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The Gauss-Eyring model: A new thermodynamic model for biochemical and microbial inactivation kinetics

H.C. Mastwijk [a,*], R.A.H. Timmermans [a], M.A.J.S. Van Boekel [b]

[a] Wageningen Food & Biobased Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands
[b] Food Quality & Design Group, Wageningen University & Research, P.O. Box 8129, 6700 EV Wageningen, The Netherlands

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ABSTRACT

A new primary model has been developed, using Gaussian distributed populations and Eyrings rate constant for the transition state, to describe inactivation kinetics of enzymes and micro-organisms subjected to heat and chemical treatment. The inactivation of both enzymes and micro-organisms could be associated with the irreversible transition to an inactivated state, as suggested by the Lumry-Eyring model for protein denaturation and enzyme inactivation. The characteristic inactivation model parameters, standard activation enthalpy and entropy, are directly related to the reference temperature and *Z*-value commonly used for kinetic analysis in food microbiology. An essential feature of the kinetic model is that its parameters, and hence the transition temperature, are treated as stochastic variables. The characteristic line shape of the primary model is the log-normal distribution. The performance of the model was validated, using literature data for enzyme and microbial inactivation over a wide range of temperature and pH.

1. Introduction

Modelling the inactivation kinetics of proteins, enzymes and micro-organisms is an important tool in biochemistry and food science to describe and predict the stability of ingredients, spoilage and the safety of foods. Several different approaches are adapted to model the observed inactivation kinetics. A common, classical approach is to assume first order kinetics (Bigelow, 1921; Van Boekel, 2008), though this is not always warranted. From the observed inactivation data at specific temperatures, a reaction rate constant *k* (or a *D*-value as its reciprocal, frequently used in microbiology) are first determined. The temperature dependence of chemical and biochemical reactions is usually established via the Arrhenius equation. A *D*-value describes the time, needed to reduce the density of a microbial population by a factor of 10 at a given reference temperature. In a secondary model, the temperature dependence of the *D*-value is analysed, expressed as a *Z*-value, which describes the temperature needed to reduce or increase the *D* value by a factor 10. The same goes for the inactivation of proteins/enzymes via *k*-values and activation energies and pre-exponential factors. This method of *D* and *Z*-values in food microbiology relies on a first order approximation of the observed inactivation kinetics and can resolve sub-populations if sufficient, accurate data are available. One of the implications of a first-order approximation is that the plot of the surviving population vs. time should be a straight line to confirm that a first-order approximation holds. Whether this is true in practice is a debatable issue; growing evidence suggests that log-linear survival curves are the exception rather than the rule, both for inactivation of enzymes and micro-organisms (Van Boekel, 2009).

A second approach to model inactivation kinetics is to use an empirical line shape as a mathematical model. Typical line shapes that are used for kinetic modelling are Weibull (Van Boekel, 2002), log-logistic (Anderson, McClure, Baird-Parker, & Cole, 1996) and modifications thereof (Bevilacqua, Speranza, Sinigaglia, & Corbo, 2015; Metselaar, den Besten, Abee, Moezelaar, & Zwietering, 2013). The choice of a suitable line shape depends on the actual shape of the observed inactivation. In case of suspected heterogeneity (Abee, Koomen, Metselaar, Zwietering, & den Besten, 2016), e.g., when biphasic inactivation curves are observed both for enzymes (e.g., Van Boekel, 2009) and microbes (e.g. Metselaar et al., 2013), the choice of a specific line shape or the number of assumed sub-populations may differ for a particular subset of the experimental data series, even though the same initial sample was used that was subjected to similar environmental conditions (Metselaar, Abee, Zwietering, & Den Besten, 2016). Nevertheless, parameter estimates in empirical models are extensively used for

* Corresponding author.
E-mail addresses: hennie.mastwijk@casema.nl (H.C. Mastwijk), rian.timmermans@wur.nl (R.A.H. Timmermans), tiny.vanboekel@wur.nl (M.A.J.S. Van Boekel).

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List of symbols

$\Delta^{\ddagger}G^{\theta}$	standard Gibbs energy, for activation J mol^{-1}
$\Delta^{\ddagger}H^{\theta}$	standard activation enthalpy for activation, J mol^{-1}
$\Delta^{\ddagger}S^{\theta}$	standard activation entropy, for activation J mol^{-1} K^{-1}
τ	reference time, min or s
σ	width (uncertainty in the transition temperature) of the survival function, °C
D-value	decimal reduction value, min or s
e	Eulers number, 2.71828
h	Planck's constant, 6.626 × 10^{-34} J s
I	inactivated stated of protein
k	rate constant
k_b	Boltzmann's constant, 1.38 × 10^{-23} J K^{-1}
K	the equilibrium constant of chemical reaction
m	the order of pH equilibrium
N	native, folded state of protein
n	initial fraction
U	unfolded, denaturated state of protein
R	gas constant, 8.314 J mol^{-1} K^{-1}
T	temperature, °C or K
T_o	temperature at standard normal conditions: 300 K, K
Tr	reference temperature, °C
t	time, min or s
Z-value	temperature increase needed to reduce the decimal value of D or Tr by a factor 10 (°C)

statistical analysis of the experimental data and to determine confidence levels for predictions (Bevilacqua et al., 2015). The advantage of an empirical line shape is that the primary model is readily available and parameters can be estimated using widespread available, non-linear fitting routines. A major drawback of this method is found in the interpretation of the empirical parameters and the relatively large confidence intervals of parameters and predictions that are usually obtained (Van Boekel, 2009).

A third approach is modelling by a suitable combination of mass balance-, chemical rate- and thermodynamic equations. Arrhenius' equation and the Eyring model have been used as a basis for secondary models for the thermodynamic modelling of temperature and pressure treated enzymes (Fachin, Van Loey, Indrawati, Ludikhuyze, & Hendrickx, 2006). By using this method, accurate estimates for inactivation have been obtained with predictions for the combined effect of pressure and temperature (Fachin et al., 2006).

In this communication we will provide an alternative approach by exploring an *ab initio* stochastic model for biochemical inactivation kinetics. The difficulty to overcome is to define a primary model with physically relevant parameters that accurately and consistently fits to kinetic data series collected over a wide range of environmental conditions. Our point of departure is the state-of-the-art Lumry-Eyring model for protein denaturation and inactivation (Lumry & Eyring, 1954). This model provides a rate constant for inactivation that is derived from first principles in Transition State Theory. The rate constant, formulated for a molecular complex in transition, was built on quantum mechanics and statistical physical theory (Eyring, 1935). It has been consistently shown (Ahern & Klibanov, 1985; Lumry & Eyring, 1954; Schokker & Van Boekel, 1997; Yoshioka, Aso, Izutsu, & Koijma, 1994) that the Lumry-Eyring model for denaturation and inactivation of proteins in solution satisfies the basic scheme:

$$N \underset{k_2}{\overset{k_1}{\rightleftarrows}} U \overset{k_3}{\rightarrow} I \qquad (1)$$

where the native, active (folded) state (N) is in equilibrium with the unfolded (denatured) state (U) via its activated state, characterized by the rate constant k_1 for unfolding, k_2 that for refolding and k_3 the one characterizing inactivation. An irreversible transition to the inactive state (I) proceeds through a transition state with its energy just above the energy of the unfolded state.

Typical standard activation enthalpies for protein unfolding are in the range of 500–600 kJ/mol while the typical standard activation entropies are also high, in the range of 500–1000 J/mol/K, thus compensating for the high activation enthalpies, as a result of which typical activation free energies are still moderate (because $\Delta^{\ddagger}G^{\theta} = \Delta^{\ddagger}H^{\theta} - T\Delta^{\ddagger}S^{\theta}$; an extensive discussion on these parameters is given in Van Boekel (2009)).

The equilibrium state that is established before entering the transition state is generally not known. Proteins may switch between a large number of different conformations (Creighton, 1988). The pathway of unfolding and inactivation may therefore proceed through a large number of different intermediate states. This is especially true for complex proteins found in food systems. In practice a large number of pathways have to be considered before kinetic model parameters of unfolding and inactivation can be determined (Schokker, 1997). In the present work we will not assume any specific reaction pathway beforehand, but estimate the standard activation enthalpy $\Delta^{\ddagger}H^{\ominus}$ and entropy $\Delta^{\ddagger}S^{\ominus}$ of the transition state from experimental data. The experimentally observed rate constant is in most cases a lumped parameter, being the resultant of k_1, k_2 and k_3 but always referring to the rate limiting step in the reaction pathway leading to inactivation. If unfolding is rate limiting, then high activation enthalpies and entropies will be found, if the subsequent inactivation step is the rate limiting (chemical) process, then the activation enthalpy will be more in the range of a chemical reaction (typically between 50–150 kJ/mol) and activation entropies (typically in the range of 0–30 J/mol/K).

Our target is the formulation of a global, thermodynamic model for the inactivation of both enzymes and micro-organisms exposed to heat and chemicals. From a microbiological point of view, it may be unconventional to consider an inactivation model for living cells that has been developed mainly for chemical reactions and isolated proteins. However, proteins contribute to a large extent to the total microbial biomass; typically 55% of the dry weight for an *E. coli* bacterium (Neidhardt & Umbarger, 1996). In this respect, the difference between an isolated protein system and a micro-organism is only found in the number of protein systems present. It has been estimated that the bacterium *E. coli* comprises of 4300 different proteins with standard Gibbs free energies (determining the stability of the native state compared to the unfolded state) in the range of 2–50 kJ/mol, which determines the functionality of the organism in a relevant temperature range of interest (Dill, Ghosh, & Schmit, 2011). Protein systems found in micro-organisms are complex and highly dynamical. Gene expression in response to environmental induced stress conditions are known to occur within seconds, and an average-size protein can be synthesized within 30 s whereas synthesis of larger proteins, as for example galactosidase, requires 2.5–3 min (Ryall, Eydallin, & Ferenci, 2012). On the other hand, it is recognized that lethal conditions of temperature for micro-organisms correspond to conditions where the majority of proteins have denatured in an irreversible state (Dill et al., 2011). Acknowledging that all individual proteins in the proteome, no matter how many or complex, are

subject to the same thermodynamic laws as isolated proteins, and given the crucial role of the proteome in the survival of the organisms, it seems therefore reasonable to explore the static Lumry-Eyring model as a model for microbial inactivation. This approach has been suggested previously (Kemeny & Rosenberg, 1973; Qin, Balasubramanian, Pearce & Bischofl, 2014; Rosenberg, Kemeny, Switzer, & Hamilton, 1971). The approach has, however, also been criticized by, for instance, Peleg, Normand, and Corradini (2012), the criticism being that the laws of Arrhenius and Eyring have never been developed for such complicated systems as living cells, only for simple reactions. Whilst assuming that protein/enzyme inactivation is a critical step in death of cells, we will acknowledge this criticism by referring to the activation parameters as apparent, since we do not know which enzymes/proteins are targeted.

This paper is organised as follows: we will first investigate the correspondence between protein inactivation and the classical approach to model microbial inactivation. Here we link molecular parameters of standard activation entropy and enthalpy in the Lumry-Eyring inactivation model of proteins to the concepts of reference temperature and Z-value for inactivation that are known in microbiology, and occasionally also used in enzyme inactivation (Bigelow, 1921). The second task in our work is devoted to incorporate the stochastic nature of molecular transitions in the model, as to define the new model. Third, the developed survival model, designated as Gauss-Eyring, will be presented and evaluated as a global model for inactivation data of enzymes and micro-organisms.

2. Materials and methods

The model parameters were estimated by the methods of Maximum Likelihood Estimation (MLE) and Sum of Least Squares (SSR) using 'R' language and environment for statistical computing (R, 2008). Models were coded as a single primary model to include all experimental variables and model parameters. The (global) optimisation and parameter estimates were performed using the method of Simulated Annealing using the GenSA package (Xiang, Gubian, Suomela, & Hoeng, 2013). Global optimisation over the entire, relevant domain of parameters was carried out using box constraints, to avoid guessing of starting values and rendering a sub-optimal model.

Confidence intervals, correlation coefficients and fit statistics were calculated by standard procedures using routines and libraries available in 'R'. Selection of redundant parameters and a reduction in the number of model parameters was carried out using the Bayesian Information Criterion (BIC) (Van Boekel, 2009). Optimized models with a reduction in the value of BIC of more than 3 are considered significantly better in a statistical sense. The models presented in this work were evaluated using published data. Inactivation data of proteinase was obtained by digitizing published graphs (Driessen 1983; Schokker & Van Boekel, 1997) using GraphClick 3.0.3 (Arizona Software 2012), and for *L. monocytogenes* the original raw data was used (Metselaar et al., 2013 and Metselaar, Abee, Zwietering, & Den Besten, 2016).

3. Deriving the Gauss-Eyring model

3.1. Temperature-time of inactivation: correspondence relations

The rate constant in Eyring's transition state equation (Van Boekel, 2009) provides a means to define equivalent temperature-time combinations to reach the same level of inactivation. For an unfolding protein system, characterised by its parameters standard activation enthalpy $\Delta^{\ddagger}H^{\ominus}$ and entropy $\Delta^{\ddagger}S^{\ominus}$, the temperature-time combination for inactivation with temperature T (°C or K) and time t, is given in first order approximation by (Supplementary data):

$$T(t) = \frac{\Delta^{\ddagger}H^{\theta}}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau}{h}\right]} - \frac{\Delta^{\ddagger}H^{\theta}}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau}{h}\right]} \times \frac{R}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau e}{h}\right]} \log\left[\frac{t}{\tau}\right] \quad (2)$$

where $R = 8.3$ J/K mol is the gas constant, $k_b = 1.4{\cdot}10^{-23}$ J/K Boltzmann's constant, $h = 6.63\ 10^{-34}$ Js Planck's constant, $\tau = 1$ min (or 1 s) defining the unit of time that is used, $T_o = 300$ K is the (arbitrarily chosen) temperature at standard normal conditions (300 K, 100 kPa and pH 7) and $e = 2.71828$.

Eq. (2) is identified as the definition of the reference temperature T_r and the Z-value known in microbiology (Bigelow, 1921). The Z-value is the required increase in temperature relative to the reference temperature needed to achieve the same level of inactivation when decreasing the exposure time, as:

$$T(t) = T_r - Z\log\left[\frac{t}{\tau}\right] \quad (3a)$$

with

$$T_r(\Delta^{\ddagger}H^{\theta}, \Delta^{\ddagger}S^{\theta}) = \frac{\Delta^{\ddagger}H^{\theta}}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau}{h}\right]} \quad (3b)$$

$$Z(\Delta^{\ddagger}H^{\theta}, \Delta^{\ddagger}S^{\theta}) = \frac{\Delta^{\ddagger}H^{\theta}}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau}{h}\right]} \frac{R}{\Delta^{\ddagger}S^{\theta} + R\log\left[\frac{k_b T_o \tau e}{h}\right]} \quad (3c)$$

Eq. (3) are the correspondence relations of the classical reference temperature and Z-value (Bigelow, 1921), expressed in the molecular parameters standard activation enthalpy and entropy in the Eyring transition state model (Eyring, 1935). The inverse relations give the standard activation enthalpy and entropy in terms of the classical reference temperature T_r and Z-value (keeping in mind that the values for enthalpy and entropy are apparent in the case that they do not refer to a specific protein):

$$\Delta^{\ddagger}H^{\theta}(T_r, Z) = \frac{RT_r^{\,2}}{Z} - RT_r \quad (4a)$$

$$\Delta^{\ddagger}S^{\theta}(T_r, Z) = \frac{RT_r}{Z} - R\log\left[\frac{k_b T_o \tau}{he}\right] \quad (4b)$$

The meaning that can be assigned to these correspondence relations is that the classical description of inactivation kinetics in microbiology can be made equivalent to the Lumry-Eyring model that deals with the thermodynamics of protein denaturation and inactivation (or any other chemical reaction for that matter).

3.2. Stochastical parameters; probability function for survival

Since at thermal equilibrium the specific, momentary folding state of a protein is subject to random changes, the activation parameters $\Delta^{\ddagger}H^{\theta}$ and $\Delta^{\ddagger}S^{\theta}$ should be treated as stochastic variables. This implicates that in an experiment the precise, momentary values of $\Delta^{\ddagger}H^{\theta}$ and $\Delta^{\ddagger}S^{\theta}$ are uncertain, and as a result, the temperature and time combinations (Eq. (2)) where inactivation takes place are no longer exactly defined. If we treat the standard enthalpy and entropy for activation as stochastic variables, there is no longer a need to specify its actual value better than within the limits of uncertainty. Since the temperature-time combination needed to reach a certain level of inactivation is a function of the parameters

($\Delta^{\ddagger}H^{\theta}, \Delta^{\ddagger}S^{\theta}$), the temperature has to be treated as a stochastic variable likewise.

In a typical experiment (Metselaar et al., 2016; Schokker & Van Boekel, 1997), the number of micro-organisms or proteins that are inactivated is very large ($\sim 10^9$ cfu/ml or $\sim 10^{16}$ proteins/ml). Under these circumstances, the survival function, that is defined by the outcome of repeated sampling of a very large number of random temperature-time combinations leading to inactivation, is well approximated by the normal distribution (Central Limit Theorem).

Since the temperature-time function has a $\log(t)$ dependence we can hypothesize the (cumulative) log-normal distribution (Weisstein, 2016a) as the bivariate probability function $S(T,t)$ for the survival of an ensemble of proteins or micro-organisms:

$$S(T,t) = \frac{1}{2}\mathrm{erfc}\left[\frac{T - T_r + Z\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma}\right] \quad (5)$$

where erfc(x) is the complementary, cumulative normal (Gaussian) distribution (Weisstein, 2016b) and σ the width (uncertainty in the transition temperature) of the survival function (°C).

3.3. Extension to include chemical treatment

The Eyring model is based on fundamental thermodynamics and the rate constant is defined by the standard free activation energy term $\Delta^{\ddagger}G^{\theta} = \Delta^{\ddagger}H^{\theta} - T\Delta^{\ddagger}S^{\theta}$ of the transition state under consideration. As such, the Gauss-Eyring model can be modified by including the thermodynamic free energy of the system.

For the general case of chemical treatment, the standard free energy term at equilibrium yields:

$$\Delta G^{\theta} = -RT\log(K) \quad (6)$$

where K is the equilibrium constant of the chemical reaction under consideration (Reiff, 1965).

The Eyring rate equation has been modified for chemical treatment by including the standard thermodynamic free energy for chemical equilibrium (Supplementary data). Remarkably, the extended temperature-time function defines a unique Z-value to account for changes both in temperature and pH. The temperature change to access the transition state by a change in pH, relative to standard normal conditions, is given by:

$$T(t,\Delta pH) = T_r - Z\log\left[\frac{t}{\tau}\right] - Z\Delta pH \quad (7)$$

where Z is the commonly known Z-value as defined for thermal treatment as in Eq. (2), and ΔpH is the change in pH. Note that the reference state for pH can be chosen arbitrarily, leading to a corresponding shift in the reference temperature.

The validity of this predicted pH dependence was investigated using experimental inactivation data of lysozyme at different pH provided by Ahern & Klibanov (1985). They studied the inactivation of lysozyme at pH 4, 6 and 8 at a fixed temperature of 100 °C and observed first order kinetic response. The standard first order Eyring model, with the addition of the free energy term for the shifted chemical equilibrium by pH, was fitted as a global model to the kinetic data series. It was confirmed (Supplementary data) that the extension given by Eq. (6) can be used to model lysozyme inactivation as a function of pH in the range of 4–8. From this we could conclude that the free energy term for chemical equilibrium, as defined by Eq. (6), is properly incorporated into the temperature-time function of the Gauss-Eyring model (Supplementary data), leading to a temperature shift as in Eq. (7).

4. Results and discussion

4.1. Inactivation model for extracellular protease

To validate the proposed Gauss-Eyring model as given in Eq. (5), the model was fitted to reported data sets by Driessen (1983), Schokker (1997), and Schokker and Van Boekel (1997). These authors studied the thermal inactivation of an extracellular protease from *Pseudomonas fluorescens* 22F, together covering the inactivation measured over a temperature range of 48–130 °C. Interestingly, inactivation of this protease at reduced temperatures, known as Low Temperature Inactivation (LTI) was discriminated from inactivation at the high temperature regime (90–130 °C) (Kroll & Klostermeyer, 1984; Schokker, 1997). By modelling reaction pathways, it was concluded that the mechanism for LTI (auto digestion) could be discriminated from chemical inactivation in the high temperature region (Schokker, 1997).

Schokker and Van Boekel (1997) have investigated inactivation of protease in the temperature range of 90–110 °C in detail and observed non-linear inactivation kinetics. Several refined models were investigated, all based on the basic Lumry-Eyring model (Eq. (1)), to deal with the observed curvature in the data. In the pre-equilibrium models of the unfolding protein, it was required to assume a minimum of two enzyme states in order to obtain a reasonable fit of the model to the data (Schokker & Van Boekel, 1997). The different rate equation models were all based on an equilibrium model for the partly unfolded protein and an irreversible transition to the inactive state, its rate quantitatively defined by Eyrings Transition State Theory.

Model parameters for the activation enthalpy and entropy, that have been determined for a series of models with increasing complexity, show relatively large confidence intervals (Schokker 1997). In the original work of Schokker and Van Boekel (1997) it was concluded that a univocal reaction pathway leading to inactivation could not be determined, but that the thermal inactivation of proteinase from *P. fluorescens* 22F is complex and comprises a sequence of at least two reactions (Schokker & Van Boekel, 1997).

Reactions that trigger irreversible transitions in proteins are highly diverse and include hydrolysis of peptide bonds, reconfiguring of disulfide bonds, destruction of amino acid residues, aggregation and conformational changes of the tertiary structures (Ahern & Klibanov, 1985). Given the large number of possible molecular reactions, modelling the pre-thermal equilibrium of the partial unfolded protein by chemical rate equations, to establish the pathway for inactivation, seems to be a challenging task, especially for complex proteins found in foods. To deal with this nonetheless, we suggest to rely on the stochastic argument that has been made (preceding Eq. (5)) to account for the uncertainty of the partially unfolded state at equilibrium.

The proposed Gauss-Eyring model for thermal inactivation of extracellular protease is:

$$S(T,t) = \begin{cases} \frac{n_1}{2}\mathrm{erfc}\left[\frac{T - T_1 + Z_1\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma_1}\right] + \frac{(1-n_1)}{2}\mathrm{erfc}\left[\frac{T - T_2 + Z_2\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma_2}\right] & 48 < T < 52\,^{\circ}\mathrm{C}\ (\mathrm{LTI}) \\ \frac{1}{2}\mathrm{erfc}\left[\frac{T - T_3 + Z_3\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma_3}\right] & 70 < T < 80\,^{\circ}\mathrm{C} \\ \frac{1}{2}\mathrm{erfc}\left[\frac{T - T_4 + Z_4\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma_4}\right] & 90 < T < 110\,^{\circ}\mathrm{C}(\mathrm{HTI}) \\ \frac{1}{2}\mathrm{erfc}\left[\frac{T - T_5 + Z_5\log\left[\frac{t}{\tau}\right]}{\sqrt{2}\sigma_5}\right] & 120 < T < 130\,^{\circ}\mathrm{C}(\mathrm{UHT}) \end{cases} \quad (8)$$

where the model parameters, the standard activation enthalpy $\Delta^{\ddagger}H^{\theta}$ and entropy $\Delta^{\ddagger}S^{\theta}$, are given in terms of T_r and Z by Eq. (3), σ is the width of the temperature distribution (°C) and where n_1 and $n_2 = 1 - n_1$ are the initial fractions of two iso-enzymes that can be distinguished in the LTI region. The independent variables are tem-

perature (T) and time (t), while the response is the measured (total) activity of the sample under investigation.

The model in Eq. (8) is defined for different temperature domains. It was found that a two iso-enzyme model (7 parameters) is required to fit the data in the LTI region, while the other regimes are best modelled by a single enzyme (3 parameters). This scheme was found after model optimisation of the overall BIC statistic. Optimisation includes the determination of the different temperature domains and number of iso-enzymes. Thus, on the basis of objective selection criteria, it was found that Eq. (8) is the best performing model to describe the combined set of data by Driessen (1983), Schokker (1997), and Schokker and Van Boekel (1997) for inactivation of protease in the temperature range of 48–130 °C.

The results of the fit to the data are shown in Fig. 1. Parameter estimates and 95% confidence intervals are given in Table 1. Despite that the optimised Gauss-Eyring model is a single isoenzyme model, the curvature in the observed data is reproduced for the high temperature region. Other models that have been tried were first order Eyring with 1 and 2 subpopulations (Yoshioka et. al., 1984). However, alternative models either required a larger number of isoenzymes, or just have poorer performance in BIC statistics (data not shown).

In essence, the model in Eq. (8) is a basic Gauss-Eyring model describing the selection of a single, heat stable isoenzyme in the low temperature region, which unfolds in the LTI regime. The rapid decline of activation enthalpy and entropy as a function of temperature (Table 1) suggest the unfolding of an enzyme at lower temperature (unfolding is here the rate determining process), followed by 'normal' chemical inactivation at higher temperatures (chemical inactivation is the rate determining process), which explains the drastic decrease in value of the standard activation enthalpies and entropies in going from the LTI region to the UHT region. The standard activation entropy and enthalpy of proteases form *Pseudomonas* spp. in the high temperature regions, shown in Table 1, are in good agreement with results typically found for heat resistant proteases (Stoeckel et al., 2016).

The Gauss-Eyring model incorporates two types of heterogeneity. Firstly, heterogeneity that is defined by the presence of multiple iso-enzymes which requires different sets of parameters for activation enthalpy and entropy for each iso-enzyme. Secondly, heterogeneity is defined for a single enzyme system, expressed by the width of the Gaussian temperature distribution for survival. This kind of heterogeneity is assumed to relate to the specific folding state of a single protein. In case that the protein engages in a very specific reaction pathway, the activation enthalpy and entropy are well defined and hence, the temperature width is relatively small. If, on the other hand, the enzyme can access a large number of conformations, the activation enthalpy and entropy are uncertain and a broader temperature distribution can be expected.

In the LTI region, the Gauss-Eyring model was able to discriminate for two iso-enzymes (Table 1) on the basis of differences in the standard activation entropy for inactivation i.e., two rate determining processes could be resolved. Global modelling and direct parameter estimation resulted in an activation entropy and enthalpy that are apparently highly precise (Table 1). The direct inactivation pathway in the LTI region, that proceeds directly from the native to the inactive state (Schokker, 1997) can be identified with the presence of a temperature sensitive isoenzyme in the LTI region (Table 1). Schokker (1997) has reported a standard activation entropy and enthalpy for inactivation of $\Delta^{\ddagger}H^{\theta}$ = 504 ± 51 kJ/mol and $\Delta^{\ddagger}S^{\theta}$ = 1232 ± 165 J/K mol. This result was obtained by kinetic modelling of the inactivation by rate equations and subsequent thermodynamic analysis (Arrhenius plot). The activation enthalpy and entropy estimates for the heat labile enzyme, according to the Gauss-Eyring model, yield respectively, $\Delta^{\ddagger}H^{\theta}$ = 604.6 kJ/mol and $\Delta^{\ddagger}S^{\theta}$ = 1597.9 J/K mol (Table 1). These numbers are in reasonable agreement with the parameter estimates for the rate determining, direct inactivation pathway made by Schokker (1997).

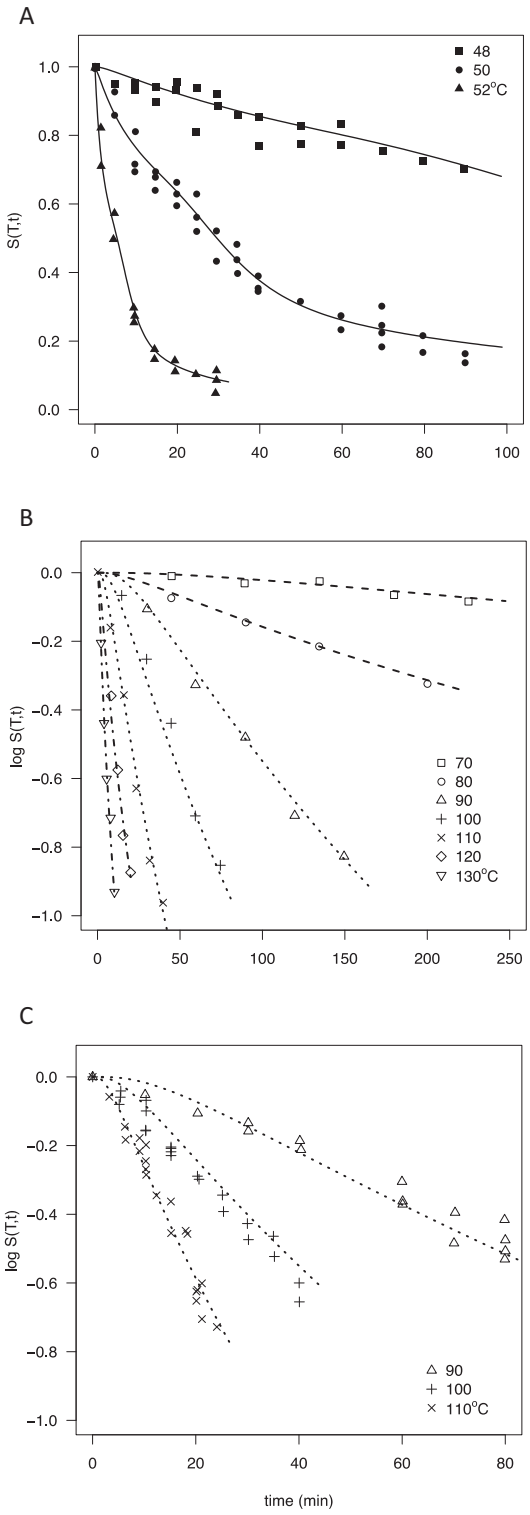

Fig. 1. Global modelling of the inactivation of extracellular proteinase from *P. fluorescence* 22F in the range of 48–130 °C. A) Inactivation data from Schokker (1997) at temperatures 48–52 °C and the model fit (—) in the LTI regime for a 2-isoenzyme system. B) Inactivation data from Driessen (1983) and model fit for intermediate temperatures 70–80 °C (– –), the high temperature regime HTI 90–110 °C (--) and at ultra-high temperatures (UHT) 120–130 °C (– –) for a single isoenzyme. C) Data from Schokker (1997) and model fit in the high temperature regime HTI 90–110 °C (--).

Table 1
Model parameter estimates for inactivation of extracellular proteinase of *P. fluorescence* 22 F for temperatures in the range of 48–130 °C according to the Gauss-Eyring model. The 95% confidence intervals of the parameter estimates are given in square brackets.

Model parameter	LTI region 48–52 °C (Schokker, 1997) i = 1	Intermediate 70–80 °C (Driessen, 1983) i = 3	HTI region90-110 °C (Driessen, 1983; Schokker, 1997) i = 4		UHT region 120–130 °C (Driessen, 1983) i = 5
			(Driessen, 1983)	(Schokker, 1997)	
n_i (%)	79 [76–83]	100 [–]	100 [–]	100 [–]	100 [–]
$\Delta^{\ddagger}H_i^{\theta}$ (kJ/mol)	916.5 [916.4–916.6]	185.5 [185.3–185.6]	112.4 [112.2–112.6]	111.2 [111.1–111.3]	111.4 [111.3–111.6]
$\Delta^{\ddagger}S_i^{\theta}$ (J/K mol)	2565 [2564–2566]	232 [231–233]	24.4 [24.1–24.8]	23.3 [23.0–23.6]	22.6 [22.2–23.0]
σ_i (°C)	1.24 [1.16–1.31]	8.7 [8.3–9.2]	11.2 [12.6–13.5]	12.0 [11.3–12.7]	14.3 [13.8–14.9]
z_i (Eq. (3b)) (°C)	2.16	15.8	30	31	31
n_2 (%)	21 [17–24]	Not detected/not resolved			
$\Delta^{\ddagger}H_2^{\theta}$ (kJ/mol)	604.6 [604.4–605.0]				
$\Delta^{\ddagger}S_2^{\theta}$ (J/K mol)	1598 [1597–1599]				
σ_2 (°C)	0.34 [0.21–0.50]				
z_1 (Eq. (3b)) (°C)	3.31				

From the unfolding and folding reaction constants, determined by chemical rate modelling, the equilibrium constant for the N ⇄ U(unfolding) reaction could be quantified as a function of temperature (Schokker, 1997). The estimated enthalpy and entropy for the unfolding reaction is $\Delta^{\ddagger}H^{\theta}$ = 1128 kJ/mol and $\Delta^{\ddagger}S^{\theta}$ = 3481 J/K mol and denaturation temperature T_d = 324 K (Schokker, 1997). Note that these are not activation parameters but refer to the differences in thermodynamical state of the native and unfolded state. These results come with the remark that the procedure of secondary modelling (van 't Hoff plot) is prone to large errors. Notwithstanding, these thermodynamic parameter estimates for (rate determining) unfolding are consistent to the parameter estimates for the activation energy of the heat stable isoenzyme complex in the LTI region (Table 1) yielding $\Delta^{\ddagger}H^{\theta}$ = 916.5 kJ/mol and $\Delta^{\ddagger}S^{\theta}$ = 2565 J/Kmol and a reference temperature defined by Eq. (3b) of 326 K.

A relatively large temperature width (σ = 1.24 °C) was found (Table 1) for the survival of the heat stable iso-enzyme when compared to the heat labile directly inactivated iso-enzyme (σ = 0.34 °C) in the LTI region. This is consistent with the view that a large number of changes in the conformational state of the protein is required to reach an unfolded pre-equilibrium transition state prior to inactivation.

To summarise: the Gauss-Eyring model provides a consistent estimation for standard activation enthalpy and entropy for thermal inactivation of extracellular protease from *P. fluorescence* 22F over the temperature range of 48–130 °C. Upon quantitative comparison of the standard activation parameters for the molecular inactivation pathways known to date, the sub-populations in the Gauss-Eyring model that are coined as 'iso-enzymes', are likely to represent two alternative pathways that lead to inactivation.

4.2. Inactivation of Listeria monocytogenes by heat and pH

As a second test, the Gauss-Eyring model was tested as the primary model for inactivation of a microbial population as a function of temperature, time and pH by combining Eq. (5) and Eq. (7).

Highly accurate kinetic inactivation data for *Listeria monocytogenes* at different temperatures and pH, were provided by Metselaar et al. (2013), Metselaar et al. (2016). Metselaar et al. (2013) have studied the inactivation of *L. monocytogenes* LO28 wild type (WT) and an acid resistant variant 14, at two different growth stages (late exponential and stationary phase) towards combinations of heat and acidification. They have modelled the observed (non-linear) inactivation kinetics piecewise using a biphasic model with a shoulder and a reduced model (Metselaar et al., 2016). Following this approach, the observed non-linear inactivation kinetics could be associated to heterogeneities in the initial population.

The Gauss-Eyring model was fitted as a global model to the inactivation data of Metselaar et al. (2013), Metselaar et al. (2016) using temperature, pH and time as the independent variables and assuming two Gaussian subpopulations (8 parameters) according to:

$$S(T,t,\Delta\mathrm{pH}) = \frac{n_1}{2}\operatorname{erfc}\left[\frac{T - T_1 + Z_1\left[\log\left[\frac{t}{\tau}\right] - m\Delta pH\right]}{\sqrt{2}\sigma_1}\right] + \frac{1-n_1}{2}\operatorname{erfc}\left[\frac{T - T_2 + Z_2\left[\log\left[\frac{t}{\tau}\right] - m\Delta pH\right]}{\sqrt{2}\sigma_2}\right] \quad (9)$$

where m is the order of pH equilibrium (Supplementary data).

The result of the global fit of the Gauss-Eyring model and the parameter estimates are shown in Fig. 2 and Table 2, respectively. The reference level for pH was chosen at pH = 0. When choosing an intermediate reference level e.g. pH = 4.0 we observed numerical instabilities as the fitting algorithm needs to cross a zero point during the optimization routine. This could be avoided by choosing the reference state for pH either higher than 7.3 or less than 2.5. Since the region of interest is the survival in the low pH range, pH = 0 was chosen as the reference level in this work.

It can be observed in Fig. 2, that the Gauss-Eyring model fits both to the inactivation data of *L. monocytogenes* LO28-WT as well to the data for the acid-resistant variant 14, either harvested in the late exponential or stationary growth phase. Remarkably, the model according to Eq. (9), which is defined by a single line shape, fits to the data for all combinations of pH and temperature under consideration.

The striking difference of the empirical model of Metselaar et al. (2016) in comparison to the Gauss-Eyring model is that the empirical model accounts for a majority (99.99%) and a minority (0.01%) population. Instead, the Gauss-Eyring model consistently fits to an optimal global solution for two sub-populations that have nearly equal concentrations (Table 2). Comparison of the biphasic model with shoulder (Metselaar et al., 2013) (15 parameters,

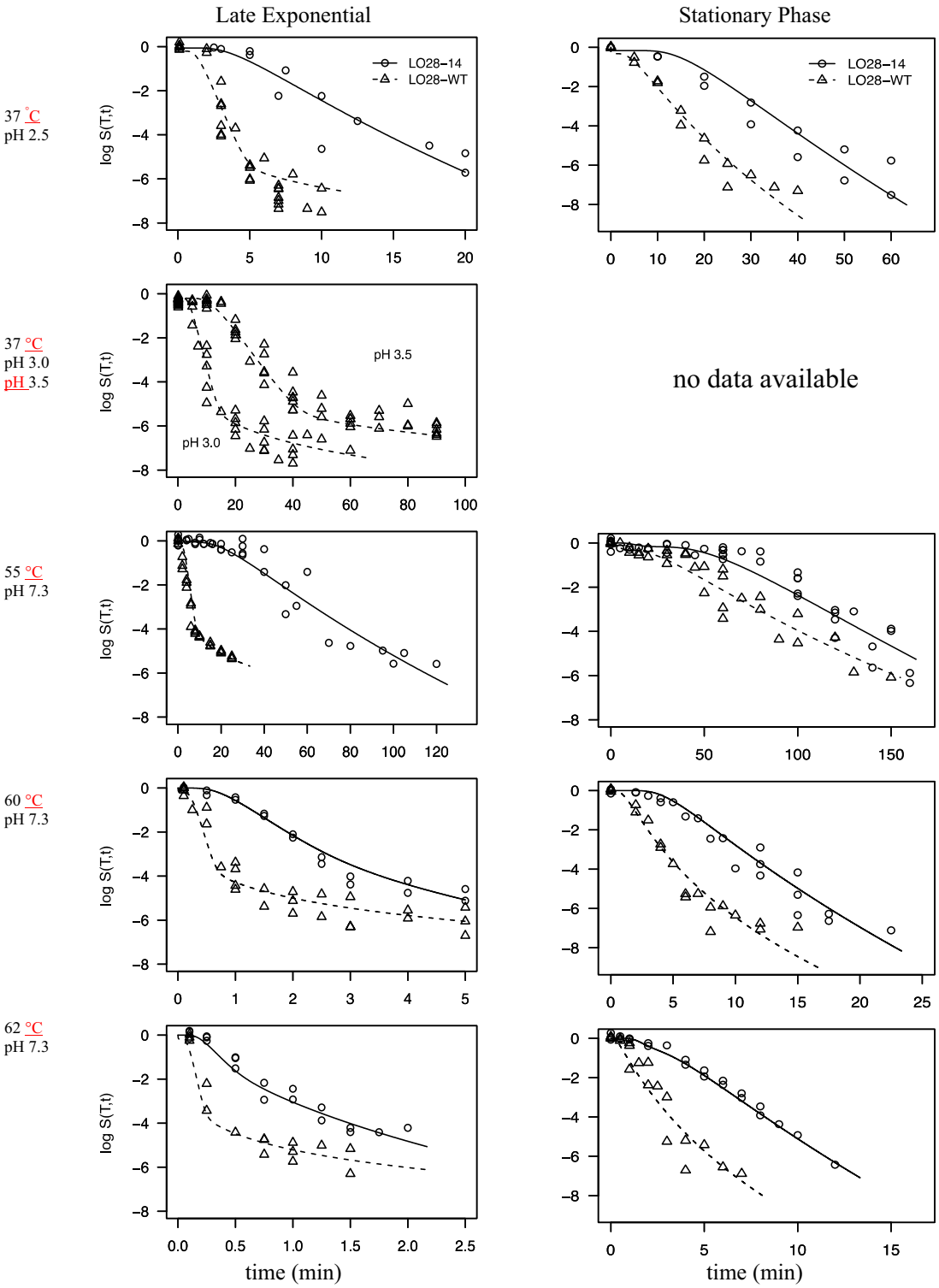

Fig. 2. Global fit of the Gauss-Eyring model to the inactivation data of *L. monocytogenes* (Metselaar et al., 2013, 2016) for different temperature, pH, exposure time and growth phase. The estimates of the model parameters and the 95% confidence intervals are shown in Table 1.

BIC = 247) and the Gauss-Eyring model (8 parameters, BIC = 201) for a series of three different pH's at a fixed temperature of 37 °C, showed that the Gauss-Erying model performs considerably better (ΔBIC = −46).

An even more relevant difference is found in the number of model parameters; the Gauss-Eyring model has 8 parameters, whereas the piecewise biphasic model requires 30 parameters. Another important observation is that the (relative) confidence intervals for parameter estimates for the Gauss-Eyring model (~5%) are significantly smaller than for the biphasic model (~25%). A smaller number of fit parameters are always to be preferred (Occam's razor principle) and smaller confidence intervals indicate that critical model parameters are more precisely determined. Based on statistical arguments, it is therefore concluded

Table 2
Parameter estimates of the Gauss-Eyring model for *L. monocytogenes* when exposed to heat and pH. The estimates are for a global fit to the inactivation data (Metselaar et al., 2013, 2016) for LO28-WT and the variant 14 in the late exponential (LE) and stationary phase (SP). In each isolate two sub-populations were resolved that differ in reference temperature T_r and Z-value. The fit parameters are given for pH 0 as the (arbitrary chosen) reference level.

Model parameter	LE-LO28-14	SP-LO28-14	LE-LO28-WT	SP-LO28-WT
T_1 (°C)	33.9 [33.8–34.0]	38.7 [38.6–38.8]	35.0 [34.9–35.1]	34.5 [34.4–34.7]
T_2 (°C)	12.8 [12.5–13.0]	7.3 [7.1–7.5]	20.9 [20.7–21.1]	0.2 [0.1–0.3]
Z_1 (°C)	3.53 [3.52–3.54]	4.45 [4.43–4.47]	4.6 [4.5–4.7]	3.74 [3.72–3.77]
Z_2 (°C)	6.21 [6.17–6.25]	11.1 [11.0–11.2]	5.2 [5.1–5.3]	8.71 [8.67–8.76]
σ_1 (°C)	0.3 [0.52–0.56]	0.37 [0.34–0.38]	0.42 [0.40–0.43]	0.44 [0.40–0.47]
σ_2 (°C)	1.14 [1.07–1.20]	1.04 [1.01–1.07]	2.75 [2.72–2.78]	1.29 [1.24–1.34]
n_1 (%)	87 [82–94]	66 [55–79]	62 [53–71]	48 [29–69]
n_2 (%)	13 [7–21]	34 [23–47]	38 [29–47]	52 [33–73]
m	1.30 [1.29–1.31]	0.858 [0.855–0.861]	0.939 [0.936–0.942]	1.237 [1.234–1.241]

that the performance of the global Gauss-Eyring model is better than for the biphasic model on this data set.

However, a decisive preference for one model over an alternative is found in the ability to incorporate basic mechanisms that explain the observed dynamics. The key question that is brought to attention by the results of Metselaar et al. (2013), Metselaar et al. (2016) is whether or not the observed non-linear inactivation kinetics originates from a possible selection of a sub-population that is initially present, or is the result of adaptation of the population during treatment as a whole (Abee et al., 2016). In the case of selection, the observed inactivation can be decomposed into two, static sub-populations of micro-organisms, whereas in the case of adaptation, the model should include dynamical effects (Corradini & Peleg, 2009), invoked by the treatment that adjusts the properties of a single population during the time of exposure.

The empirical biphasic model with a shoulder was assumed to contain two sub-populations, each including an explicit time shift (Metselaar et al., 2013). Therefore, both static and dynamical aspects are accounted for. The Gauss-Eyring model does not require an explicit time delay to mimic a shoulder. Remarkably, the Gauss-Eyring model is able to deal with both the observed shoulder and tail (Fig. 2), without introduction of an additional model parameter or other modification. To understand this phenomenon, the intrinsic time delay in the Gauss-Eyring model was studied in more detail.

In Fig. 3, the dynamics of inactivation in the Gauss-Eyring model are exemplified by decomposition of the two underlying Gaussian sub-populations. Decomposition was carried out for the inactivation of late exponential cells of LO28-WT treated at 37 °C, pH 3.5. In Fig. 3a, the Gaussian distributions for the two subpopulations are shown, using the fit parameters given in Table 2. Subpopulation I is characterised by T_r = 19.7 °C, Z_1 = 5.5 °C, σ = 1.8 °C and subpopulation II by T_r = 31.1 °C, Z_2 = 3.9 °C, σ = 0.8 °C. The different values of T_r and σ for the subpopulations I and II, clearly show their contribution into the Gaussian distribution in Fig. 3a, with T_r as the mean of the 'bell-shaped' curve and σ as the width of the distribution. The Gaussian distribution is directly related to the survival function (cumulative distribution) given by Eq. (5), and represents the number of organisms initially present in a (small) temperature interval $[T,T + dT]$.

Fig. 3a shows that when the time of exposure of *L. monocytogenes* LO-28WT to pH 3.5 at 37 °C is increased, the degree of inactivation, represented by a blackened area, is increasing. Whereas inactivation for sub-population I starts within the first seconds after exposure, inactivation of subpopulation II is delayed for 17 minutes. In Fig. 3b, the corresponding cumulative distributions (survival) are shown, plotted on a logarithmic scale. This graph confirms that the inactivation is initiated in sub-population I immediately at $t = 0$. The change in curvature of the survival function of organisms in sub-population I shows that the initial inactivation rate is high, but declines in time. Also shown is the contribution of sub-population II to the total inactivation. The inactivation of sub-population II is delayed in time, known as a 'shoulder'. However, once the inactivation in sub-population II has started, the rate of inactivation remains constant, leading to a nearly straight line.

The dynamics of inactivation in the Gauss-Eyring model is determined by its three parameters, T_r, Z-value and σ. The onset for inactivation occurs for the subpopulation with the lowest reference temperature. A shoulder is the result of a time delay in inactivation that is defined by the term $T_r - Z\log(t)$ in the survival curve (Eq. (5)). A large reference temperature, or a small Z value, leads to a broader shoulder. The tailing of the inactivation curve is determined by the ratio of Z/σ where σ is the temperature width of the Gaussian distribution. A large temperature width, or small Z-value leads to a longer tail. The behaviour at intermediate times (between the onset and tailing), and hence the shape of the survival curve, depends on the actual values of the three model parameters. In the case that multiple sub-populations are present, the observed kinetics for the total inactivation is the sum of multiple survival curves (superposition principle), which may appear quite complex (Figs. 1a, 2 and 3).

As in the case of heat inactivation of protease, which was discussed in the previous section, the Gauss-Eyring model entails two types of heterogeneity. For the inactivation of *L. monocytogenes,* heterogeneity has been attributed to the presence of distinct sub-populations that have different reference temperatures and Z-values. Using the corresponding relations of the model parameters according to Eq. (3), a difference in reference temperature may be associated to differences in the denaturation temperature of certain proteins, that are included in the proteome and are critical for survival. The survival of micro-organisms with a distinct, static feature resembles the principle of selection.

A second kind of heterogeneity originates from the uncertainty in the transition temperature that is contained within the Gaussian distribution representing the subpopulation. As

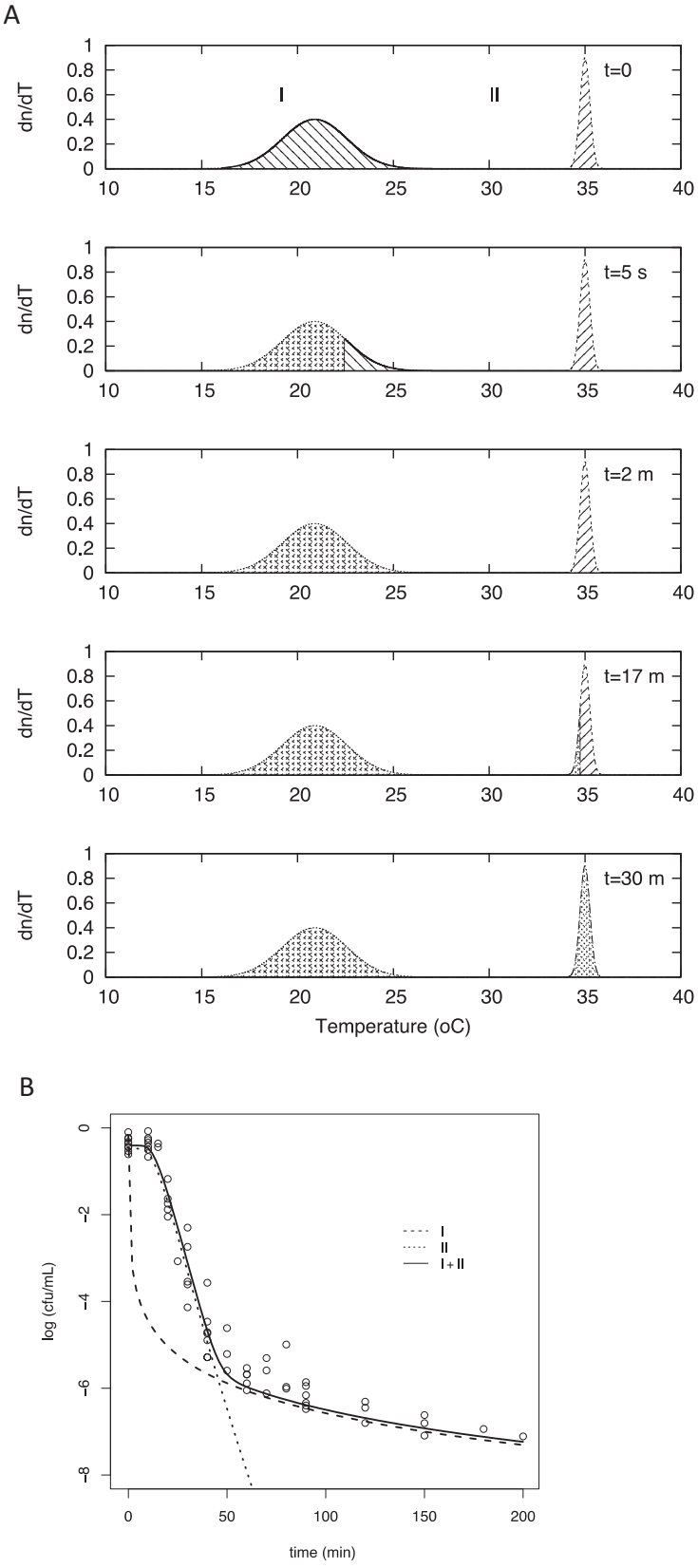

Fig. 3. Inactivation dynamics by acid treatment of two sub-populations resolved in Listeria LO28WT by the Gauss-Eyring model. Upper pane: The initial culture consists of two Gaussian distributed subpopulations with distinct T and *Z*-values. Subpopulation I has a reference temperature of 20.9 °C and *Z*-value of 5.2 °C. The acid resistant subpopulation II has a reference temperature of 35.0 °C and *Z*-value of 4.6 °C. Inactivation (indicted as blackened area) in subpopulation I is initiated immediately after exposure to pH = 3.5 at 37 °C. Lower pane: Inactivation of cells in subpopulation II is delayed for 17 min but proceeds thereafter at a higher rate than for organisms in subpopulation I. The survivors that are found in the tail originating from subpopulation I, are getting less sensitive during exposure and therefore can survive for treatment times longer than 50 min.

discussed throughout this work, the width of this associated Gaussian distribution reflects the uncertainty of the reference temperature and Z value for an ensemble of micro-organisms. For proteins it was hypothesised that an uncertainty in the standard activation entropy and enthalpy for the activated complex leaves more choice to proceed along alternative reaction pathways. So, this kind of heterogeneity suggests that a system is subject to dynamical changes which resembles the principle of adaptation.

Clearly, the mathematical and basic thermodynamic framework of the Gauss-Eyring model has potential to falsify and validate predictions made for thermal and chemical inactivation of protease and *L. monocytogenes*. This is impossible, or at least very difficult, in the case that phenomenological models are considered. Although the Gauss-Eyring model was successfully demonstrated to include complex inactivation kinetics for both enzymes and micro-organisms, it should be noted that only a limited amount of data available in literature has been considered. Therefore, the Gauss-Eyring model should be, for the moment at least, hailed as nothing more than a concept to deal with fundamental uncertainties in biochemistry and microbiology; further evaluation of the performance of the model is definitely needed.

5. Conclusions

A new primary model has been formulated for complex inactivation kinetics of proteins and micro-organisms. It has been shown that a one-to-one correspondence exists between the standard activation enthalpy and entropy in the Lumry-Eyring model for protein inactivation and the reference temperature and Z-value defined in the kinetic heat inactivation model used in food chemistry and classical microbiology. The temperature-time combination for inactivation is identified as a stochastic variable that reflects the uncertainty in the momentary folding state of proteins in thermal equilibrium. The primary line shape for inactivation is given by the cumulative probability distribution of the temperature-time function of inactivation.

As the model is the result of merging the Eyring rate constant for the transition state with the Gaussian distribution, the model is denominated Gauss-Eyring. The thermodynamics covered by the Gauss-Eyring model are primarily based on heat treatment, but can be extended to any treatment by adding the proper free energy term and recalculation of the temperature-time combination for inactivation.

The validity of the Gauss-Eyring model has been evaluated on the basis of a global fit to experimental data. Performance of the model was tested for inactivation of both enzymes and micro-organisms. Complex inactivation kinetics have been accurately modelled as a single primary model over a broad range of temperatures and pH. In both cases it was demonstrated that the Gauss-Eyring model has improved statistical performance over state-of-the art kinetic models for inactivation and in both cases more accurate estimates for model parameters were obtained.

Conflict of interest

The authors report no conflict of interests.

All authors participated in the research, manuscript assembly, revision and discussion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.05.070.

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