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Description of *Aspergillus flavus* growth under the influence of different factors (water activity, incubation temperature, protein and fat concentration, pH, and cinnamon essential oil concentration) by kinetic, probability of growth, and time-to-detection models

Carlos E. Kosegarten, Nelly Ramírez-Corona, Emma Mani-López, Enrique Palou, Aurelio López-Malo *

Departamento de Ingeniería Química, Alimentos y Ambiental, Universidad de las Américas Puebla, Cholula, Puebla 72810, Mexico

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ABSTRACT

A Box-Behnken design was used to determine the effect of protein concentration (0, 5, or 10 g of casein/100 g), fat (0, 3, or 6 g of corn oil/100 g), a_w (0.900, 0.945, or 0.990), pH (3.5, 5.0, or 6.5), concentration of cinnamon essential oil (CEO, 0, 200, or 400 μL/kg) and incubation temperature (15, 25, or 35 °C) on the growth of *Aspergillus flavus* during 50 days of incubation. Mold response under the evaluated conditions was modeled by the modified Gompertz equation, logistic regression, and time-to-detection model. The obtained polynomial regression models allow the significant coefficients ($p < 0.05$) for linear, quadratic and interaction effects for the Gompertz equation's parameters to be identified, which adequately described ($R^2 > 0.967$) the studied mold responses. After 50 days of incubation, every tested model system was classified according to the observed response as 1 (growth) or 0 (no growth), then a binary logistic regression was utilized to model *A. flavus* growth interface, allowing to predict the probability of mold growth under selected combinations of tested factors. The time-to-detection model was utilized to estimate the time at which *A. flavus* visible growth begins. Water activity, temperature, and CEO concentration were the most important factors affecting fungal growth. It was observed that there is a range of possible combinations that may induce growth, such that incubation conditions and the amount of essential oil necessary for fungal growth inhibition strongly depend on protein and fat concentrations as well as on the pH of studied model systems. The probabilistic model and the time-to-detection models constitute another option to determine appropriate storage/processing conditions and accurately predict the probability and/or the time at which *A. flavus* growth occurs.

1. Introduction

Molds are spoilage microorganisms that may produce mycotoxins. Particularly, *Aspergillus* species have the ability to grow in a range of environmental conditions and foods, are responsible of many worldwide food spoilage problems, and cause important economic losses. Mold growth in food products depends on several factors such as product composition, pH, a_w, temperature, composition of the atmosphere, presence and concentration of preservatives, as well as storage time. Since a_w and temperature are among the most important factors for *Aspergillus* growth response, several approaches (García et al., 2011; Gougouli and Koutsoumanis, 2010) have taken into account these two factors for estimation of time to growth or time to fail (spoilage). However, most available models ignore factors such as food composition and structure, as well as potential microbial interactions and the presence of antifungal agents (Gougouli et al., 2011). *Aspergillus flavus* has been associated to the production of aflatoxins, carcinogenic metabolites that are greatly regulated in most countries. Although maximum mold growth does not necessarily coincide with the most favorable conditions for aflatoxin production, it is very important to find the combination of factors to prevent mold growth and development (Klich, 2007a, 2007b; Yue et al., 2011).

Mathematical models together with experimental data generated under different combinations of factors that influence microbial growth, may be utilized to properly define the formulation, processing, and storage conditions for selected foods, especially for those that are minimally processed (Tienungoon et al., 2000). Most common predictive mycology growth models are the linear model, Baranyi's model, and Gompertz's equation (Gougouli and Koutsoumanis, 2013). A simple model is always preferred due to ease of interpretation and use. Mold growth models for mycelium diameter data with no transition between the lag and the growth phase and no stationary phase have been utilized for their simplicity (Gougouli and Koutsoumanis, 2013). When a stationary phase is observed, Baranyi's and Gompertz's models can be applied. Some authors prefer Baranyi's model; however, Gompertz's

* Corresponding author.
E-mail address: aurelio.lopezm@udlap.mx (A. López-Malo).

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modified equation has been sometimes selected based on its flexibility to different asymmetrical growth data (Char et al., 2005). Secondary growth models for fungi, describe the individual or combined effects of various factors on parameters estimated from primary models, such as radial growth rate and lag phase duration; in addition, they can describe the influence of factors on the growth/no growth boundary (Deschuyffeleer et al., 2013).

In order to develop mathematical models able to describe the interface between growth and no growth, it is necessary to gather a lot of data with regards to the diverse involved factors to outline this transition zone (Vermeulen et al., 2007), which may require a significant experimental effort. An interesting approach to solve this drawback is the use of probabilistic models, which may be useful for obtaining a reliable estimation of such interface in a fast way (Masana and Baranyi, 2000), aiding to define the combination of factors that prevent the growth of microorganisms (Polese et al., 2011). Among these kinds of models, the logistic regression is a useful tool for defining the combination of factors that helps to prevent the growth of microorganisms within food systems (Sosa-Morales et al., 2009), exhibiting better adjustments than those obtained with linear models for growth/no growth data (Zhao et al., 2001).

Additionally, time-to-visible growth (time-to-detection, TTD) models allow estimating the time at which microbial visible growth occurs. Failure (TTD) times have been reported for *Zygosaccharomyces bailii* growth in acidified products (Jenkins et al., 2000) as well for *Aspergillus niger* in dried tomatoes (Gómez-Ramírez et al., 2013).

In the last two decades, the use of natural bactericidal and fungicidal agents, such as essential oils, for food processing and preservation has acquired a growing importance (Gamboa-Alvarado et al., 2002). García et al. (2006) tested the antifungal action of cinnamon essential oil (CEO) on pecans during storage. Other authors (Montes-Belmont and Carvajal, 1998; Morozumi, 1978; Nguefack et al., 2004; Tantaoui-Elasaki and Beraoud, 1994; Yousef and Tawil, 1980) have evaluated the effect of different essential oils on mold growth, showing that either alone or in combination with other preservation factors, they can be successfully used as antifungal agents.

In this work, the growth response of *A. flavus* in a food model system under selected conditions was obtained, in order to be modeled using the modified Gompertz equation. A probabilistic model that considers the combinations of studied preservation factors (a_w, pH, fat, protein, essential oil of cinnamon, and incubation temperature) was developed to predict the growth boundary for *A. flavus*. In addition, the time at which mold growth occurred was recorded and fitted to a TTD model. Then, performance of tested Gompertz, probabilistic, and TTD models were compared. Few studies include the use of three approaches for modeling fungal growth; a comprehensive analysis of data will allow to better describe fungal behavior under tested conditions.

2. Materials and methods

2.1. Experimental design

A Box-Behnken design was utilized to evaluate the effect of selected factors on the lag time and radial growth of *A. flavus*. Box-Behnken design considers more experiments at central point than other kinds of surface response type designs, in this study 25 °C is the middle point thus more experiments are established by the design around this temperature than for 15 or 35 °C. Most studies focus on food factors such as a_w, temperature, and essential oils. However, foods are complex systems that contain protein and fat, which could affect the antimicrobial effect of cinnamaldehyde (the main active antimicrobial compound of CEO) due to protein- or fat-cinnamaldehyde interactions (Weibel and Hansen, 1989). Furthermore fat and protein could potentially affect mold growth, and these two components have been less studied than other food components. In this work, the presence of protein and fat effects were evaluated in order to simulate some dairy products like cheese and bakery products. Thus selected concentrations of protein and fat are similar to those contained in such products. Cinnamon essential oil is more expensive than traditional antimicrobials; though, potential application is possible in these products during storage or manufacture. Studied factors were incubation temperature (15, 25, or 35 °C), protein concentration (0, 5, or 10 g casein/100 g), fat concentration (0, 3, or 6 g corn oil/100 g), water activity (0.900, 0.945, or 0.990) adjusted with sucrose, CEO (0, 200, or 400 μL/kg) and pH (3.5, 5.0, or 6.5) adjusted with hydrochloric acid or sodium hydroxide (both 0.1 mol/L). Every studied system was evaluated by triplicate and incubated during 50 days.

2.2. Model systems preparation

For each model system, 100 g were prepared by weighing the sucrose solution (to adjust a_w), and/or casein (Sigma Chemical Co., Steinheim, Germany), and/or corn oil (Mazola, Monterrey, Mexico), and 3.9 g of potato dextrose agar (PDA, Becton Dickinson de Mexico, Cuautitlan, Mexico). The pH was adjusted with hydrochloric acid solution (Mallinckrodt Baker, Xalostoc, Mexico) or sodium hydroxide (Merck, Darmstadt, Germany) 0.1 mol/L solutions. For systems containing corn oil, Tween 20 (Chemical Meyer, Tlahuac, Mexico) at 2 mL/100 g was added as emulsifier. Each tested system was heated to boil for 1 min, sterilized (110 °C, 2 min), cooled to 45 °C, added with 1 mL of Tween 20 and the corresponding concentration of CEO (Aromáticos Químicos Potosinos SA de CV, San Luis Potosi, Mexico), emulsified for 2 min using a mechanical homogenizer (Silverson model L4R, Silverson Machines Ltd., Chesham, England), and poured into Petri dishes.

2.3. Determination of pH and water activity

The pH was determined with a potentiometer (model 50, Beckman, Brea, CA, USA) previously calibrated in buffer solutions of pH 4, 7, or 10. Water activity was determined with a hygrometer (AQUALAB, series 3B, v.3.0. Decagon, Pullman, WA, USA) calibrated and operated according to the procedure described by López-Malo et al. (1993). In order to determine pH and a_w of each medium formulation and after 50 days of incubation, dishes without fungal inoculation were measured. Measurements were performed by triplicate.

2.4. Preparation of the inoculum and inoculation

A. flavus (ATCC 200026), obtained from the Food Microbiology Laboratory of Universidad de las Americas Puebla, was grown in PDA at 25 °C during 7 days. The culture surface was washed and spores recovered to obtain a spore suspension of ≅ 10^6 spores/mL. Solidified agar systems were inoculated with 5 μL of the spore suspension in order to form a circular inoculum in the center of each tested dish, and incubated at studied temperatures in sealed containers (avoiding anoxic conditions).

2.5. Mold radial growth

A. flavus radial growth in the inoculated plates was monitored and once growth of studied mold was detected, the diameter of the colonies was measured daily with a digital caliper (Mitutoyo Corp., Kawasaki, Japan), through the cover of the dish in two directions at right angles to each other.

2.6. Modeling growth response

2.6.1. Modified Gompertz equation

In spite of the linear model being the most widely used model to describe mold growth, the modified Gompertz equation can be also utilized due to its flexibility (Char et al., 2005) and ability to describe sigmoidal curves. The use of Baranyi's model has been proposed by some researchers because of its accurate prediction (Marín et al.,