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Food Research International 32 (1999) 677–682

FOOD RESEARCH INTERNATIONAL

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Combined effect of temperature and propionic acid concentration on the growth of *Aspergillus parasiticus*

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Received 29 April 1999; accepted 11 November 1999

Abstract

Effects of temperature (25, 30 and 36°C) and propionic acid concentration (129, 258 and 516 ppm) on the growth of *Aspergillus parasiticus* in solid media were analyzed. Growth rate, expressed as the increment of colony diameter per unit of time, was studied as a function of storage time employing the Arrhenius model. The inverse of the lag phase was fitted to an Arrhenius-type equation and the inhibition index was also calculated. A linear relationship between the lag phase and the reciprocal growth rate at different propionic acid concentrations was assessed by linear regression analysis. Fungi behavior was modeled considering the main controlling factors and a response surface methodology was established in terms of propionic acid and temperature. The proposed model could be used in food microbiology to predict the growth of toxicogenic fungi growth. © 2000 Canadian Institute of Food Science and Technology. Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Aspergillus parasiticus*; Predictive modelling; Propionic acid; Fungal growth

1. Introduction

Molds commonly contaminate crops and foods and cause significant yield reduction and economic losses. They render contaminated foods not only unpalatable because of changes in appearance, taste, texture and odor but also unsafe for human consumption by production of mycotoxins. Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning or mycotoxicoses, sometimes resulting in death. Species in *Aspergillus* section *flavi* (Grams, Samson, Pitt, Onions & Christensen, 1985) previously incorrectly known as the *Aspergillus flavus* group (Raper & Fennell, 1965) are among the most commonly occurring food spoilage fungi. They are of particular interest because some species produce aflatoxins. The presence of aflatoxins in food products has been of public health concern due to their carcinogenic, mutagenic and teratogenic effects on living organisms (Ellis, Smith, Simpson & Oldham, 1991).

An aid in preventing fungal spoilage of mildly processed foods would be the application of the hurdle concept or combined preservation method (Gould, 1995). This method comprises the uses of various hurdles which separately may not give adequate preservation but which, when combined, will give proper preservation. These hurdles may include lowering the temperature, pH, water activity (a_w by addition of NaCl or sugars), or the addition of preservatives. If the hurdle concept is to be applied successfully, the influence of the various environmental factors on fungal growth need to be quantified.

Propionic acid as well as its calcium or sodium salts has long been recognized as the most powerful mold-inhibiting chemical on the market. Although propionic acid exhibits antimycotic activity, its use is limited to foods in which the pH is fairly acidic, since it has virtually no activity at neutral or near neutral pH values.

Predictive modeling of filamentous fungal growth has not received as much attention as bacterial growth, probably due to the inherent complexities of fungal growth quantification. Measurement of hyphal extension rate, usually reported as radial growth rate, is probably the simplest and most direct measure, but it does not necessarily represent the true nature of fungal

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PII: S0963-9969(99)00146-5

growth (Gibson & Hocking, 1997). A prerequisite for producing a useful and reliable model must be a data base containing large amount of relevant data, preferably in the form of growth or survivor curves. Bratchell, Gibson, Truman, Kelly and Roberts (1989) previously discussed the problems associated in collecting data from bacterial growth curves. Moreover, difficulties in obtaining reproducible and similar quality growth curves for fungi are much more complicated.

Some more mechanistic models of mold growth have been described (Pitt, 1993). In practice, however, simple empirical approaches to modeling are frequently preferable (Box & Draper, 1987).

The objectives of the present study were (a) to evaluate the simultaneous effect of temperature (25, 30 and 36°C) and propionic acid concentration (129, 258 and 516 ppm) on the growth of *A. parasiticus* in solid media at pH 5.5 and a_w 0.95, (b) to obtain the parameters growth of *A. parasiticus* by fitting adequate equations and (c) a response surface model that could be used to determine treatment conditions that inhibit *A. parasiticus* growth.

2. Materials and methods

2.1. Preparation of spore inoculum

A. parasiticus NRRL 2999 was obtained from Cátedra de Microbiología de Alimentos, Facultad de Ciencias Exactas, UBA, Argentina. Fungi were maintained at 4°C on potato dextrose agar (PDA) slants (Merck, Germany) and transferred weekly. The inoculum was prepared by growing the fungi on PDA slants for 7 days at 30°C. Cultures were washed with 5 ml of 0.01% (w/w) sodium lauryl sulfate in 1% (w/w) sodium chloride solution. Spores were loosened by gently scraping with a spatula. The number of spores, about 10^7 per ml, was assessed by a haematocytometer.

2.2. Culture media

The basal medium contained malt extract (1%), yeast extract (2%) and agar (2%). Media of $a_w = 0.95$ were prepared according to Gonzalez, Resnik and Vaamonde (1987) supplementing the necessary amount of glucose to reach the desired a_w. The a_w level was determined with a Novasina Thermoconstanter Humidat TH2/TH1 (Novasina, Zurich, Switzerland) which was calibrated against saturated salt solutions with known a_w. The pH of the medium was adjusted to 5.5 with NaOH.

Temperature and propionic acid concentrations were examined by means of a full factorial design. Combinations between 25, 30 and 36 ± 0.1°C and 0 (control) 250, 750 and 1500 ppm of ammonium propionate/propionic acid/inert solid (Fungistop) were assayed ($3 \times 4 = 12$ experiments). The maximum value assayed (1500 ppm) is within the highest concentration of propionic acid used in foods. All experiments were performed in duplicate.

2.3. Cultivation and examination

Cultures were grown on standard size Petri dishes (90 mm diameter) containing approximately 15 ml of solid medium. For each combination, three plates of medium were inoculated with 10 μl of the spore suspension ($a_w = 0.95$), dispensed from a micropipette. The diameter of the circular inoculum obtained was assumed as the colony initial diameter. Inoculated plates were incubated in an upright position at 25, 30 and 36°C inside plastic boxes containing dishes of saturated solutions of acid disodium phosphate ($a_w = 0.95$) to prevent changes in a_w. Colony diameters of macroscopic colonies were measured 2 to 3 times each day by placing the Petri dishes on a millimeter scale illuminated from beneath by a light box. Two diameter measurements were taken at right angles from each colony and the results were calculated from the mean diameter of the replicate colonies (Horner & Anagnostopulos, 1973).

2.4. Determination of propionic acid concentration

Commercial propionic acid (Fungistop) was used as preservative. This product is a fungicide composed of propionic acid/ammonium propioniate (14.7%/27.3%) prepared on a granular solid carrier. To know the exact amount propionic acid added, preservative levels were determined by high performance liquid chromatography (HPLC) and expressed as ppm of propionic acid. Twenty-five ml of 0.009 N H_2SO_4 (mobile phase) were added to 7 g agar media and extracted for 1 h with agitation in a shaker (Rolco S.R.L.). Samples were centrifuged at $700 \times g$ for 5 min according to a modification of the method of Bevilacqua and Califano (1989). The supernatant was filtered once through filter paper and twice through a 0.45 μm membrane filter (Milipore Waters Associates SM N11306). Duplicate analyses were performed on all samples. A HPLC (Shimadzu) with Aminex HPX-87-Biorad column was used operating under the following conditions: 0.009N H_2SO_4 as mobile phase, 0.7 ml/min flow, 580–600 psi, between 58 and 62°C.

2.5. Statistical analysis

The statistical analysis was carried out with SYSTAT software (SYSTAT Inc, 1990 version 5.0). ANOVA provided the coefficients and the corresponding standard deviations. Response surface analysis (RSA) was applied to study interactions between the factors affecting the growth (temperature and propionic acid concentration).

A stepwise procedure was used to analyze the simultaneous dependence of the growth rate and lag phase on temperature and propionic acid concentration. The stepwise selection method inserts variables until the regression equation is satisfactory ($P<0.05$). This method has been generally recommended as one of the best for variable selection (Draper & Smith, 1981).

The percent variance between observed and predicted values is given by Snedecor and Cochran (1969):

$$\%V = 1 - \left(\left(1 - r^2\right)(n-1)/(n-N-1)\right) \quad (1)$$

where n = number of observations, N = number of terms and r^2 = multiple regression coefficient.

3. Results and discussion

3.1. Determination of propionic acid concentration

Propionic acid is added to foodstuffs mixed with specific carriers in an inert solid. These carriers gradually liberate propionic acid gas, enhancing its inhibitory power. The main advantage is that the vapors are not as corrosive as liquid propionic acid. In the present work, 250, 750 and 1000 ppm of commercial propionic acid were added to the media and the corresponding levels of propionic acid measured by HPLC were 129, 258 and 516 ppm in the agar media, respectively.

3.2. Determination of growth rate and lag phase

Filamentous molds grow on solid media forming circular colonies. Figs. 1a–c shows the effect of propionic acid concentration on the radial growth of *A. parasiticus* as a function of storage time at 25, 30 and 36°C, respectively. Plots of colony diameter versus time showed a straight line, after an initial lag period. The growth rate (K_D) can be calculated from the regression slope of colony diameter versus time during the linear growth phase. The lag phase (lag) is the time required for the colony to growth beyond the inoculation zone (typically, 5 mm). This point corresponded to the intersection of the regression line with the horizontal line corresponding to the initial inoculation zone diameter (Cuppers, Oomes & Brul, 1997). The increased propionic acid concentration showed that K_D decreased and lag phase increased. Similarly, as temperature increased at the same level of added preservative, K_D increased and lag phase decreased (Table 1). ANOVA showed significant differences in K_D and lag phase duration ($P<0.05$) due the effect of the temperature and addition of propionic acid on the growth of *A. parasiticus*.

An early report by Cooper (1963) stated that, in some cases, the ratio of growth rate to generation time was

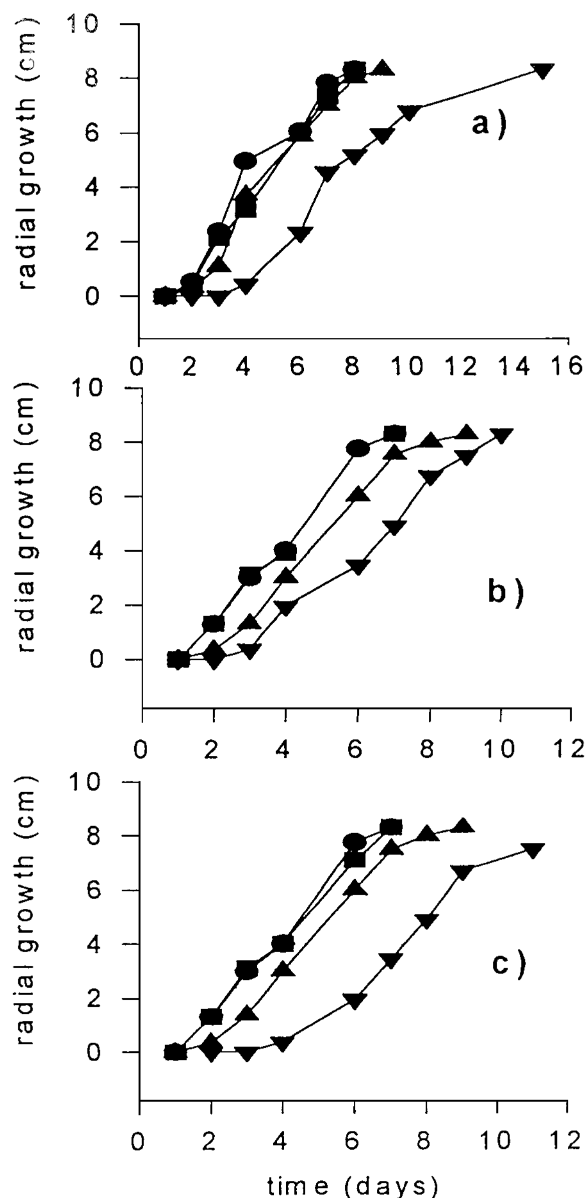

Fig. 1. *A. parasiticus* growth in solid media supplemented with different concentrations of propionic acid: ● control, ■ 129 ppm, ▲ 258 ppm, ▼ 516 ppm; (a) 25°C, (b) 30°C and (c) 36°C.

approximately constant. This observation suggested a linear relationship between lag phase and the reciprocal growth rate. A proportional relation between the lag time and the generation time on explicity assumed by many microbiologists, but observations of this phenomenon on experimental data have rarely been published (Baranyi & Roberts, 1994; Cooper; Delignette-Muller, 1998; McKellar, 1997). In the present work, we confirmed that the lag phase showed a linear behavior with the reciprocal growth rate for *A. parasiticus* (Fig. 2). The correlation coefficient (R^2) ranged between 0.889 and 0.999.

Table 1
Effects of propionic acid (ppm) and temperature on radial growth rate (K_D) and lag phase duration (lag)

Propionic acid (ppm)	K_D (25°C) (mm/h)	K_D (30°C) (mm/h)	K_D (36°C) (mm/h)	Lag (25°C) (hs)	Lag (30°) (hs)	Lag (36°) (hs)
Control	0.47 ± 0.02	0.52 ± 0.03	0.58 ± 0.02	13.00 ± 2.01	8.10 ± 3.12	7.00 ± 2.56
129	0.47 ± 0.03	0.51 ± 0.02	0.53 ± 0.01	21.01 ± 2.45	11.30 ± 2.56	9.40 ± 3.11
258	0.45 ± 0.01	0.47 ± 0.03	0.49 ± 0.03	24.00 ± 2.00	16.00 ± 3.66	20.00 ± 3.01
516	0.32 ± 0.02	0.35 ± 0.02	0.36 ± 0.01	32.00 ± 1.97	21.00 ± 2.45	21.00 ± 3.87

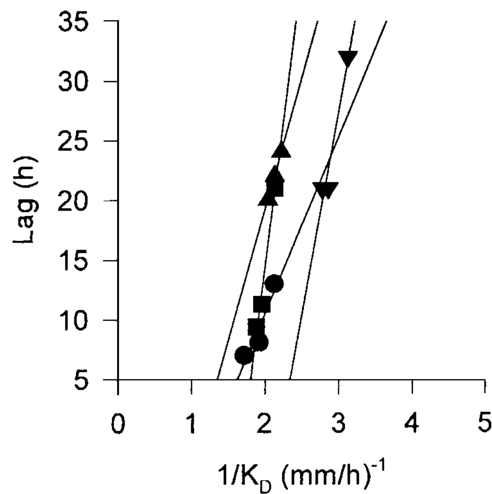

Fig. 2. Correlation of lag time and inverse K_D for *A. parasiticus* as a function of temperature and propionic acid concentration. ● control, ■ 129 ppm, ▲ 258 ppm, ▼ 516 ppm.

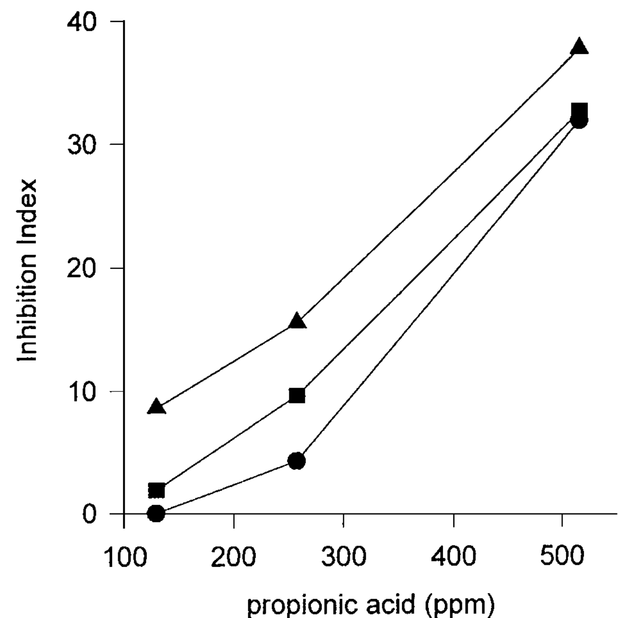

Fig. 3. Inhibiting index corresponding to different propionic acid concentrations at three temperatures: ● 25°C, ■ 30°C, ▲ 36°C.

3.3. Inhibition index

The effect of different concentrations of propionic acid and temperature on the mycelial growth was studied though the inhibition index (*II*). The inhibition index was defined as:

$$II = 7((K_{D\ control} - K_{D\ treated})/K_{D\ control})100 \quad (2)$$

where $K_{D\ control}$ and $K_{D\ treated}$ are the growth rate for the control and treated samples, respectively. *II* is 100 when molds are in the lag phase ($K_D = 0$) during storage. *II* is 0 when treated and control samples show the same growth rate. *II* varies from 0 to 100 due to the preservative action.

Fig. 3 shows the II of *A. parasiticus* obtained according to Eq. (2) as a function of different concentrations of propionic acid. Inhibition index values increased with the increasing residual content of propionic acid in the media, indicating antifungal action. *II* decreased as the temperature decreased. Values of *II* at 25 and 30°C in the range of propionic acid concentration tested did not show significant differences ($P > 0.05$). At 36°C, a greater inhibition was observed due to the combined effect of propionic acid and storage temperature.

3.4. Combined effect of temperature and propionic acid concentration on the growth of A. parasiticus

When applying the Arrhenius model to the growth parameters for each propionic acid concentration separately, good fits were obtained. Inspection of the residuals revealed that the logarithm of the growth rate could be used to stabilize the variance (Zwietering, Cuppers, de Wit & van't Riet, 1994).

For combined temperature and propionic acid concentration, we chose the model of the Arrenhius-type temperature dependence for each individual propionic acid level as the starting point for developing a model that included both temperature and propionic acid effect. In the present work, different models were fitted and those with the highest correlation coefficients and the lowest errors in the estimated parameters were selected. The following equations were obtained by stepwise analysis with statistical SYSTAT software. They described both the temperature effect (25, 30 and 36°C) and the propionic acid concentration (129, 258 and 516 ppm) dependence of the K_D and lag

Table 2
Coefficients of Eqs. (3) and (4) that give mycelial growth rate K_D and 1/lag phase dependence on control factors and statistical parameters of the regressions[a]

	$K1$	$K2$	$K3$	RSS[b]	F[c]	R^2
Eq. (3)	3.13 (4.80×10^{-1})	−1.11 (1.44×10^{-1})	-1.55×10^{-6} (6.71×10^{-8})	7.86	4293	0.99
Eq. (4)	1.01×10^{1} (5.87×10^{1})	−4.05 (1.78)	-3.06×10^{-6} (8.20×10^{-7})	9.13×10^{1}	342	0.99

[a] Standard deviation of the coefficients given in parentheses; degree of freedom = 3; $P < 0.001$ in all cases.
[b] RSS, sum of square of the regression.
[c] F ratio, Mean-square$_{regression}$/Mean-square$_{residual}$.

$$\ln K_D = K1 + K2/T + K3\ (\text{propionic acid})^2 \quad (3)$$

$$\ln(1/\text{lag}) = K1 + K2/T + K3\ (\text{propionic acid})^2 \quad (4)$$

Coefficients ($K1$, $K2$, $K3$), their standard deviations and other statistical parameters are shown in Table 2. The percent variance accounted for Eq. (1) was very high at 98.8%, indicating a very good fit of the model to the data. The parameter $K2 = E_a/R$ where E_a is the activation energy of K_D and lag phase duration (KJ/mol) applied Eqs. (3) and (4), respectively, and R is the gas constant (8.31 KJ/°K mol). In the present work, $E_{a_{KD}} = 9.25$ KJ/°K mol and $E_{a_{lag}} = 33.66$ KJ/°K mol. Fig. 4a and b shows examples of surface plots corresponding to Eqs. (3) and (4), obtained by fitting K_D and 1/lag phase of *A. parasiticus* versus temperature and propionic acid concentration. Cuppers et al. (1997) found that the logarithm of the growth rate of fungi showed a parabolic relationship with the square root of NaCl concentration. Davey (1989) used the Arrhenius approach to develop a modified or "additive" Arrhenius model to predict the combined effects of temperature and a_w on bacteria growth rate. Davey states that his equations are referred to as "additive" because they contain no interaction term between temperature and water activity. Davey (1991) also used his Arrhenius equation to predict microbial lag phase. Advantages of a linear Arrhenius model include its accuracy of prediction and ease of formulation, and demonstrated wide application to some 55 years of independent and published data (Davey, 1993).

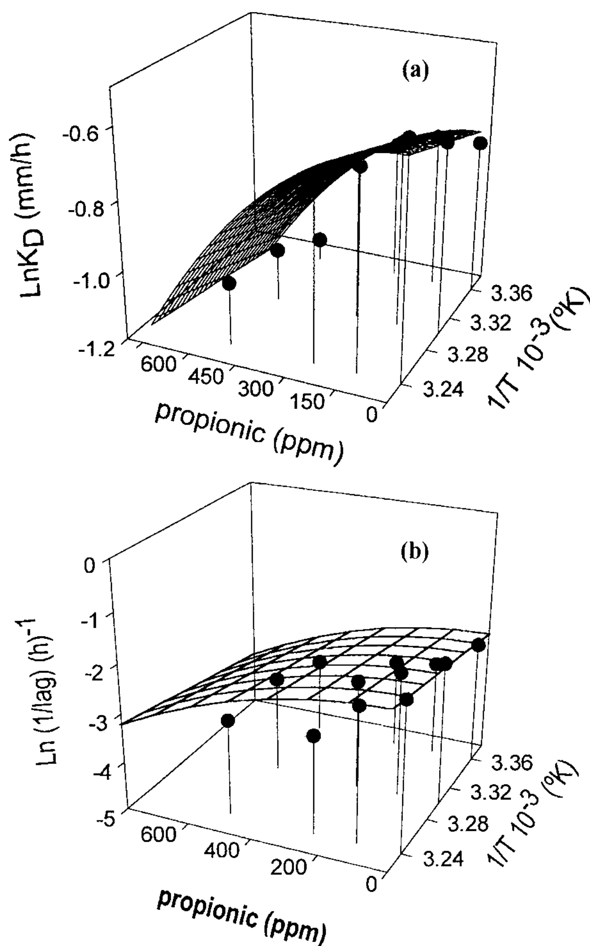

Fig. 4. Surface response plots showing the dependence of growth parameters of Aspergillus parasiticus on temperature and propionic acid: (a) K_D, (b) 1/lag phase.

This model described in the present work would allow the prediction of fungal growth under different conditions from those tested experimentally in this work, but within the studied range of temperatures and propionic acid concentration. Although we model only though temperature and propionic acid concentration, it is important to extend the modelling to other factors such as pH and a_w. With the aid of predictive models including as many hurdles as possible, reliable prediction of the fungal vulnerability of products will be possible in the future.

Acknowledgements

The authors acknowledge the financial support of the University of La Plata, Argentina; Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET) and Agencia de Promoción Científica y Tecnológica.

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