Journal of Food Protection, Vol. 68, No. 6, 2005, Pages 1203–1207

Modeling the Effect of Ethanol Vapor on the Germination Time of *Penicillium chrysogenum*

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MS 04-440: Received 29 September 2004/Accepted 9 February 2005

ABSTRACT

The influence of ethanol vapor on germination of *Penicillium chrysogenum* was determined on yeast nitrogen base plus glucose agar medium at 25°C. Ethanol vapors were generated by 0 to 6% (wt/wt) ethanol solutions at the bottom of hermetically closed petri dishes. The logistic equation was used to describe the data as the percentage of germination versus time and to estimate the germination time. The effect of ethanol concentration on germination time was described by a new reparameterized equation, resulting in an estimated limiting ethanol concentration of 4.3%. Up to 3% ethanol, all spores germinated, and the germination time increased with increasing ethanol concentration. At 3.5 and 4%, some spores formed abnormal germ tubes and others were inhibited at the swelling stage. The inhibiting effect of ethanol was reversible under these experimental conditions.

Under favorable environmental conditions, molds can grow on a wide variety of substrates. Among the physicochemical hurdles cited in the literature, ethanol is recognized as a mold inhibitor *(11).* It increases the mold-free shelf life of bread when added after baking and cooling at concentrations from 0.5 to 3.5% (wt/wt) of loaf weight *(8, 16, 22, 23, 25).* The inhibitory effect can be obtained by adding ethanol directly to the product or by using an encapsulated ethanol pouch (Ethicap, Freund Industrial Co. Ltd., Tokyo, Japan). This ethanol emitter extended the shelf life of packaged apple turnovers *(24),* pita bread *(1),* packed sliced rye bread *(17),* English-style crumpets *(3),* and prebaked buns *(7).*

When using ethanol vapor generators, a decrease in the ethanol in the headspace occurs *(3)* due to absorption of ethanol by the products. Therefore, it is difficult to assess the influence of ethanol when the concentrations in the headspace and in the product are not constant. Most of the previous studies were concerned with natural microflora, including bacteria, yeasts, and molds.

Since the earlier work of Lesage *(12),* who demonstrated the effect of ethanol vapor on the germination of *Penicillium glaucum* and *Sterigmatocystis nigra* (*Aspergillus niger*), no more detailed studies have been published. However, spore germination deserves focused attention. A product will be spoiled shortly after spores are germinated; therefore, prevention of germination will prevent fungal growth and subsequent spoilage. Through the example of *Penicillium chrysogenum,* the present study provided a predictive model for estimating the effect of ethanol vapor on spore germination time. The potential applicability of ethanol vapors for preserving foods and raw products is discussed.

MATERIALS AND METHODS

Mold. *P. chrysogenum* was isolated from a spoiled pastry product and identified based on the descriptions of Samson et al. *(18).* The mold was maintained on potato dextrose agar medium (PDA; bioMérieux, Marcy l'Etoile, France) at room temperature (18 to 25°C).

Media. The basal medium used for spore production was PDA. The germination medium (YNB-GA; water activity [a_w] = 0.99) consisted of yeast nitrogen base (6.7 g/liter; Difco, Becton Dickinson, Sparks, Md.) supplemented with glucose (20 g/liter) and agar (15 g/liter). The initial pH for all the experiments was 5.7 ± 0.1. All plates were incubated at 25°C. All trials were performed in triplicate for a maximum of 3 weeks.

Experimental set up. The device used in each experiment was made from a petri dish, as described previously *(19, 20).* In each dish, three patches of germination medium (16 mm diameter, 1 mm thick) were applied to the lid, and ethanol solutions were poured into the bottom. To apply the medium to the lid, a sterile dish was opened in a laminar flow cabinet. Three small glass cylinders were placed on the internal side of the lid and filled with sterile germination medium to a thickness of about 1 mm. After the medium had solidified, the glass cylinders were removed and each surface was ready for inoculation. To control the ethanol vapor pressure, 15 ml of ethanol-water solution at 0 to 6% (wt/wt) was poured into the petri dish. The ratio of medium to solution (vol/vol) was about 0.04.

Calculation of ethanol vapor pressure. The ethanol vapor pressure P_e (kPa) in equilibrium with the ethanol solution can be calculated according to the Raoult equation: $P_e = X_e\gamma_e Pt$, where X_e is the molar fraction of ethanol in the solution, γ_e is the activity coefficient (which can be taken as 1 for low ethanol concentrations), and Pt (kPa) is the vapor pressure at 25°C in equilibrium with ethanol (Pt = 7.87 kPa) *(13).*

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The molar fraction of ethanol can be calculated for 100 g of solution, which contained E grams of ethanol and $100 - E$ grams of water:

$$X_e = \frac{\frac{E}{46}}{\frac{E}{46} + \frac{100 - E}{18}}$$

Substituting X_e in the Raoult equation:

$$P_e \text{ (kPa)} = \frac{\frac{E}{46}}{\frac{E}{46} + \frac{100 - E}{18}} \times 7.87$$

This relation can be approximated to P_e (kPa) = 0.03126E for E in the range 0 to 6% (wt/wt) ($r^2 = 0.99$). Given an atmospheric pressure of P_{atm} = 101.3 kPa in the experimental device, the concentration of headspace ethanol is P_e (%, vol/vol) = [100(0.03126E)]/101.3 = 0.03086E.

Spore preparation. After incubation of the PDA growth medium at 25°C for 7 ± 1 days, *P. chrysogenum* spores were collected by flooding the surface of the plates with sterile saline solution (NaCl, 9 g per liter of water) containing Tween 80 (0.1% vol/vol; Prolabo, Paris, France).

Spore germination. The surface of each patch of YNB-GA medium was inoculated with 10^2 *P. chrysogenum* spores (1 μl of a suspension of 10^5 spores·ml^{-1}). After inoculation and addition of ethanol, petri dishes were hermetically closed with adhesive tape to maintain a constant ethanol concentration throughout the experiment. Without opening the dishes, spores were examined daily through the lid with a Labophot-2 (×400) microscope connected to a CoolPix990 camera (Nikon, Champigny sur Marne, France). To ensure that the total number of spores examined, N_{total}, was close to 100, about 10 different pictures were taken. A spore was considered germinated when the length of the germ tube was equal to half of the spore diameter *(15).* The percentage of germinated spores was calculated as P (%) = (N_{germ}/N_{total}) × 100. To monitor the germination of the same spores throughout the experiment, petri dishes were marked.

Primary model. The logistic function as described previously *(6)*

$$P \text{ (\%)} = P_{max}/\{1 + \exp[k(\tau - t)]\}$$

was used to model the percentage of germinated spores P (%) as a function of time t (h). P_{max} (%) is the asymptotic values of P, k (h^{-1}) is the slope term at the inflection point, and τ (h) is the germination time for $P = P_{max}/2$. For each ethanol concentration, the correlation coefficient r was determined by performing a linear regression of the variance, Var (P) against the mean, Mean (P) *(26).* The Student t test did not exhibit any significant correlation:

$$\left| t_{stud} = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}} \right| < T_{table}$$

and $n = 8$ was the number of observations. Therefore, there was no need for transforming the data for percentage of germination to stabilize the variance.

Secondary model. The influence of ethanol on the fungal growth rate was described previously by a reparameterized Monod-type equation *(5)*:

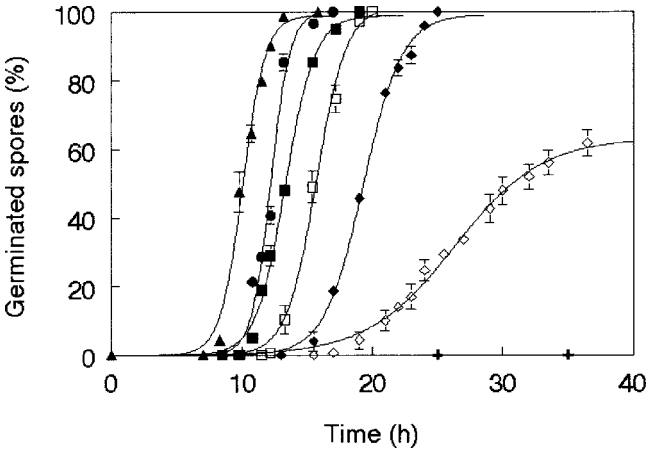

FIGURE 1. *Germination kinetics of* P. chrysogenum *on YNB-GA medium (0.99 a_w, pH 5.4, 25°C) in the presence of ethanol solutions (wt/wt) of 0% (▲), 1% (●), 2% (■), 2.5% (□), 3% (♦), 3.5% (◊), and 4% (+) and logistic model (——) fit. Error bars are standard deviations. Data obtained at 0.5 and 1.5% were omitted for representation purposes.*

$$\mu = \mu_{opt}\frac{K(E_{max} - E)}{K(E_{max}) - 2KE + E(E_{max})}$$

For modeling the effect of ethanol (E) on the germination time τ (h), the reciprocal of this equation can be used as follows:

$$\tau = \tau_{opt}\frac{K(E_{max}) - 2KE + E(E_{max})}{K(E_{max} - E)}$$

where τ_{opt} (h) is the germination time at $E = 0$ (%, wt/wt), K (%, wt/wt) is the ethanol percentage at which $\tau = 2\tau_{opt}$, and E_{max} (%, wt/wt) is the percentage of ethanol in solution at which no germination occurs. Data where no germination was observed were not included in the adjustment but were used for validation of the model. All model equations were fitted to the data using nonlinear regression software (SlideWrite 4.1, Advanced Graphics Software, Carlsbad, Calif.) based upon the Levenberg-Marquardt algorithm as described previously *(4).*

RESULTS

Germination. All spores germinated at ethanol concentrations ≤3.0% (wt/wt) (Fig. 1). At 3.5% (wt/wt), about 60% of the inoculated spores were capable of germinating after 40 h, eventually forming a mycelium. The other spores were inhibited at the swelling stage or had their germinating tube blown up at the apex level. One spore (Fig. 2, arrow) was photographed at different times in 3.5% ethanol. An abnormal germinating tube that was formed at 40 h eventually blew up at 64 h (Fig. 2). Two spores located at the bottom left in Figure 2c remained at the swelling stage. These findings were also observed in 4% ethanol. After 3 weeks of incubation, no germination had occurred at ethanol concentrations of 4% and higher.

The germination curves are shown in Figure 1 for different ethanol concentrations. A close fit was provided by the logistic function. All adjusted regression coefficients were close to 1 (Table 1). At 0 to 3% (wt/wt) ethanol, all spores were capable of germinating. Under these conditions, the asymptotic estimated values, P_{max}, were not significantly different from 100% (Table 1). At 3.5% (wt/wt)

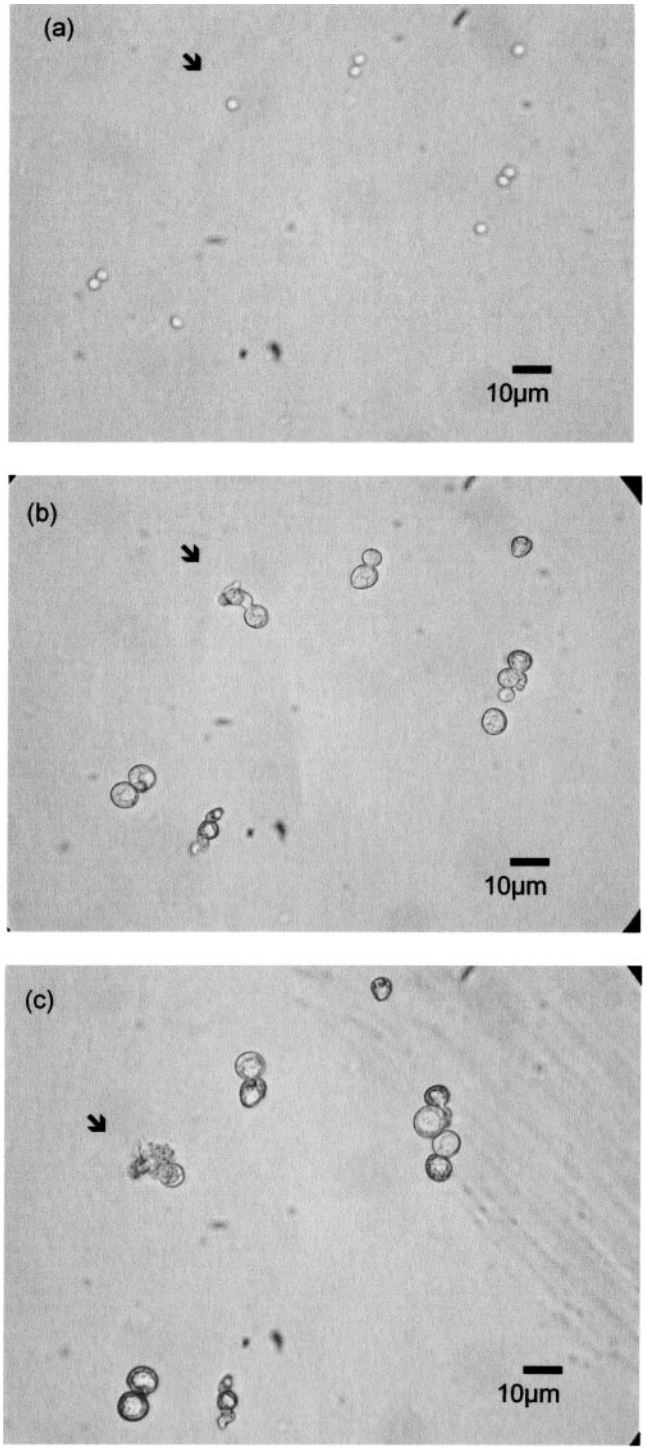

FIGURE 2. *Microscopic observations of* P. chrysogenum *conidia on YNB-GA medium at 25°C, 0.99 a_w, and pH 5.4 in the presence of ethanol vapor generated by a 3.5% (wt/wt) ethanol-water solution at (a) 15 h, (b) 40 h, and (c) 64 h.*

ethanol, the asymptotic estimated P_{max} was 63.1% (Table 1). The slope term k did not differ significantly up to 1.5% (wt/wt) ethanol but then decreased with increasing ethanol concentration (Table 1). The τ value obtained at 0% ethanol represented the smallest germination time, τ_{opt}, that can be obtained on YNB-GA medium. The water activity, temperature, and pH of the medium were optimal. At 0.5 to 1.5% (wt/wt) ethanol, τ was constant and then increased with increasing ethanol concentration.

Modeling the effect of ethanol on germination time. The germination time was modeled as a function of the ethanol concentration. The estimated parameters are listed in Table 2. All parameters were characterized by narrow confidence intervals. The value of τ_{opt} was in accordance with the value obtained under optimal conditions (i.e., 0%, wt/wt, ethanol). At 4% (wt/wt) ethanol, no germination was observed after 3 weeks of incubation. Therefore, the germination time was greater than 504 h, but the model predicted a value of only 58.3 h. The parameter K was closer to the E_{max} value than to E_0 (i.e., $K/E_{max} = 0.75$). A twofold increase in the germination time was observed with increasing ethanol concentration from E_0 to K (75% of the ethanol range). The effect of ethanol was really effective (i.e., more than a twofold increase in the germination time) from K to E_{max} (25% of the ethanol range). A sharp increase in τ occurred at 3 to 4% (wt/wt) ethanol (Fig. 3).

DISCUSSION

Ethanol exerts its effect through disruption of the cell membrane and subsequent interference and/or inhibition of normal cell metabolic functions *(9).* Ethanol concentrations of 3.5 and 4% were critical for conidial homeostasis. At higher ethanol concentrations, spores did not initiate germ tubes; they were blocked at the swelling stage or the native stage. Empty ghostlike cells (i.e., cells that were collapsed and devoid of intracellular contents) were observed in cultures of *Clostridium botulinum* grown with either 2 or 4% (wt/wt) ethanol but not in cultures grown with 0% ethanol *(2).* Our results also support the hypothesis that ethanol is responsible for the leakage of solutes across the membrane or for cell lysis following decreased peptidoglycan cross-linking in the growing cell wall *(10).*

After 3 weeks, the cultures that failed to germinate were allowed to continue to incubate. The ethanol solutions of 4 to 6% (wt/wt) were replaced with physiological water, and the cultures were incubated at 25°C. In all cases, visible mycelium appeared during the next days, indicating that many conidia had remained viable. However, we did not determine whether all spores or only a fraction of spores (either at the swelling or the native stage) were capable of germinating. Under these experimental conditions, the inhibitory effect of ethanol was clearly reversible, but longer exposure to ethanol may have prevented spores for germinating. This point should be examined in future studies.

One of the objectives of the proposed secondary model was the estimation of the percentage of ethanol capable of inhibiting germination. Accordingly, a model capable of estimating this value, E_{max}, was developed. However, the estimation of E_{max} should be considered with caution. The lowest ethanol concentration that inhibited germination for 3 weeks was 4% (wt/wt). E_{max} was obtained by extrapolation; therefore, a better estimate can be obtained if germination times at E values close to E_{max} can be determined. These determinations can be made by continuing trials at 3.5 to 4% ethanol provided that germination times are less

TABLE 1. *Parameter estimates of the logistic model for the germination data of* P. chrysogenum *on YNB-GA medium (0.99 a_w, pH 5.4, 25°C) at different ethanol concentrations*

Ethanol (%, wt/wt)	Estimates for each parameter: P_{max} (%) Value	95% CI[a]	t	k (h^{-1}) Value	95% CI	t	τ (h) Value	95% CI	t	Adj r^2
0	99.0	95.0–103.0	51.1	1.15	0.97–1.34	12.9	10.1	9.9–10.2	131.8	0.988
0.5	100.2	97.0–103.3	65.0	1.23	1.09–1.38	17.4	12.2	12.1–12.3	226.9	0.992
1.0	100.2	94.8–105.6	38.4	1.35	1.09–1.61	10.7	12.2	12.0–12.4	149.9	0.980
1.5	98.9	96.8–101.0	97.8	1.33	1.20–1.46	21.0	12.4	12.3–12.4	299.3	0.995
2.0	98.9	95.6–102.2	62.5	0.89	0.80–0.99	19.1	13.3	13.2–13.5	164.2	0.994
2.5	101.5	96.3–106.7	41.2	0.87	0.72–1.01	12.3	15.7	15.4–15.9	131.6	0.990
3.0	99.2	96.0–102.3	65.1	0.67	0.60–0.74	19.8	19.3	19.1–19.5	197.1	0.994
3.5	63.1	61.4–64.8	74.6	0.30	0.27–0.33	20.3	26.3	25.9–26.7	134.1	0.983

[a] CI, confidence interval.

than 3 weeks or by extending the incubation time at 4% ethanol until the germination time can be calculated. A concentration of 4% (wt/wt) ethanol was probably not sufficient to prevent germination for a longer period of incubation. Therefore, the estimate of E_{max} was greater than 4% (wt/wt).

The estimated limiting ethanol value for inhibiting the growth of the same mold in a previous study was 3.93% (wt/wt) *(5)*. But comparison with E_{max} is difficult because the media in the two studies were different and the growth inhibition was observed by having ethanol added directly to the medium. However, the limiting ethanol values for germination and for growth of *P. chrysogenum* had the same order of magnitude.

A disadvantage of using ethanol vapor is its absorption from the package headspace by the product. The ethanol absorbed by a medium depends on the water activity of the medium and the concentration of ethanol in the headspace. In our experiments, ethanol vapors were generated by a large amount of solution and the germination medium was very thin; the ratio of medium to solution was about 4%. Accordingly, the amount of ethanol absorbed by the YNB-GA medium was negligible and the headspace ethanol concentration was considered constant. This assumption was supported by the fact that after 3 weeks of incubation the ethanol concentrations did not differ significantly from the initial concentrations.

Ethanol vapor appears to be effective at controlling fungal development in high-moisture bakery products, thus ensuring the safety of these products at ambient temperature *(3)*. However, when cake and bread were treated with >2% (wt/wt) ethanol, alcoholic flavors were very noticeable and products were rejected on the basis of flavor and/or odor *(21)*. Thus, ethanol vapor treatment has been used in combination with other factors such as water activity and gas packaging for controlling growth of *Saccharomyces cerevisiae (24)*. The effects on mold germination of treatments involving combinations of ethanol and other stress factors are currently being examined.

Another interesting but unexplored application is the use of ethanol vapor for protecting stored grains from mold spoilage and mycotoxin production. In contrast to liquid products or powders, ethanol vapor has the advantage of reaching remote places in the silo. Ethanol is a very hydrophilic molecule that can be absorbed especially in the more humid places where fungal development is more likely to occur. A headspace ethanol concentration of about 0.13% (vol/vol) was sufficient to inhibit the germination of *P. chrysogenum* on synthetic medium (a_w of 0.99) at 25°C.

TABLE 2. *Parameter estimates of the reparameterized Monod equation for modeling the influence of ethanol on germination time of* P. chrysogenum

Parameter	Estimate	t	95% confidence interval
τ_{opt} (h)	10.77	28.0	9.78–11.76
K (%, wt/wt)	3.23	42.0	3.03–3.43
E_{max} (%, wt/wt)	4.30	27.5	3.90–4.70

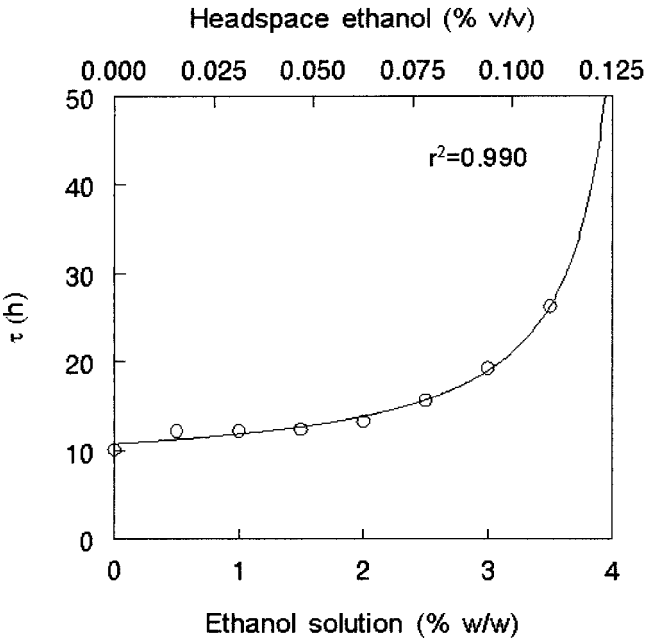

FIGURE 3. *Plot of germination time versus ethanol concentration fitted by a reparameterized Monod-type model.*

This concentration is well below the lower flammability limit for ethanol, which is 3.5% (vol/vol) *(14)*. More studies should be conducted to determine whether ethanol vapor could be used at a sufficient concentration without risk to prevent development of toxigenic molds during grain storage. This application would have a great impact on both the economy and food safety of stored grains.

ACKNOWLEDGMENT

This research was partially supported by ACTIA, contract 00.06.

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