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Aspergillus ochraceus spores inactivation with high-pressure carbon dioxide

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Abstract

The aim of this research was to study the inactivation of Aspergillus ochraceus spores exposed to high pressure carbon dioxide (HPCD) treatments and to estimate the kinetic and the thermodynamic parameters. A synergistic effect of pressure and temperature was noticed in the range from 5.4 to 7 MPa and from 30 to 50°C. Weibull model is the best primary kinetic model to describe the inactivation of A. ochraceus in the range from 5.4 to 7 MPa and from 30 to 50°C. The statistical indices show better correlations between the experimental and the predicted values for Weibull equation compared with Gompertz and Logistic models. The values of enthalpy, enthropy and total free energy were estimated for the experimental domain using nonlinear regression analysis. There was no significant variation in the values of the Gibbs free energy for the experimental domain. This work contributes to the general understanding of mould inactivation using high pressure carbon dioxide (HPCD) treatment.

Keywords: supercritical carbon dioxide, inactivation, mould, kinetics, thermodynamics.

1. Introduction

The increasing consumers demand for natural, fresh food, free of chemical preservatives, and the current trends to avoid over-processing the foods which may produce a loss of flavor, color and nutrients, have led the food industry to developing an interest in non-thermal processing (C. ORTUÑO & al., 2012 [1]). In the last two decades, applications of high pressure carbon dioxide (HPCD) using either sub- or supercritical CO_2 emerged as promising for pasteurization of foods (L. GARCIA-GONZALEZ & al., 2009 [2]). This process effectively inactivates vegetative microorganisms and, because a low temperature can be applied, HPCD processing can produce high-quality, pasteurized food products, meeting the consumer's expectations (L. GARCIA-GONZALEZ & al., 2007 [3]).

Supercritical carbon dioxide (SC-CO_2) has a liquid-like density, gas-like diffusivity and viscosity, and a zero surface tension. It is, therefore, capable of penetrating into complex structures, and has the ability to inactivate microorganisms (C. ORTUÑO & al., 2012 [1]).

Numerous microbial strains have been studied in order to evaluate their sensitivity to HPCD treatments. The species that have been investigated ranged from Gram-negative bacteria like *Salmonella typhimurium, Escherichia coli* or *Yersinia enterocolitica* to Gram positive or yeasts, such as *S. cerevisiae*, *L. innocua* or *L. monocytogenes*. However, until now less work was reported for moulds (M. SHIMODA & al., 2002 [4]), although the total microbial flora inactivation by HPCD was reported (G. FERRENTINO & al., 2013 [5]).

Fungal contamination is one of the main sources of pre- and post harvest grains deterioration. Members of the *Aspergillus spp*., amongst many other toxigenic fungi, have

been associated with important contamination problems in cereals caused by the biosynthesized secondary metabolites also known as mycotoxins. Ochratoxin A (OTA) is a nephrotoxic mycotoxin produced by *A. ocharceus* with carcinogenic, immunosuppressive and teratogenic properties (R. BHAT & al., 2010 [6]).

Considering the potential implications of *A. ochraceus* presence in different food matrixes, the aim of this work was to estimate the kinetic and thermodynamic parameters of *A. ochraceus* inactivation with supercritical carbon dioxide in a model system.

2. Material and methods

2.1. *Preparation of Aspergillus ochraceus spores*

Aspergillus ochraceus 151 strain was obtained from USAMV (University of Agronomic Science and Veterinary Medicine, Bucharest, Romania) collection. The mould strain was activated on potato dextrose agar (PDA; Merck, Darmstadt) slant and a stock culture was prepared on the PDA slant by incubation at 25°C for 3 days. The mould cultures for experiments were subcultured from stock culture using PDA plates at 25°C for 6 days. The spores were collected by washing the surface of the agar plate cultures using potato dextrose media (PDB; Difco, Detroit) containing 0.1 % Tween 80 (pH=5.6). The spore suspension was filtered through three layers of sterile cheesecloth to remove the hyphae under aseptic conditions. Finally, the number of spores in the filtrate was brought to about 10^6 spores x ml^{-1} by adding the necessary amount of physiological saline solution. The initial number of spores in the suspension was counted by direct microscopic counting method using Neubauer improved chamber (O. ERKMEN, 2001 [7]). The spore suspension used in all experiments was freshly prepared every day and stored in a refrigerator at 4°C during the experiments.

2.2. *Equipment*

The high-pressure installation used for experimental treatments comprises a cylindrical pressure vessel (maximum pressure tolerance level of 100 MPa) with the internal volume of 266 ml used for CO_2 pressurization as previously described by (O. ERKMEN, 2000 [8], O. ERKMEN, 2001 [9]).

2.3. Pressure treatments

Two samples containing 5 ml of daily prepared spore suspension (10^6 spores × ml^{-1}) were placed into loosely capped (sterile metallic cap standing at fixed position) sterile test cylindrical tubes (110 × 14 mm) and the tubes were gently shacked. The tubes were placed into the pressure vessel and the vessel was tightly closed and immersed in a thermostatic water bath (ST-402; Nüve, Sanayii ve Malzemeleri İmalat ve Ticaret A.Ş., İstanbul, Turkey) at constant temperatures (30, 40 and 50°C). When the temperature was equilibrated (within 1 min) and all tubing connections were secured, commercially available CO_2 (purity 99.990%, FNA 84-37-611; Koçerler, Industrial and Medical Gas Producing and Marketing Commercial Limited Company, Gaziantep, Turkey) was injected through the gas inlet valve from the gas cylinder into the vessel, reaching the desired pressures (5.4, 6 and 7 MPa) within 1 min.

After being exposed to HPCD treatment for a designated time period at a temperature of 30, 40 or 50°C, the pressure was lowered to atmospheric pressure (within 1 min) by opening the gas outlet valve slowly and the duplicate tubes were pulled out. A blank tube containing 5 ml of sample with the same concentration of spore suspension as the regular samples was incubated under atmospheric pressure in the water bath. After the treatment, 1 ml of spore suspension was immediately taken out from each of the two tubes and the samples were examined for surviving *A. ochraceus* spores. The experiments were done in triplicate.

2.4. Enumeration of A. ochraceus spores

Each treated sample was serially diluted with sterile physiological saline solution. The initial and the surviving *A. ochraceus* spores were counted by spread plating 1 ml of diluted and non diluted samples on duplicate plates of PDA (pH = 5.6). The plates were incubated at 25°C for 3 days, after which all the visible *A. ochraceus* colonies on PDA plates were counted (O. ERKMEN, 2000 [8]). The number of survivors was expressed as log colony forming unit (CFU) ml^{-1}.

2.5. Inactivation kinetics and statistical analysis

The inactivation kinetics of *A. ochraceus* spores was assessed using three mathematical nonlinear regression models that take into account the presence of a lag-phase previous to the inactivation period.

To describe the inactivation of the spores Logistic, Weibull model and modified Gompertz equations were considered and compared.

The Weibull model takes into account the biological variation among spores with respect to the inactivation and it is a statistical model of the distribution of inactivation times. The model considers lethal events as probabilities and survival curves as a cumulative form for the distribution of lethal events (M.A.J.S.VAN BOEKEL, 2008, [10]; M. PELEG, & M.D NORMAND 2004 [11]).

$$\log\left(\frac{N}{N_0}\right) = -b \cdot t^n \tag{1}$$

where b -the non-linear rate parameter; n -the parameter responsible for the curve shape; N - the CFU after treatment, t –the inactivation time (min) and N_0 -the initial CFU when t = 0. The inactivation curve will have a concave upward semilogarithmic shape for n<1 and a convex shape for n>1. If the n=1 than Weibull model will reduce to Bigelow (1921) equation.

Modified Gompertz was used for describing sigmoidal curves that includes the lag phases and the asymptotic phases. The model parameters have biological meaning and describe the microbial cell inactivation (C. ORTUŇO & al., 2012 [1]):

$$\log\frac{N}{N_0} = A \cdot \exp\left\{-\exp\left[\frac{-k_d \cdot e}{A}(\lambda - t) + 1\right]\right\} \tag{2}$$

where A -the lowest asymptotic value, k_d- the maximum inactivation rate (min^{-1}), t – the inactivation time (min), λ – phase of disappearance of the lag phase, and e = 2.718.

The Logistic model was used by many researchers to describe the non-liner inactivation of microorganisms in different non-thermal processes (J. WANG & al., 2010 [12]; H.CHEN, & HOOVER 2003 [13]).

$$\ln\left(\frac{N}{N_0}\right) = \frac{a}{1 + \exp(b - ct)} \tag{3}$$

a,b,c are fit parameters and t- the time (min.).

Using non-linear regression procedure (SAS software, version 9.1, Cary, NC, USA) the parameters of equations (1), (2) and (3) were estimated.

The thermodynamics of the activated complex for the sporogenic cell of *Aspergillus ocharaceus* was expressed according to Eyring equation:

$$k_i = k\, x \frac{k_b\, x\, T}{h} x e^{-\Delta G^{0\neq} / RT} \tag{4}$$

where k_i -the transmission factor (dimensionless), k_b -the Boltzmann constant (1.38 x 10^{-23} $J \cdot K^{-1}$), h -the Plank`s constant (6.63 x 10^{-34} J·s), k-the rate constant, T-the temperature (K) and $\Delta G^{0\neq}$ the Gibbs free energy of activation (kJ/mol).

$$\Delta G^{0\neq} = \Delta H^{\neq} - T\Delta S^{\neq} \tag{5}$$

where $\Delta H^{\neq}$ is the enthalpy of activation and $\Delta S^{\neq}$ is the enthropy of activation

By combining Eq. (4) and (5) followed by linearization one will obtain:

$$\ln\left(\frac{k_i}{T}\right) = \left\{\ln\left(\frac{k_b}{h}\right) + \left(\frac{\Delta S^{\neq}}{R}\right)\right\} - \left\{\left(\frac{\Delta H^{\neq}}{R}\right)\left(\frac{1}{T}\right)\right\} \tag{6}$$

To estimate $\Delta H^{\neq}$ and $\Delta S^{\neq}$ values nonlinear regression analysis was applied to Eq. (6).

Two statistical indices were examined as a basis for model discrimination and for assessing the fitting capacity of the tested models:

RMSE (root mean square error)

$$RMSE = \sqrt{\frac{\sum_{i=1}^{ni}\left(y_{\exp}(t_i) - y(t_i, p_{is})\right)^2}{n_t - n_p}} \tag{7}$$

where $y_{exp}(t_i)$ denotes the experimental observations, $y(t_i,p_{is})$ the predicted values, n_t the total number of data points and n_p the number of estimated model parameters.

R_{adj}^2 (adjusted R square) for the model

$$R_{adj}^2 = 1 - \left(\frac{n_t - 1}{n_t - n_p}\right) \cdot \frac{SSE}{SSTO} \tag{8}$$

where n_t is the total number of data points and n_p the number of estimated model parameters, SSE is the sum of square errors and SSTO is the total sum of square errors. Standard deviation of the estimated parameters was also a good indicator for the contribution of the individual parameters to the model.

3. Results and Discussion

Fig. 1 a), b) and Fig 2 show the inactivation of *A. ochraceus* spores with HPCD. At 5.4 MPa and 30°C the complete inactivation of the spores takes place slowly, after 320 min, while at 40°C and 50°C the inactivation rate increases and a total inactivation of the sporogenic cells is achieved after 120 min of treatment (Fig. 1a). At 6 MPA the inactivation takes place faster for all the studied temperatures compared with 5.4 MPa and it takes 180 min of HPCD treatment to complete the inactivation at 30°C, 75 min at 40°C and 60 min at 50°C (Fig. 1b). At 50°C and 7 MPa the inactivation of *A ochraceus* spores is achieved after 20 minutes of HPCD treatment (Fig.2). A 4-log reduction of the initial *A. ochraceus* count was achieved at 5.4 MPa and 30°C in 120 min of treatment, and in 90 min at 50°C. A 3-log reduction of the initial count was reached after 105 min at 7 MPa combined with 30°C. Almost the same inactivation (3-log) was achieved after 90 min at 5.4 MPa combined with 40°C and after 10 min at 7 MPa and 50°C. In the studied experimental range a synergistic effect of pressure and temperature on *A. ocharceus* HPCD inactivation could be noticed. The

results from this study are in line with the ones reported by M. SHIMODA & al., 2002 [4] for the death kinetics of *Aspergillus niger* spores.

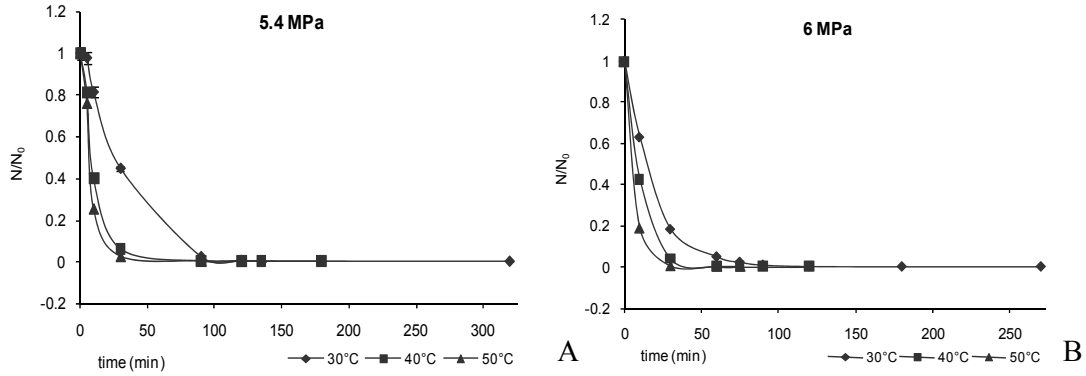

Figure 1. Inactivation of *A. ochraceus* spores with HPCD at: A) 5.4 MPa and B) 6 MPa

In order to analyze the inactivation curves of *A. ocharceus* spores, obtained with the HPCD treatment, the kinetic data were fitted to three mathematical models: Weibull, modified Gompertz and Logistic equations (Eq. (1), Eq. (2) and Eq. (3)). These three models were largely reported in the literature for describing nonlinear microbial inactivation kinetics (M.A.J.S.VAN BOEKEL, 2008, [10]).

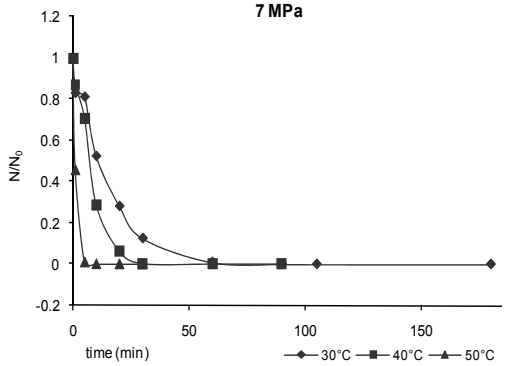

Figure 2. Inactivation of *A. ochraceus* spores with HPCD at 7 MPa

Aimed at finding the best model that accurately describes the *A. ocharecus* death the statistical indices (RMSE and $R^2_{adj.}$) were calculated for each inactivation curve and enabled the selection of the best fit model. Table 1, 2 and 3 presents these estimated parameters of the nonlinear regression equations Eq. (1), Eq. (2) and Eq. (3).

The estimated coefficient of the Weibull equation Eq. (1) at 5.4 MPa and 30°C but also at the same pressure and 50°C, that determines the shape of the curve (n) has a value higher than 1, suggesting that for these inactivation conditions the shape of the curve is convex. At 40°C the value of the n-parameter indicate a slightly concave curve (n = 0.9932). The lag phase λ estimated by the Gompertz equation (Eq. (2)) has a large value (40.18) at 5.4 MPa and 30°C, indicating a slow inactivation and, as the estimated parameters indicate (Table 1), the lag phase at 40°C (18.08) is smaller than at 50°C (37.29) for the treatment at 5.4 MPa.

Table 1. Mathematical models of *A. ochraceus* spores inactivation with SC-CO_2 at 5.4 MPa

Temperature	30 (ºC)	40 (ºC)	50 (ºC)
Weibull model			
b	0.0160	0.0380	0.0316
n	1.0480	0.9932	1.1083
RMSE	0.1426	0.0911	0.3471
$R_{adj.}^2$(%)	99.78	99.93	99.06
Equation	$\log\left(N/N_0\right) = -0.02 \cdot t^{1.05}$	$\log\left(N/N_0\right) = -0.04 \cdot t^{0.99}$	$\log\left(N/N_0\right) = -0.03 \cdot t^{1.11}$
Gompertz modified model			
A	-7.6683	-8.9453	-20.5498
λ	40.1861	18.0893	37.2927
k_d	0.0295	0.0440	0.0796
RMSE	0.1324	0.2684	0.4502
$R_{adj.}^2$(%)	99.82	99.49	98.42
Equation	$\log(N/N_0) = -7.67 \cdot \exp\{-\exp[0.01 \cdot (40.19 - t) - 1$	$\log(N/N_0) = -8.94 \cdot \exp\{-\exp[0.01 \cdot (18.09 - t) - 1]\}$	$\log(N/N_0) = -20.55 \cdot \exp\{-\exp[0.01 \cdot (37.29 - t) - 1]\}$
Logistic model			
a	-15.7101	-16.7886	-24.6868
b	3.1687	2.8474	3.1559
c	0.0203	0.0276	0.0303
RMSE	0.6235	0.8491	1.149
$R_{adj.}^2$(%)	99.15	99.05	98.07
Equation	$\log\left(N/N_0\right) = \frac{-15.71}{1 + \exp\left(3.17 - 0.02 \cdot t\right)}$	$\log\left(N/N_0\right) = \frac{-16.79}{1 + \exp\left(2.85 - 0.03 \cdot t\right)}$	$\log\left(N/N_0\right) = \frac{-24.69}{1 + \exp\left(3.16 - 0.03 \cdot t\right)}$

Table 2. Mathematical models of *A. ochraceus* spores inactivation with SC-CO_2 at 6 MPa

Temperature	30 (ºC)	40 (ºC)	50 (ºC)
Weibull model			
b	0.0200	0.0341	0.0735
n	1.0424	1.1104	1.0412
RMSE	0.0775	0.3142	0.1331
$R_{adj.}^2$(%)	99.71	99.42	99.96
Equation	$\log\left(N/N_0\right) = -0.02 \cdot t^{1.04}$	$\log\left(N/N_0\right) = -0.03 \cdot t^{1.11}$	$\log\left(N/N_0\right) = -0.07 \cdot t^{1.04}$
Gompertz modified model			

A	-8.0835	-8.6867	-9.1978
λ	26.0447	16.9194	24.4123
k_d	0.0322	0.0739	0.0461
RMSE	0.2184	0.2672	0.2672
$R_{adj.}^2$(%)	99.97	99.46	99.66
Equation	$\log(N/N_0) = -8.08 \cdot \exp\{-\exp[0.01 \cdot (26.04 - t) - 1]\}$	$\log(N/N_0) = -8.69 \cdot \exp\{-\exp[0.02 \cdot (16.92 - t) - 1]\}$	$\log(N/N_0) = -9.19 \cdot \exp\{-\exp[0.01 \cdot (24.41 - t) - 1]\}$
Logistic model			
a	-16.1275	-16.6441	-16.9431
b	2.8230	3.1878	2.6694
c	0.0208	0.0476	0.0618
RMSE	0.5263	0.5278	2.9318
$R_{adj.}^2$(%)	99.51	99.62	98.48
Equation	$\log\left(N/N_0\right) = \frac{-16.13}{1 + \exp(2.82 - 0.02 \cdot t)}$	$\log\left(N/N_0\right) = \frac{-16.64}{1 + \exp(3.19 - 0.05 \cdot t)}$	$\log\left(N/N_0\right) = \frac{-16.94}{1 + \exp(2.67 - 0.06 \cdot t)}$

Table 3. Mathematical models of A. ochraceus spores inactivation with SC-CO2 at 7 MPa

Temperature	30 (ºC)	40 (ºC)	50 (ºC)
Weibull model			
b	0.0184	0.0779	0.6041
n	1.1339	0.9954	0.7030
RMSE	0.0855	0.3521	0.1945
R^2(%)	99.95	99.17	99.76
Equation	$\log\left(N/N_0\right) = -0.02 \cdot t^{1.13}$	$\log\left(N/N_0\right) = -0.07 \cdot t^{0.99}$	$\log\left(N/N_0\right) = -0.60 \cdot t^{0.70}$
Gompertz modified model			
A	-9.1978	-7.1776	-6.9475
λ	24.4123	8.8145	-0.1242
k_d	0.0461	0.1090	0.2932
RMSE	0.1862	0.2392	0.5691
R^2	99.56	99.48	98.01
Equation	$\log(N/N_0) = -9.19 \cdot \exp\{-\exp[0.01 \cdot (24.41 - t) - 1]\}$	$\log(N/N_0) = -7.18 \cdot \exp\{-\exp[0.04 \cdot (8.81 - t) - 1]\}$	$\log(N/N_0) = -6.95 \cdot \exp\{-\exp[0.11 \cdot (-0.12 - t) - 1]\}$
Logistic model			
a	-16.7045	-15.2843	-15.1662
B	3.1214	2.9347	2.1310
c	0.0305	0.0716	0.1800
RMSE	0.6187	0.9580	1.6985
R^2(%)	99.08	98.42	96.66
Equation	$\log\left(N/N_0\right) = \frac{-16.70}{1 + \exp(3.12 - 0.03 \cdot t)}$	$\log\left(N/N_0\right) = \frac{-15.28}{1 + \exp(2.93 - 0.07 \cdot t)}$	$\log\left(N/N_0\right) = \frac{-15.16}{1 + \exp(2.13 - 0.18 \cdot t)}$

The statistical indices show that the weakest model among the three analyzed that describes *A. ochareus* inactivation at 5.4 MPa combined with the temperature range 30-50°C is the Logistic model that presents the smallest R^2_{adj} values: 98.07% at 50°C and 99.05% at 40°C and the highest RMSE values. Comparing the statistical indices it results that Weibull model is the best one to describe the inactivation at 5.4 MPa because it has better indices at 40 and 50°C compared with Gompertz that has better indices only at 30°C. At 6 MPa the Weibull model showed n-values higher than 1 for all the studied temperatures and the b values increased with temperature from 0.0200 at 30°C to 0.0735 at 50°C (Table 2). When analyzing the parameters obtained from the Gompertz equation at 6 MPa it can be noticed the same pattern of the lag phase as for the 5.4 MPa. At 30°C the lag phase is 26.04 min indicating a slow disappearance of the spores, at 40°C the lag phase is shorter (16.92 min) but a slight bounce back can be observed for 50°C (24.41 min). Although, in general good correlations could be observed between the experimental values and the predicted ones for the Logistic model, this model had, compared to Weibull and Gompertz equations, lower statistical indexes. Moreover, the highest RMSE value for the entire experimental domain was obtained for the Logistic model at 6 MPa and 50°C (2.9318) indicating a higher difference between the experimental values and the ones predicted by the model, thus a less accurate model than Gompertz and Weibull. Weibull equations at 6 MPa had better statistical indices at 30°C and 50°C while Gompertz equation registered better correlations indices for 40°C.At 7 MPa the lag phase for the Gompertz equation sharply decreased from 24.4123 min at 30°C to a negative value at 50°C (-0.1242 min) indicating an almost instantaneous inactivation of *A. ochraceus* spores at 50°C. The parameter n of the Weibull equation had a value higher than 1 at 30°C, however for 40°C and 50°C the n-value was less than 1. The best statistical indices were obtained for Weibull equation for 30°C and 50°C while at 40°C Gompertz equation showed better indices. When evaluating the entire experimental domain it results that Weibull model is the best one to describe *A. ochraceus* inactivation kinetics using HPCD because it has better statistical indices than Gompertz and Logistic model.

3.1. Thermodynamic parameters

Considering the importance of understanding the *A. ochraceus* inactivation mechanism from the perspective of thermodynamic changes we estimated the parameters with the Eq. (4), Eq. (5) and Eq. (6).

Table 4. Estimated thermodynamic parameters for the irreversible HPCD denaturation of sporogenic *A.ochraceus*

P (MPa)	T(K)	$\Delta H^{\neq}$ (kJ·mol^{-1})	$\Delta S^{\neq}$ (J mol^{-1}K^{-1})	$\Delta G^{\neq}$ (kJ·mol^{-1})
5.40	303.15	15.61	-193.77	74.32
5.40	313.15	15.53	-193.77	76.18
5.40	323.15	15.44	-193.77	78.03
6.00	303.15	50.31	-112.15	84.29
6.00	313.15	50.23	-112.15	85.33
6.00	323.15	50.14	-112.15	86.37
7.00	303.15	139.22	180.05	84.67
7.00	313.15	139.14	180.05	82.78
7.00	323.15	139.05	180.05	80.90

The increase in enthalpy was noticed for the pressure increase for all the experiments however, at the same pressure, there was no significant change for the temperature variation

(Table 4). This increase can be explained by the faster dissociation of the H_2CO_3 with pressure that lowers the extracellular pHex. The response of the microorganisms is increased energy consumption to maintain pH homeostasis by the proton motive force (L. GARCIA-GONZALEZ & al., 2007 [3]).

The negative sign for the change in enthropy noticed in the current study and observed by other authors for enzymes (R. HERNÁNDEZ-MARTÍNEZ & al. 2011 [14]; G.S.N. NAIDU & T. PANDA, 2003 [15]) could be related to the transition form a high entropy state to a low entropy state, in relation with the changes in the environment. The formation of charged particles around the spores, the orientation of the solvent, or the reduced proton motive force across the membrane could cause the negative sign of the change in enthropy. The values of $\Delta G^{\neq}$ calculated from Eq. (5) are presented in Table 4, but no significant change in the free enthalpy could be noticed with the increase of temperature and pressure.

4. Conclusions

Inactivation of the *A. ochraceus* spores in PDA media with HPCD was obtained in the pressure range of 5.4-7 MPa combined with temperatures from 30 to 50°C. The rate of inactivation and the parameters that describe the shape of the curves were estimated applying nonlinear regression analysis. Statistical indices (RMSE and $R^2_{adj.}$) enabled the selection of the best model that describes mould inactivation with HPCD. Weibull model was found to be the best one to predict the inactivation of *A. ochraceus* using high pressure carbon dioxide when compared with other kinetic models such as Logistic and Gompertz equation.

The thermodynamic approach enabled the estimation of the enthropy, enthalpy and Gibbs free energy and also some considerations on the inactivation mechanism of *A. ochraceus*..

The present study contributes to the understanding of spores` inactivation during HPCD treatments from a kinetic and thermodynamic perspective and the conditions that can influence the inactivation.

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