APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1996, p. 3745–3749 Vol. 62, No. 10
0099-2240/96/$04.00+0

Tailing of Thermal Inactivation Curve of *Aspergillus niger* Spores

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Received 11 March 1996/Accepted 15 July 1996

The nonlinear thermal inactivation of *Aspergillus niger* spores in phosphate-citrate buffer was studied. The thermal inactivation pattern of the spore consisted of a shoulder, an accelerated decline, and a tail at various constant temperatures around 60°C. The pattern fitted a thermotolerant subpopulation model. In the model, we postulated that some spores in the initial population had become thermotolerant at a certain ratio during heating. The model parameters including the rate coefficients, the time lag, and the existence ratio of thermotolerant cells were analyzed at various temperatures. The tailing was not observed at an initial concentration below 10^3 cells per ml. Cells cultured from thermotolerant cells showed an inactivation pattern similar to that of the original cells. Also, cells at the second heating showed the same thermotolerance as or were slightly more thermosensitive than the original cells. Intermittent heating was found to be effective to inactivate cells at a high concentration.

The thermal inactivation of microorganisms obeys the first-order (FO) model (1, 12). The *D* value in sterilization is also based on the same assumption. However, we have often observed a shoulder at the beginning of the thermal process and a tail after an accelerated decline in the inactivation curve of an organism. These are observed not only for bacteria but also for fungi and yeasts (2, 3, 5, 9, 12). Moreover, *D* values are generally obtained from the linear portion of a (sigmoidal) survival curve (2, 5, 8, 12). Thus, thermal sterilization of products such as foods or drugs in factories calculated from *D* values or the FO model only might lead to insufficient inactivation.

Cerf (3) reviewed the tailing of thermal inactivation of bacterial spores. Several investigators have proposed kinetic models to describe nonlinear inactivation of bacterial cells (2, 6, 7, 11). On the other hand, the inactivation kinetics for fungal spores have been little studied (5). Tailing in the survival curve for fungal spores is often striking compared with bacteria and their spores. It is very difficult to completely destroy the viability of fungal spores in foods by pasteurization, even if they had a *D* value shorter (a higher sensitivity to heat) than that of bacterial cells. In this study, therefore, we investigated the nonlinear thermal inactivation of fungal spores, especially the tailing. *Aspergillus niger* was used as a test strain because it is a common contaminant of foods and other products (10).

MATERIALS AND METHODS

Fungal strain. *A. niger* 326 was isolated from food at our laboratory. The strain had an intermediate thermal sensitivity with respect to several *A. niger* strains in our collections.

Spore suspension. The strain was cultured on potato dextrose agar plates (Eiken Chemical, Tokyo, Japan) with 0.01% (wt/vol) chloramphenicol at 25°C at a humidity of 94% for 1 week. After a portion (about 15 ml) of 0.1 M dibasic sodium phosphate adjusted with 0.05 M citric acid monohydrate to pH 7.0 (PC) with 0.005% (vol/vol) Tween 80 was poured onto the plate, spores were suspended in PC buffer by rubbing gently with a glass rod. Clumped spores in PC buffer were scattered by sonication for 2 min. The suspension was filtered twice through a glass wool column to remove hyphae. The spore concentration of the suspension was estimated with a hemocytometer. The suspension was diluted with PC buffer to make 2×10^6 cells per ml.

Temperature history measurement of spore suspension. A 3.5-ml portion of the spore suspension prepared as above was equilibrated at 25°C in a Pyrex test tube (10 mm in inner diameter and 100 mm high) with a tight plastic cap. The sample was heated at a constant temperature of 56 to 62°C in a water bath unit (Jr-100; Taitec Corporation, Koshigaya, Japan). The surface of the sample was positioned about 20 mm under the surface of the circulating water in the bath. The temperature of the sample was monitored with a digital thermometer (HL-2; Anritsu Meter Co., Ltd., Tokyo, Japan). The head of the thermometer probe was positioned at the geometric (cylindric) center of the sample. The heating period, t at a constant temperature was defined as $t = t_r + t_h$, where t_r is the temperature-rising period and t_h is the temperature-holding period. From the temperature history patterns, the period t_r was 2 min for all temperatures investigated. During the period t_r, the sample substantially reached the designated temperature, with a difference of less than 0.2°C.

Heat treatment of spore suspension. The cell suspension was heated in the same manner as for the temperature measurement. Immediately after the treatment, the sample was cooled in ice water.

Estimation of viable-cell count. The heated cell suspension was diluted serially with sterile saline. A 0.5-ml portion of the dilution was spread on a potato dextrose agar plate in duplicate and incubated at 25°C for 1 week. The numbers of fungal colonies grown on the plates were then counted.

Thermotolerant-subpopulation (TTSP) model. The thermal inactivation curve of *A. niger* spores consisted of a shoulder, an accelerated decline, and a tail. We postulated that during heating, thermotolerant cells would be generated as a proportion, p, of the total cell population. Here, $0 < p \ll 1$. The rest of the population, $1 - p$, would consist of thermally normal, sensitive cells. Then we postulated that both tolerant and normal cells would be inactivated following the first-order kinetics with rate coefficients of k_1 and k_2, respectively. Here, $k_1 < k_2$. In this model, the tolerant cells would dominate the surviving cells at the tailing portion. The shoulder of the inactivation curve is treated as a time delay, t_d, of the normal cell inactivation. The kinetics of the whole spore population of the model, therefore, is shown by the following equation:

$$N/N_0 = p \exp(-k_1 t) + (1 - p) \exp[-k_2 (t - t_d)] \quad (1)$$

where N and N_0 are the numbers of living cells per unit volume at times t and 0, respectively.

The model was applied during the temperature-holding period, t_h. The thermal inactivation during the temperature-rising, period t_r, was not analyzed in this study, because the inactivation during this period could not be analyzed. Thus, N_2, the number of surviving cells heated for 2 min (t_r), was substituted for N_0 in equation 1 for each experiment.

Estimation of model parameters. The TTSP model describes a biphasic curve in a semilogarithmic plot of the survival rate, N/N_0, and t. The value of k_1 was estimated from the slope in the linear portion of the curve (4). All experimental results in this study showed that the tailing was kept at a constant survival rate, as shown below. This meant that $k_1 = 0$ in those experiments. Thus, the survival ratio at the tail was directly equal to p. Then the values of k_2 and t_d were estimated by a linear regression method for ln (N/N_0) and t.

Estimation of rate coefficients of the FO model. The rate coefficient, k, of a conventional FO model for a thermal inactivation curve shown in the following equation was estimated (1): $-dN/dt = kN$. That is, k was calculated by a linear regression method from the linear portion of the curve showing the slope of decline (2, 8)

Heating of cultured thermotolerant cells. The spore suspension (2×10^6 cells per ml) was heated at 58°C for 30 min and cooled in ice water. Some portion of

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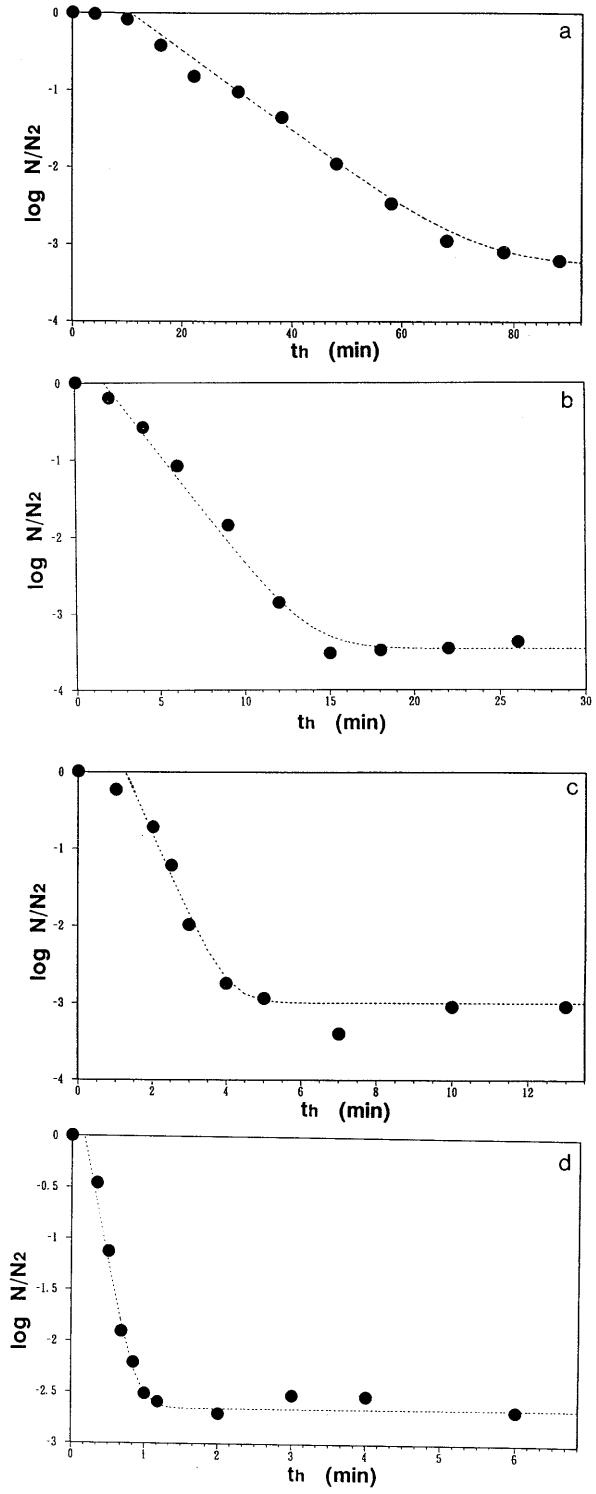

FIG. 1. Thermal inactivation curve of *A. niger* cells at constant temperatures. (a) 56°C; (b) 58°C; (c) 60°C; (d) 62°C. Dashed lines are lines fitted by the TTSP model.

the heated suspension was spread on potato dextrose agar plates and incubated at 25°C for 1 week. The cultured spores were suspended in PC buffer and heated at 58°C in the same manner as for the temperature measurement then cooled.

Reheating of thermotolerant cells. The spore suspension (2×10^6 cells per ml) was heated at 58°C for 30 min and cooled. After different intervals at 25°C, the spore suspension was heated again at 58°C in the same manner as for the temperature measurement then cooled. As a control, previously unheated spores at a concentration of about 10^3 cells per ml were also heated.

Heating at intervals. The spore suspension (about 10^7 cells per ml) was heated at 58°C for 30 min in the same manner as for the temperature measurement and cooled. After 3 h at 25°C, the spore suspension was heated again for 30 min and cooled. Finally, after 3 h at 25°C, the cell suspension was heated for 30 min and cooled. For each heating, the thermal inactivation pattern of the spores was investigated.

Statistical analyses. The experimental data were statistically analyzed by using Student's *t* test. The statistically processed values were expressed as the mean and standard deviation.

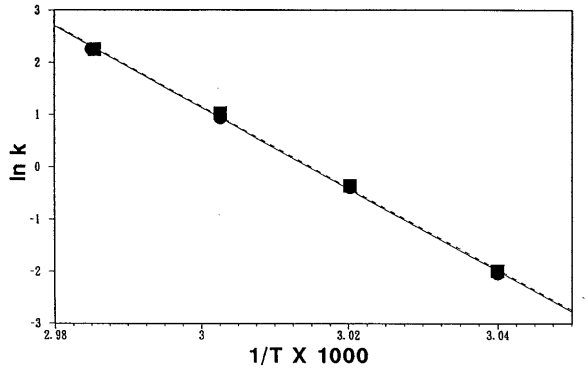

FIG. 2. Arrhenius plot for the rate coefficient k_2. Symbols: ●, TTSP model; ■, FO model. Solid and dashed lines are linear regression lines for the TTSP and FO models, respectively.

RESULTS

Inactivation patterns at different temperatures. The thermal inactivation pattern of *A. niger* spores at a constant temperature of 56 to 62°C consisted of a shoulder, an accelerated decline, and then a tail (Fig. 1). The shoulder (or the time lag) was smaller at higher temperatures. The slope of the decline portion was steeper at higher temperatures. The tail was unchanged during heating at all temperatures. The TTSP model accurately described these inactivation patterns of the spores (Fig. 1).

The parameter values of the model for these patterns were then analyzed. The k_1 value was zero at these temperatures, because the tail was horizontal. The Arrhenius plot of k_2 was linear with a high correlation coefficient ($r = 0.9985$) (Fig. 2). From the slope of the plotted line, the activation energy of inactivation was $6.50 \times 10^5 \pm 2.47 \times 10^4$ J/mol. The rate coefficients of the FO model for these inactivation patterns were also analyzed for comparison. The Arrhenius plot for this model was also highly linear ($r = 0.9981$), and the activation energy was $6.49 \times 10^5 \pm 2.82 \times 10^4$ J/mol (Fig. 2). No significant differences in the rate coefficients and the activation energy were found between the models at a level of 0.05.

The survival ratio, *p*, was then analyzed (Table 1). *p* was categorized into p_1 and p_2, which are the survival ratios N/N_2

TABLE 1. Survival ratios of the TTSP model

Temp (°C)	p_1 (mean ± SD)[a]	p_2 (mean ± SD)[a]
56	$2.83 \times 10^{-4} \pm 1.78 \times 10^{-4}$	$2.76 \times 10^{-4} \pm 1.68 \times 10^{-4}$
58	$3.87 \times 10^{-4} \pm 1.03 \times 10^{-4}$	$3.56 \times 10^{-4} \pm 9.80 \times 10^{-5}$
60	$8.00 \times 10^{-4} \pm 2.00 \times 10^{-4}$	$5.17 \times 10^{-4} \pm 1.23 \times 10^{-4}$
62	$2.55 \times 10^{-3} \pm 3.50 \times 10^{-4}$	$3.64 \times 10^{-4} \pm 3.79 \times 10^{-5}$

[a] Values were obtained from four experiments at each temperature.

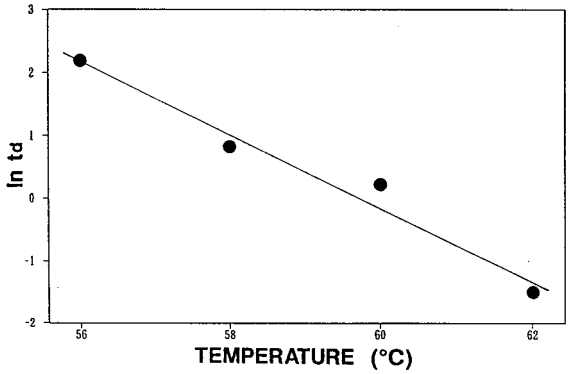

FIG. 3. Semilogarithmic plot of the time lag, t_d. A solid line is a linear regression line for the time lag.

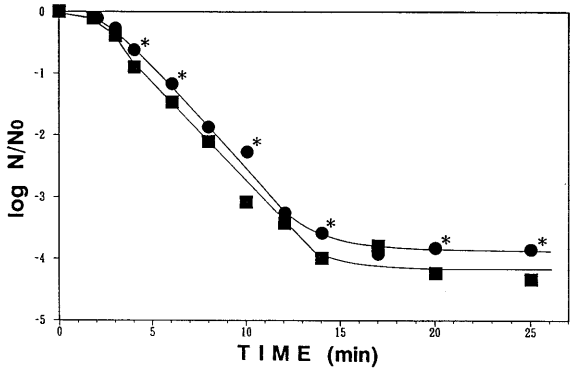

FIG. 5. Thermal inactivation curve of *A. niger* cells cultured from thermotolerant cells. Symbols: ●, cells cultured from surviving cells; ■, original cells. Asterisks show values that are significantly different at a level of 0.025.

and N/N_0, respectively. p_1 was higher at higher temperatures. Interestingly, the p_2 values were almost constant, around 3×10^{-4} to 5×10^{-4}, at these temperatures.

The delay, t_d, was longer at a lower temperature. The semologarithmic plot of t_d versus the temperature was linear ($r = 0.9826$) (Fig. 3).

Inactivation patterns at different concentrations. Thermal inactivation profiles of *A. niger* spores at different initial cell concentrations were then studied. At a concentration higher than 10^4 cells per ml, the inactivation pattern consisted of a shoulder, an accelerated decline, and a tail (Fig. 4). At those initial concentrations, the inactivation curves were almost in parallel, suggesting that the rate coefficient of the decline portion and the survival ratio at the tail of these curves were almost invariable. On the other hand, at concentrations lower than about 10^3 cells per ml, the tail of the curve was not clear, and during the heating phase, the cell concentration was finally reduced to zero (Fig. 4).

Inactivation pattern of thermotolerant cells. The thermal inactivation of thermotolerant spores was analyzed. The cells surviving at the tail portion in the inactivation curve were grown again on an agar plate. Those cells had an inactivation pattern similar to that of the original cells (Fig. 5). This indicated that those cells were as thermosensitive as the original cells.

The cells that survived at the tail portion in the inactivation curve were heated again at different intervals. The reheated cells had the same thermotolerance as or were slightly more thermosensitive than the original cells (Fig. 6). This showed that the thermotolerance was easily lost once the cells were heated. The duration of the interval had no effects on the thermal inactivation of the cells (Fig. 6).

Inactivation of cells at a high concentration. Since the tailing in the thermal inactivation curve of the cells was marked at a high cell concentrations, a set of thermal treatments at intervals was conducted for complete sterilization (Fig. 7). First, cells at a high concentration of about 10^7 cells per ml were heated at 58°C for 30 min. At that time, the cell concentration was reduced to about 10^4 cells per ml. For the second run, the inactivation pattern also had a tail. The cell concentration at the tail was about 10^1 to 10^2 cells per ml. For the third run, a slight tail was observed, and the cell concentration was finally reduced to less than 1 cell per ml. This indicated that complete sterilization could be obtained by intermittent heating at a high cell concentration.

DISCUSSION

The tailing in the thermal inactivation of *A. niger* spores was observed for long periods and at high temperatures in a preliminary study. That is, the tailing was unchanged for periods longer than 60 min at 58°C and a slight tailing was observed at high temperatures between 70 and 90°C. At these higher temperatures, the decrease in cell number during the period t_r was

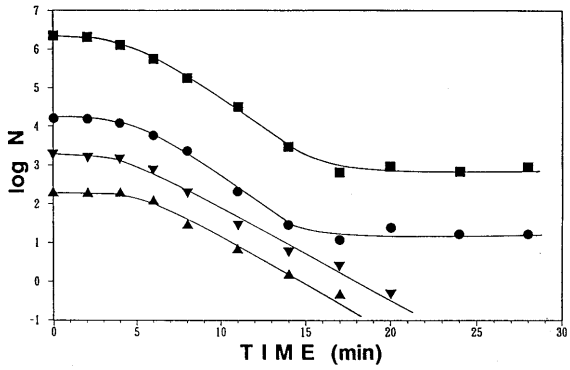

FIG. 4. Thermal inactivation curve of *A. niger* cells at various initial cell concentrations. The initial cell concentrations were about 10^6 (■), 10^4 (●), 10^3 (▼), and 10^2 (▲) cells per ml. Cells were heated at 58°C.

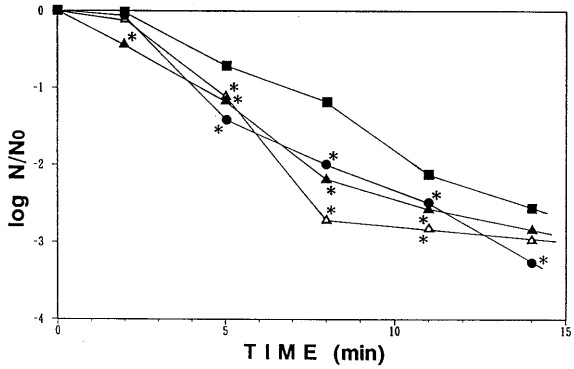

FIG. 6. Reheating of thermotolerant *A. niger* cells after an interval. Surviving cells heated at 58°C for 30 min were reheated at 58°C after intervals of 3 h (●), 6 h (▲), or 24 h (△). The control (■) was heated at an initial cell concentration of 10^3 cells per ml. Asterisks show values that are significantly different at a level of 0.025.

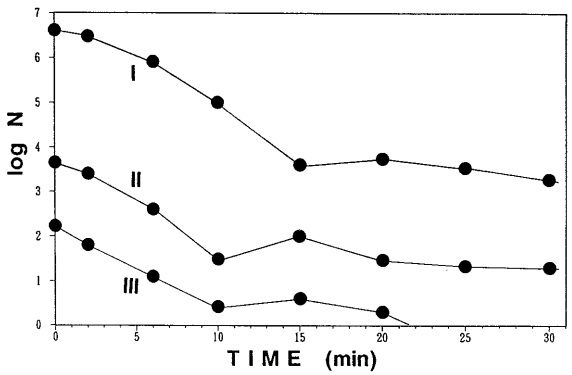

FIG. 7. Intermittent heating of *A. niger* cells. Cells were heated up to three times at 58°C for 30 min at 3-h intervals. Inactivation curves I, II, and III correspond to the numbers of heat treatments applied.

marked. Thus, the range for analyses in the kinetic study during t_h was very narrow at these temperatures, making it very difficult to estimate the model parameters. The experiments, therefore, were done at around 60°C, where the inactivation kinetics were studied with precision. On the other hand, agglomerated cells were not seen during thermal treatments. This indicated that the thermotolerance of cells was not due to the agglomeration of the cells.

In the TTSP model, thermotolerant cells were considered to appear during heating in the initial population. Cells cultured from surviving cells were not thermotolerant (Fig. 5). Also, the tolerance was not observed once surviving cells were heated (Fig. 6). Therefore, the nature of the tolerance may be transient or apparent. However, when the concentration of the surviving cells was high, i.e., $>10^3$ cells per ml, some of these cells survived at a similar ratio after the second heating (Fig. 7).

The survival ratio, p_1 was higher at higher temperatures (Table 1), because the survival ratio for the period t_r, p_r ($=N_2/N_0$), was lower at higher temperatures and p_2 was almost constant at all temperatures. Here, $p_2 = p_1 p_r$.

The TTSP model parameters in equation 1 were evaluated from the relationships between the parameters and the temperatures. k_2 was evaluated from the Arrhenius plot (Fig. 2). t_d was approximated from the semilogarithmic plot (Fig. 3) by the following equation:

$$\ln t_d = 35.0 - 0.587T \quad (2)$$

where T is the temperature. p_1 was approximated by a polynomial equation:

$$\ln p_1 = 0.000103T^2 - 0.0118T + 0.337 \quad (3)$$

The parameter values at a given temperature could be calculated from those relationships. The predicted inactivation pattern at the temperature calculated with these parameter values accurately described the experimental data (Fig. 8). This showed that the TTSP model would be useful for prediction of the thermal inactivation profile of the microbe.

The intermittent-heating experiment was performed because the cells at the tail portion were not thermotolerant once they were heated (Fig. 6) and because some cells in the original population had survived at a certain rate, p_2, after a single heating (Table 1). Intermittent heating has been used for inactivation of bacterial spores which transform into vegetative forms during the interval. However, in our study, the treatment was effective for sterilization from a different point of view. Pasteurization is used to inactivate food-borne pathogens, not to destroy all microorganisms in the products. Thus, if one needs to completely destroy contaminating fungal spores in a product and if the quality of the product is not damaged by intermittent heating, the treatment would be effective. The treatment might also be effective for elimination of bacterial cells which show tailing after a single heating.

On the other hand, the 12-D concept has been widely applied for thermal sterilization in food industries. The concept is based on the FO inactivation of microorganisms, especially thermotolerant bacterial spores. Therefore, this would not be simply compared with the intermittent heating studied in this study, which is based on the characteristics of tailing of microbial cells.

Our study revealed that when the initial spore concentration was less than 10^3 cells per ml, the tailing would not be observed in thermal inactivation at the temperatures studied. This is related to the fact that the value p_2 was about 1/3,000 (Table 1). That is, when the initial cell concentration was 10^3 cells per ml, the cell concentration at the tail portion is less than 1 cell per ml by use of the p_2 value; the tail could not be observed.

The shoulder in the thermal-inactivation pattern of the spores was described as the time lag in the TTSP model. This makes the shoulder of the predicted curve a broken line. The actual shoulder was not a broken line but a curve, as shown in Fig. 1. Thus, the model requires further improvement.

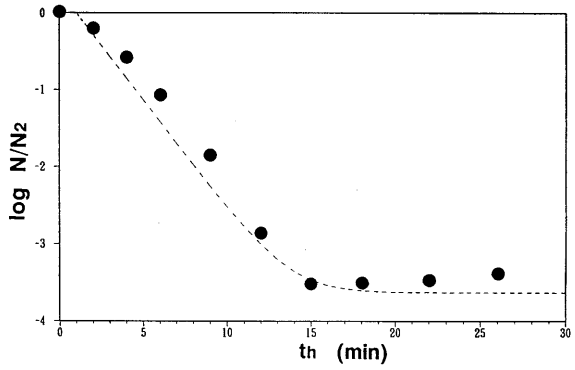

FIG. 8. Inactivation curve predicted by the TTSP model. The parameters were estimated at 58°C by the interpolation method. A dashed line was generated by the model with these estimated parameters. The microbiological experimental data were those shown in Fig. 1b.

ACKNOWLEDGMENTS

We thank S. Morozumi and T. Wauke for their helpful advice on fungi and T. Tsuchido for his valuable suggestions about the experiments.

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