (Haberbeck *et al*. 2012). In Fig. 1, each survival curve at different temperatures, is represented by the most thermo-resistant curve. Table 1 shows the mean value and the standard deviation of the inactivation parameter *b* obtained by the Weibull model with a fixed α.

The temperature dependence of $b(T)$ and the fit of the exponential equation (Eq. 8) as its model is shown in Fig. 2. Eq. (8) had a good fit to the experimental values of *b* as can be seen both visually and through the R^2 value next to 1.

$$b(T) = 2.10^{-26} \exp(-0.593T) \; R^2 = 0.98 \quad (8)$$

By means of the primary and secondary models, it was possible to establish a nonisothermal model, based on the model proposed by Peleg (2006), to predict the behavior of *B. coagulans* within the isothermal temperature range studied (90–100C), under fluctuating temperature conditions. Figure 3 shows results for the nonisothermal profile 1, 90–95C for 13.5 min with cycles of 1 min at each temperature; and Fig. 4 for profile 2, 95–90C for 10.5 min with cycles of 5 min at each temperature. The continuous lines in Figs. 3a and 4a show the simulation of microbial inactivation under a nonisothermal condition, as given by Eq. (5),

FIG. 2. THE TEMPERATURE DEPENDENCE OF THE PARAMETER (A) $b(T)$ OF *BACILLUS COAGULANS* WITH 400 PPM DESCRIBED BY EQ. (2); (B) RESCALED GRAPHIC (A) FROM TEMPERATURE 90 TO 97C

considering the secondary model for *b* shown by Eq. (8). The temperatures profiles were shown in Figs. 3b and 4b, the continuous lines represent the fit of Eq. (6) to the experimental temperature profile.
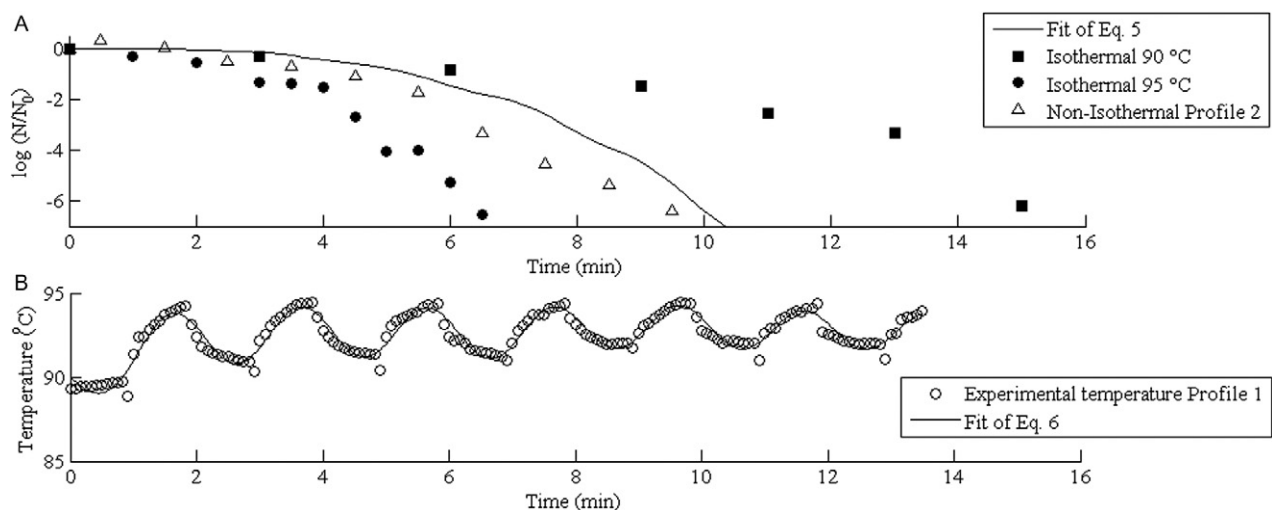
FIG. 3. (A) *BACILLUS COAGULANS* EXPERIMENTAL DATA FOR ISOTHERMAL AND NONISOTHERMAL INACTIVATION OF PROFILE 1 (90–95C/1 MIN). (B) EXPERIMENTAL TEMPERATURE PROFILE 1
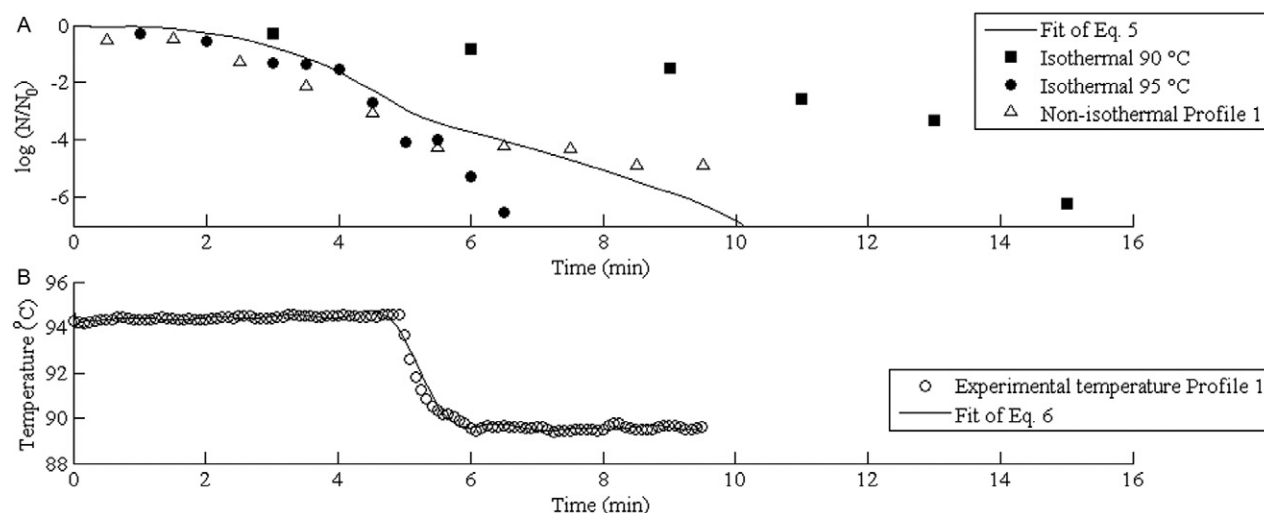
FIG. 4. (A) *BACILLUS COAGULANS* EXPERIMENTAL DATA FOR ISOTHERMAL AND NONISOTHERMAL INACTIVATION OF PROFILE 2 (95–90C/5 MIN). (B) EXPERIMENTAL TEMPERATURE PROFILE 2

DISCUSSION

An overestimation between predictions and experimental data for profile 1 is observed in Fig. 3a. The thermochemical inactivation curve with the nonisothermal profile 1 gave a MSE equal to 0.9. Fig. 3b shows the good fit of Eq. (6) to the experimental values of temperature oscillation as the correlation coefficient was equal to 0.92.

The predicted thermochemical nonisothermal curve and the experimental data for temperature profile 2 (Fig. 4a) during the first 5 min was in agreement with the thermochemical isothermal experimental data at 95C. After that, when the temperature was changed to 90C, the experimental inactivation rate became noticeably lower. The thermochemical inactivation curve with the nonisothermal profile 2 gave a *MSE* equal to 1.2. The sinusoidal equation (Eq. 6) had also fitted well to the experimental temperature for profile 2 (Fig. 3b), with R^2 equal to 0.9.

For both nonisothermal temperatures profiles, it is possible to notice that observed and predicted thermochemical nonisothermal curves are located between the thermochemical isothermal inactivation data of 90 and 95C. Therefore, the nonisothermal results are in agreement with the isothermal ones.

In order to validate a predictive model for nonisothermal treatment, the model should anticipate the outcome of experiments whose results were not used in its formulation (Peleg 2006). The majority of data for nonisothermal tests are based in heat treatments at various rates (Mattick *et al.* 2001; Periago *et al.* 2004; Valdramidis *et al.* 2006), while, this study took a different approach, where temperature fluctuated between 90 and 95C, in a range of predetermined

times. Most of the heating curves in industrial processes are smooth, the temperature variation should be, normally, between 2 and 3C. However, a faulty temperature control can cause significant oscillations, as well as sudden temperatures changes, like those produced by an interruption in the steam supply, for example. The study of predictive models for oscillating temperatures is particularly useful in the evaluation of the impact of accidents and unplanned interruptions in the process on the residual survival ratio of the target organism or spore (Peleg 2006).

In the current study, the nonisothermal inactivation model proposed by Peleg (2006) was validated by comparing the nonisothermal experimental data obtained by two temperature profiles with the nonisothermal data generated by the model. The primary Weibull model showed a good fit to the isothermal inactivation data of *B. coagulans* in NB with 400 ppm of oregano EO at isothermal temperatures (Fig. 1). The influence of the temperature on parameter *b*, resulting from the primary modeling, could be well described through Eq. (8) (Fig. 2). Likewise, a sinusoidal equation (Eq. 6) showed a good fit to both temperature profiles. Thus, from the secondary and primary models, the nonisothermal model pointed by Peleg (2006) was established and validated to predict the *B. coagulans* spore thermochemical inactivation under variable temperature conditions.

The difference between predicted and observed inactivation values can be attributed to a number of reasons. The experimental data can afford some errors, because they were not in duplicate. The sealed TDT may have had different temperatures from the TDT with thermocouples, because the latter was not sealed by a gas flame. Another

cause would be a secondary model that does not properly describe the primary parameter. However, Eq. (8) showed a good fit to the experimental values of the parameter *b*, mainly between the temperatures used for the nonisothermal inactivation, 90 and 95C, as can be seen is Fig. 2b. One more remark can be made about the two nonisothermal experiments; temperature reached values slightly above instead of exactly 95C. For temperature profile 1, the lowest temperature was between 91 and 92C, not exactly at 90C. On the other hand, for temperature profile 2, the lowest temperature was slightly below 90C.

Mattick *et al.* (2001) analyzed the nonisothermal inactivation of *Salmonella* cells in two sugar-rich medium heated at various rates to selected temperatures in the range of 65–80C. The predictive Weibull model proposed by Peleg (2006) was also adequate to describe the nonisothermal survival curves with good accuracy. Periago *et al.* (2004) observed the nonisothermal curves of *B. sporothermodurans* spores in three soups. Even though they also employed a limited database for the analysis, the survival patterns of the spores under nonisothermal conditions could be well estimated by the nonisothermal Weibull model.

Although only a limited experimental database was employed for the analyses and the predicted values slightly overestimated the observed values, the survival parameters can be used to estimate the inactivation patterns of the spores under nonisothermal heating treatments at least approximately. Future studies can be performed with another nonisothermal model, as well as with more oscillating temperature profiles and their replicates in order to better understand the behavior of *B. coagulans* spores under thermochemical nonisothermal inactivation.

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