LWT - Food Science and Technology 46 (2012) 267–273

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Bacillus coagulans spore inactivation through the application of oregano essential oil and heat

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ARTICLE INFO

Article history:
Received 24 May 2011
Received in revised form
22 August 2011
Accepted 29 September 2011

Keywords:
Bacillus coagulans
Oregano essential oil
Isothermal inactivation
Weibull model

ABSTRACT

The present study evaluated the effect of thermal (temperature) and thermochemical (temperature + oregano essential oil (EO)) inactivation of *Bacillus coagulans* spores in Nutrient Broth (NB) adjusted at 4 °Brix and pH of 4.2. Thermal treatments included temperatures between 95 and 103 °C. For thermochemical treatment, first temperature was fixed at 100 °C and EO concentration varied between 250 and 1000 μg/g. Thermochemical treatment significantly reduced the time needed to reduce a 6 log level of spores compared to thermal treatment, for example around 1.4 min with 400 μg/g of EO. Then, EO concentration was fixed at 400 μg/g and temperature varied between 90 and 100 °C. Although the first results showed a faster spore reduction with 500 μg/g, the fixed EO concentration was 400 μg/g, since it represents a lower organoleptic impact and also a significant reduction in the spores' resistance. For instance, at 97 °C and 400 μg/g, about 4.3 min was needed to reduce the spores in 6 log, without the EO this time was 5.0 min. These findings indicate that oregano EO may be used to render *B. coagulans* spores more susceptible to the lethal effect of heat.

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1. Introduction

Bacillus coagulans, a non-pathogenic, facultative anaerobic, thermotolerant and acidophilic bacteria, is an important food spoilage microorganism. In the canned vegetable industry where foods are acidified to pH values between 4 and 4.5, this bacterium is frequently found, since spores of *B. coagulans* are able to grow and germinate at pH values as low as 4 (De Clerck et al., 2004; Lucas et al., 2006). Moreover, this bacterium is capable of increasing the pH of food products to values that may allow for germination of surviving *Clostridium botulinum* spores (Viedma et al., 2010). Besides, *B. coagulans* has caused considerable economic loss for the food industry because of the "flat sour spoilage", which is a drastic acidification of the food product due to the production of lactic acid without gas formation (Lucas et al., 2006). For official protocols to validate low acidity foods heat sterilization, *C. botulinum* spores are the target microorganism and the temperature reference is 121.1 °C. Nevertheless, heat resistant mesophilic spore formers such as *Bacillus sporothermodurans* (Periago et al., 2004) and *B. coagulans* may often determine the stability of foods and safety of industrial processes.

Lately, the food industry has been concerned about the consequences of chemical preservatives in consumers' health, and also consumer demand for safe and high-quality foods is increasing. Therefore, the research for natural preservatives is facing an increase of new approaches and technologies. Particularly, essential oils from herbs and spices have demonstrated antimicrobial activity against a broad spectrum of microorganisms (Burt, 2004; Tajkarimi, Ibrahim, & Cliver, 2010). The addition of 2000 and 4000 μg/g of oregano EO in fresh octopus stored under vacuum packaging and at 4 °C, increased the shelf life in 8 and 14 days, respectively (Atrea, Papavergou, Amvrosiadis, & Savvaidis, 2009).

Mathematical models are developed and analyzed in predictive microbiology in order to describe microbial behavior (inactivation, growth and survival) as a function of environmental factors (Janssen et al., 2008) such as temperature, pH and preservative concentrations, among others. The mathematical model based on the Weibull distribution has attracted attention due to its simplicity and flexibility (Fernandez, Lopez, Bernardoa, Condon, & Raso, 2007). Different shapes of inactivation curves can be described through the Weibull model: log-linear, convex and concave (Peleg, 2006).

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doi:10.1016/j.lwt.2011.09.021

The aim of this study was to determine the thermal (temperature) and thermochemical (temperature + oregano EO) inactivation of *B. coagulans* spores in nutrient broth (4 °Brix and pH of 4.2) under isothermal conditions.

2. Materials and methods

2.1. Strain and preparation of inocula

B. coagulans ATCC7050 was pre-cultivated in NB (Himedia, India) at 37 °C for 24 h. The microorganism sporulation was performed in Petri dishes containing Nutrient Agar (Biolife, Italy) supplemented with 5 μg/g of manganese sulfate (Vetec, Brazil) (Pacheco & Massaguer, 2004). Then, plates were incubated over 10 days at 37 °C; previous studies, carried out in our laboratory, showed that these conditions resulted in the most resistant *B. coagulans* spores. After incubation, spores were harvested by flooding the medium surface with sterile distilled water and gently rubbing it with a sterile rubber rod. The collected spores were sedimented by centrifugation (2000×*g*, 15 min) and washed with sterile distilled water. The centrifugation and washing steps were accomplished five times. The final spore suspension was stored at 4 °C until used. The population density was determined by serial dilutions in 0.1 g/100 g peptone water, then dilutions were pour plated in Tryptone Dextrose Agar (TDA) (Biolife, Italy). The plates were incubated at 37 °C for 48 h to determine the initial number of bacterial spores expressed in CFU/mL.

2.2. Oregano essential oil

The oregano EO was provided by Givaudan Brazil Ltda. (Sao Paulo, Brazil). EO main compounds were identified by GC-MS analysis. The analysis was performed on GC-MS chromatograph (Varian GC-3800, MS/MS Varian 1200L), VF5-MS column (30 m × 0.25 mm, 0.25 μm) (Varian) using split injection mode with a flow ratio of 1:10. The operating conditions: injector temperature was 300 °C, carrier gas was helium (He) at a flow rate of 1 mL/min, initial column temperature was 60 °C (maintained for five min), warming at a rate of 5 °C/min up to 250 °C (maintained for 5 min). The detector and mass spectrometry in scan mode was in the range of 40–400 *m*/*z*. The compounds were identified through a data base for natural products (Standard Reference Data Series of the National Institute of Standards and Technology-NIST – Mass-Spectral Library with Windows search program-Version2), where the mass spectra were compared. Quantification of the relative amount of the individual components was performed according to the area percentage method.

2.3. Emulsion procedure

Oregano EO was emulsified in order to improve its solubility. Soy lecithin (Alfa Aesar) was used as surfactant. Initially, the organic phase (EO + soy lecithin) was stirred magnetically for 50 min, at a ratio of 4 g of soy lecithin/100 g of EO. Then, the aqueous phase (NB + distilled water) was added to the organic phase, at a ratio of 4 g of aqueous phase/g of organic phase. Then they were agitated for 20 min on a magnetic stirrer. After that, the solution underwent sonification by using an ultrasound (Fisher Scientific, Sonic Dismembrator Model 500, 400 W) for 4 min with 70% amplitude. The emulsion was stored at 4 °C until used.

2.4. Heat medium

Nutrient Broth was prepared with distilled water, and adjusted to 4 °Brix by adding glucose (Nuclear, Brazil), standardization was performed with the help of a digital refractometer (AR200, Reichert). The medium pH was standardized at 4.2 by adding citric acid solution at 1.8 g/L and measured by a pH meter (AN2000, Analion). Soluble solid concentration and pH values were chosen aiming at simulating tomato pulp, the product in which the oregano EO can be easily employed and the spoilage by *B. coagulans* is frequently reported. The heat medium was autoclaved at 121 °C for 15 min. There was no change in soluble solids and pH after this treatment.

2.5. Thermal and thermochemical inactivation

Inactivation tests were performed by using sealed thermal-death-time (TDT) tubes (8 × 120 mm glass tubes with wall thickness of 1 mm) (Stumbo, 1978). Contact time between *B. coagulans* and oregano EO before heat treatment was standardized at 15 min. NB containing appropriate concentrations of homogenized EO emulsion was inoculated with spores of *B. coagulans* and the contact time started being recorded immediately.

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); afterward, they were sealed by gas flame (LPG/O_2). After the contact time, TDT tubes were submerged into a thermostatic bath containing silicone oil. The come-up-time for the temperature in the TDT tubes has been estimated to be 2 min. Then, TDT tubes were individually removed at predetermined times and immediately cooled in an ice bath. After that, TDT tubes were aseptically opened with the aid of a diamond glass cutter. Population density was determined by serial dilutions in 0.1 g/100 g peptone water, and dilutions were pour plated in TDA. The plates were incubated at 37 °C for 48 h to determine the number of bacterial spores expressed in CFU/mL. The procedure for thermal inactivation was identical to the thermochemical one except for oregano EO addition. For thermal inactivation, tested temperatures were 95, 97, 100 and 103 °C. In order to test EO emulsion efficiency, a thermochemical resistance with 500 μg/g of EO at 100 °C was performed with the non-emulsified EO. In the case of thermochemical treatment, the studied temperatures were 95 and 100 °C, and the EO concentrations were 250, 300, 350, 400, 500 and 1000 μg/g (stage I). Subsequently, the EO concentration was fixed at 400 μg/g and the tested temperatures were 90, 95, 97 and 100 °C (stage II and III).

2.6. Mathematical modeling

For primary modeling, the Weibull distribution function (Equation (1)) was adjusted to the experimental data through the program Matlab® (The MathWorks Inc, Natick, USA).

$$\log\frac{N(t)}{N_0} = -\left(\frac{t}{\beta}\right)^{\alpha} \tag{1}$$

where N_0 is the initial number of spores (CFU/mL) and $N(t)$ is the number of spores after t(min) of heat treatment (CFU/mL); β is known as the location factor and α is the shape factor.

A general secondary model was used to describe the influence of temperature on inactivation parameters. The exponential (Equation (2)) was applied as secondary model through Excel software (Microsoft®).

$$y = a\cdot\exp(c\cdot x) \tag{2}$$

where a and c are empirical parameters of the equation; x corresponds to values of temperature (°C); and y corresponds to values of β or α or the time to reach six decimal reductions ($t6D$).

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