

# *Aspergillus flavus* dose–response curves to selected natural and synthetic antimicrobials

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## Abstract

The effects of selected concentrations of antimicrobials from natural (vanillin, thymol, eugenol, carvacrol or citral) or synthetic (potassium sorbate or sodium benzoate) origin on *Aspergillus flavus* lag time inoculated in laboratory media formulated at water activity ( $a_w$ ) 0.99 and pH 4.5 or 3.5, were evaluated. Time to detect a colony with a diameter > 0.5 mm was determined. Mold response was modeled using the Fermi function. Antimicrobial minimal inhibitory concentration (MIC) was defined as the minimal required inhibiting mold growth for 2 months. Fermi function successfully captured *A. flavus* dose–response curves to the tested antimicrobials with a highly satisfactory fit. Fermi equation coefficients,  $P_c$  and  $k$ , were used to compare antimicrobials and assess the effect of pH. Important differences in  $P_c$  and  $k$  were observed among antimicrobials, being natural antimicrobials less pH dependent than synthetic antimicrobials. A large  $P_c$  value represents a small antimicrobial effect on *A. flavus* lag time; thus, high concentrations are needed to delay growth. *A. flavus* exhibited higher sensitivity to thymol, eugenol, carvacrol, potassium sorbate (at pH 3.5), and sodium benzoate (at pH 3.5) than to vanillin or citral. MICs varied from 200 ppm of sodium benzoate at pH 3.5 to 1800 ppm of citral at both evaluated pHs. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The inactivation or inhibition pattern of microorganisms exposed to lethal or inhibitory agents or environments can be described by a dose–response curve. Microbial response is plotted as a function of the intensity of the preservation factor (Peleg et al., 1997). The dose–response curve has typically a sig-

moid shape, characterized by a region of marginal or unnoticeable inhibition or mortality followed by a clearly linear region, representing an exponential decay (Peleg, 1996). This behavior has been traditionally separated into two regions, one with marginal or no effect at low exposure intensities of the preservation factor and a second region with a linear (after a logarithmic transformation) behavior, which is modeled using first-order reaction kinetics. An alternative approach to model microbial dose–response is to consider it continuous for the entire range of the applied preservation factor, without abrupt changes in response kinetics but with a gradual transition from no or marginal effect at relatively low intensities to

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inhibitory or lethal effects at high intensities of the preservation factor. Microbial population declines as a result of exposure to lethal agents, that is, high temperature, pulsed electric fields, high-pressure treatments, radiation or antimicrobial dose, and can be described by the Fermi function (Peleg, 1996; Peleg et al., 1997; Palou et al., 1998).

Data acquisition for elucidating the interactions of multiple variables associated with food systems has been underway for several decades, particularly in relation to determining how antimicrobial activity is affected by other parameters (Alzamora and López-Malo, in press). However, there is a need for better understanding of the effects of combinations of traditionally used preservatives on microbial growth (Parish and Davidson, 1993). This lack of research is even greater when the combination includes a naturally occurring antimicrobial. In many cases, the effects of antimicrobials in combination with other microbial stress factors may produce growth after an important delay to observe it (López-Malo et al., 1998; Mata-moros-León et al., 1999).

The use of antimicrobials to enhance the safety of fresh and processed fruits and vegetables is of great interest to the food industry (Cherry, 1999). The antimicrobial activities of extracts from several plants and spices used as flavoring agents in foods have been recognized for many years. Among the natural antimicrobials more studied are those present in plants, weeds, herbs, and spices. It is recognized that the active antimicrobial components are phenolic compounds and essential oils (Davidson, 1996). According to Shelef (1983), phenolic compounds are probably the major antimicrobial component present in the essential oil of spices, and have been recognized as the active components of some essential oils as eugenol, carvacrol, thymol, and vanillin (López-Malo et al., 2000a,b). However, data on the effect of extracts alone or in combination with other factors on mold growth are scarce (López-Malo et al., 2000a,b). Additionally, there are no models to predict performance when natural preservatives are used in combination with other factors (Gould et al., 1995). The toxic effects of antimicrobials on microorganisms are usually determined by arbitrarily fixing the exposure period and determining the antimicrobial concentration required to cause a certain response (Shelef, 1983). In this type of method, the inhibitory concen-

tration is determined by measuring a microbial response such as turbidity, zone of inhibition, viable cell population, and mycelial weight, among others. Inhibition is defined after a given time period, usually 1 to few days (Parish and Davidson, 1993; López-Malo et al., 2000a,b). The result is reported as minimal inhibitory concentration (MIC), but these results can be dubious since growth in the presence of a fungistatic agent can occur later. From a food preservation point of view, it would be desirable to have a method that relates antimicrobial concentration with the time required to attain a defined level of inhibition. The objectives of this research were to evaluate at water activity ( $a_w$ ) 0.99, and pH 4.5 or 3.5 the effects of selected concentrations of antimicrobials from natural (vanillin, thymol, eugenol, carvacrol or citral) or synthetic (potassium sorbate or sodium benzoate) origin on *Aspergillus flavus* lag time, as well as to model the mold response using the Fermi function.

## 2. Materials and methods

### 2.1. Microorganism and preparation of inocula

*A. flavus* ATCC 16872 was cultivated on potato dextrose agar (PDA; Merck, Mexico) slants for 10 days at 25 °C and the spores harvested with 10 ml of 0.1% Tween 80 (Merck) solution sterilized by membrane (0.45 µm) filtration. The spore suspension was adjusted with the same solution to give a final spore concentration of  $10^6$  spore/ml and used the same day.

### 2.2. Preparation of the systems

PDA systems were prepared with commercial sucrose to reach  $a_w$  0.99, sterilized for 15 min at 121 °C, cooled, and acidified with hydrochloric acid to the desired pH. The amounts of sucrose and acid needed in each case were previously determined. The sterilized and acidified agar solutions were aseptically divided and the necessary amount (100, 200, 300, up to 1800 ppm) of vanillin, thymol, eugenol, carvacrol, citral, potassium sorbate or sodium benzoate (Sigma, St. Louis, MO) was added and mechanically incorporated under sterile conditions. Agar solutions were poured into sterile Petri dishes.

### 2.3. Inoculation and incubation

Triplicate Petri dishes of each system and for every antimicrobial were centrally inoculated by pouring 2  $\mu\text{l}$  of the spore suspension ( $\sim 2.0 \times 10^3$  spore/plate) to give a circular inoculum. For each pH, growth controls without antimicrobial were prepared and inoculated as described above. Three plates of each system were maintained without inoculation for  $a_w$  and pH measurements. The inoculated plates and controls were incubated for 2 months at 25 °C in hermetically closed plastic containers to avoid dehydration. A sufficient headspace was left in the containers to avoid anoxic conditions. Periodically, inoculated plates were removed briefly to observe them and immediately re-incubated.

The  $a_w$  was measured with a Decagon CX-1 (Decagon Devices, Pullman, WA) calibrated and operated following the procedure described by López-Malo et al. (1993). The pH was determined with a Beckman pH meter model 50 (Beckman Instruments, Fullerton, CA). Measurements were performed by triplicate.  $a_w$  and pH of the PDA systems without inoculation determined at the beginning and at the end of incubation demonstrated that the desired values remained constant for the incubation conditions.

### 2.4. Mold growth response

The inoculated systems were observed during 60 days using a stereoscopic microscope (American Optical, model Forty). The time to detect a colony with a diameter  $> 0.5$  mm was recorded and defined as the lag time. MIC was defined, as the minimal required inhibiting mold growth for 2 months.

### 2.5. Modeling mold response

The Fermi function modified to antimicrobial concentration as the inhibitory or lethal agent has the form:

$$S(P) = \left[ \frac{1}{1 + \exp\left(\frac{P - P_c}{k}\right)} \right] \quad (1)$$

where  $S(P)$  is the mold lag time in the control PDA (without antimicrobial) divided by the lag time in the PDA formulated with a selected antimicrobial con-

centration,  $P$  the antimicrobial concentration used (ppm),  $P_c$  a critical level of  $P$  where  $S(P)$  is 0.5, and  $k$  is a constant (ppm) indicating the steepness of the dose–response curve around  $P_c$ . Since about 90% of the inhibition occurs within  $P_c \pm 3k$  (Peleg, 1996), a large value of  $k$  means a wide span, while a small value a very steep decline. Parameter values of the Fermi equation were obtained by nonlinear regression (Kaleidagraph 3.08c, Synergy Software). The generated models were used to predict the minimal concentration of every antimicrobial that inhibits at least for 60 days *A. flavus* growth and were compared with those experimentally obtained.

## 3. Results and discussion

Figs. 1 and 2 compare *A. flavus* lag time in media formulated with natural (thymol) and synthetic (potassium sorbate) antimicrobials and also present the effect of pH on mold response. For media formulated with thymol (Fig. 1) at both evaluated pHs, the increase of antimicrobial concentration caused an important increase in lag time, represented by a reduction in the  $S(P)$  value. For potassium sorbate (Fig. 2), the concentration needed to obtain the same effect on mold lag time [equal  $S(P)$  value] was higher at pH 4.5 than the required at pH 3.5, demonstrating the antimicrobial action of undissociated sorbic acid (Davidson, 1996).

Fig. 3 presents mold response to selected natural antimicrobials when evaluated in media formulated at pH 3.5. The patterns observed differed depending on the antimicrobial, being citral and vanillin less effective to inhibit mold growth since higher concentrations were needed. However, a relatively high vanillin or citral concentration caused an important delay in mold growth.

The applicability of Eq. (1) to the antimicrobial dose–response curves of *A. flavus* is demonstrated in Figs. 1–3. Regression parameters are listed in Table 1. As judged by statistical criteria ( $r > 0.90$ ), the fit was highly satisfactory, hence Eq. (1) was successful in capturing *A. flavus* dose–response curves for the tested antimicrobials. The Fermi equation is an empirical model; therefore, it can only be used to describe and compare inhibition patterns.  $P_c$  and  $k$  can be used to compare antimicrobials and assess the effects of

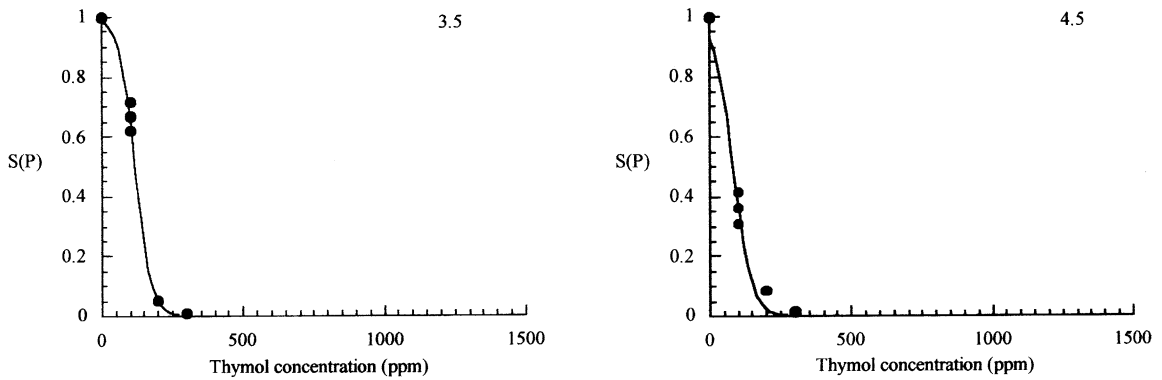


Fig. 1. *A. flavus* dose–response curves in PDA formulated with  $a_w$  0.99, selected pHs (3.5 or 4.5), and thymol. Circles are experimental data; solid lines are the fit of Eq. (1).

environmental factors as demonstrated in Table 1 and Figs. 1–3.

Longer lag times are represented by smaller  $S(P)$  values. As antimicrobial concentration increases, smaller  $S(P)$  values were obtained as exemplified in Figs. 1–3. Important differences in  $P_c$  and  $k$  were observed among antimicrobials, being in general natural antimicrobials less pH dependent than the synthetic. A large  $P_c$  value represents a small antimicrobial effect on mold lag time; thus, relatively high concentrations are needed to delay mold growth. *A. flavus* presented higher sensitivity to thymol, eugenol, carvacrol, potassium sorbate (at pH 3.5), and sodium benzoate (at pH 3.5) than to vanillin or citral. It is also noticed (Table 1), that increasing antimicrobial con-

centration in the case of thymol, carvacrol, eugenol, potassium sorbate, and sodium benzoate had a dramatic effect on *A. flavus* lag time, since their  $k$  values are small in comparison with vanillin and citral  $k$  values, which in some cases had numerical values similar to  $P_c$ .

MICs varied from 200 ppm sodium benzoate at pH 3.5 to 1800 ppm citral at both evaluated pHs. Our results demonstrate the efficacy of natural antimicrobials as antimycotic agents. Davidson (1993) reported that the exact cause–effect relation for the mode of action of phenolic compounds such as thymol, eugenol, carvacrol, and vanillin has not been determined, but they may inactivate essential enzymes, react with the cell membrane or disturb genetic material functionality.

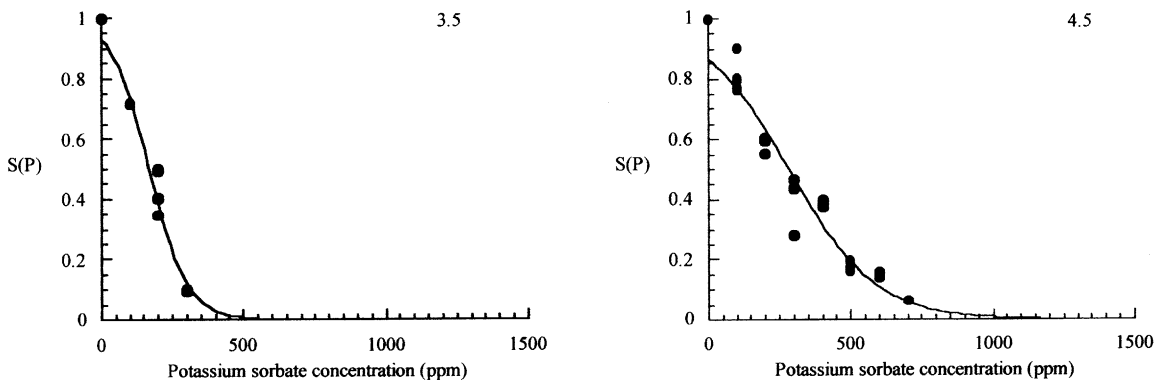


Fig. 2. *A. flavus* dose–response curves in PDA formulated with  $a_w$  0.99, selected pHs (3.5 or 4.5), and potassium sorbate. Circles are experimental data; solid lines are the fit of Eq. (1).

The generated equations predict *A. flavus* lag time with errors <10% for the evaluated antimicrobials. These errors increased as the standard errors on the

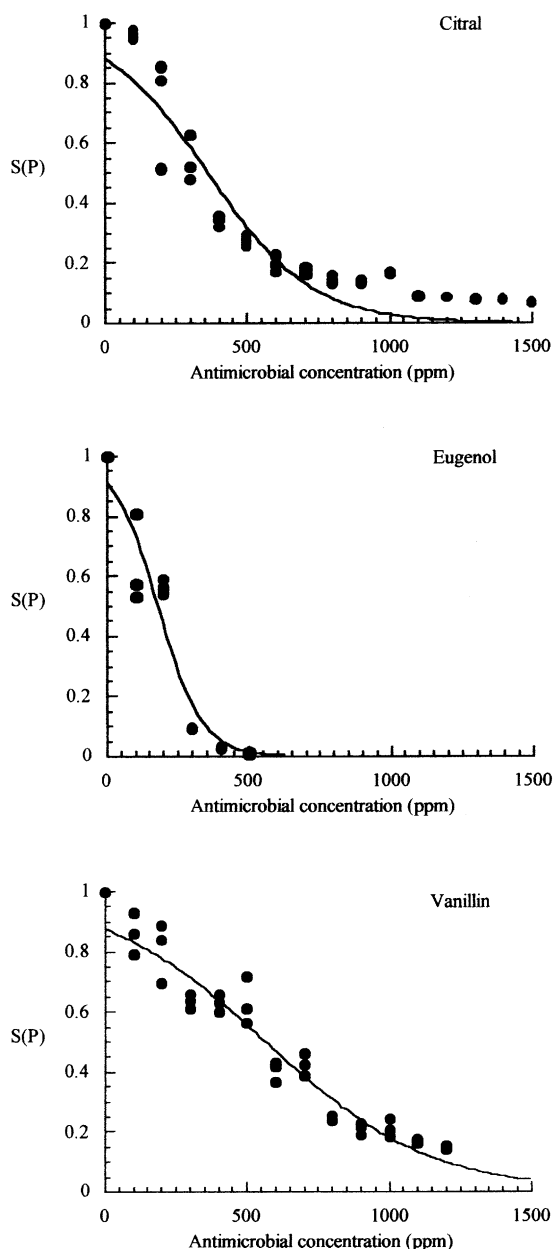


Fig. 3. *A. flavus* dose–response curves in PDA formulated with  $a_w$  0.99, pH 3.5, and selected antimicrobials. Circles are experimental data; solid lines are the fit of Eq. (1).

Table 1

Regression parameters<sup>a</sup> of Eq. (1) as a model for *A. flavus* lag time when exposed to selected antimicrobials in PDA formulated with water activity 0.99 and different pHs

Antimicrobial	pH	$P_c$ (ppm)	$k$ (ppm)	$r^b$	MIC <sup>c</sup> (ppm)
Vanillin	4.5	720 ± 27	328 ± 30	0.928	1300
	3.5	567 ± 17	289 ± 18	0.969	1300
Thymol	4.5	83 ± 5	33 ± 7	0.987	400
	3.5	120 ± 2	28 ± 2	0.988	400
Carvacrol	4.5	155 ± 2	28 ± 2	0.998	400
	3.5	90 ± 23	74 ± 27	0.901	300
Eugenol	4.5	212 ± 12	51 ± 11	0.958	500
	3.5	182 ± 13	77 ± 13	0.952	600
Citral	4.5	251 ± 23	175 ± 24	0.900	1800
	3.5	364 ± 18	181 ± 18	0.945	1800
Potassium sorbate	4.5	285 ± 13	151 ± 14	0.969	800
	3.5	170 ± 6	66 ± 6	0.987	400
Sodium benzoate	4.5	607 ± 17	382 ± 19	0.972	1500
	3.5	– <sup>d</sup>	–	–	200

<sup>a</sup> Figures ± are the standard deviations.

<sup>b</sup> Regression coefficient.

<sup>c</sup> Minimal inhibitory concentration.

<sup>d</sup> Insufficient data to fit Eq. (1).

estimates for  $P_c$  and  $k$  increased, due to error propagation, as can be seen in Table 1 and Fig. 3 for citral.

Equation parameters,  $P_c$  and  $k$ , can be used to explain differences in mold sensibility or resistance to antimicrobials, which can be applied to select them with a scientific basis, and further find applications for natural antimicrobials in real foods. Plant-derived antimicrobials are not yet fully exploited. The use of spices, herbs, plants, essential oils, and related phenolic compounds as antimicrobials is limited, due to the high MICs required in foods with high protein and/or fat contents (López-Malo et al., 1995; Castañón et al., 1999), which also may impart objectionable flavors and/or aromas. These undesirable effects can be minimized, if the natural compound is used in combination with other environmental stress factors, as in our case with a reduced pH value.

#### 4. Conclusion

*A. flavus* exhibited higher sensitivity to thymol, eugenol, carvacrol, potassium sorbate (at pH 3.5), and sodium benzoate (at pH 3.5) than to vanillin or citral.

Fermi equation described adequately dose–response curves for the tested antimicrobials, and can be used to evaluate critical antimicrobial concentrations ( $P_c$ ), as well as to model, via the  $k$  values, flat (vanillin, citral, and sodium benzoate at pH 4.5) or very sharp drops (thymol, carvacrol, eugenol, and potassium sorbate) in inhibition.

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