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Temperature/growth relationships for psychrotrophic food-spoilage bacteria

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The growth of psychrotrophic food-spoilage bacteria in pure cultures and in binary mixtures at chill temperatures (2–15°C) was monitored using colony counts. Sigmoidal curves of best fit were calculated for the data with a computer program. Growth/temperature relationships were studied using two mathematical equations: the Arrhenius relationship ($k = Ae^{-\mu/RT}$) and the Square Root relationship ($\sqrt{r} = b(T - T_o)$). The Square Root equation was found to be the better description of the microbial growth/temperature relationship at chill temperatures and was also applicable to mixtures of organisms. Implications of these results in relation to food analysis are discussed.

Introduction

A knowledge of the relationship between temperature and growth rate is important in the prediction of the likely levels of organisms after a known time at a specific temperature. In most cases, organisms have been studied which are capable of growing at chill temperatures and thus of causing spoilage of cold-stored, perishable foods (Splittstoesser 1976, Kraft and Rey 1979). There has been considerable dispute over the nomenclature of this group of organisms. Throughout this paper the term 'psychrotroph' will be used (Eddy 1960, Morita 1975) rather than the term 'psychrophile', which implies a low optimum temperature for growth.

Two equations have been used to relate growth rate of organisms to temperature – the Arrhenius equation (Ingraham 1958) and the Square Root equation (Ratkowsky et al. 1982). The linearity of the Arrhenius plot is a matter of dispute as several authors have obtained varying results. Straight lines were obtained by Ingraham (1958) and Janota-Bassalik (1963) but some authors obtained curved plots (Scott 1937, Ward and Cockson 1972), whilst others obtained plots with two linear portions joining at a critical temperature (Shaw 1967, Mohr and Krawiec 1980).

There is also considerable dispute about the significance of μ, the temperature characteristic of an organism derived from the slope of the Arrhenius plot. Ingraham (1958) claimed that μ values were lower for psychrotrophs than for mesophiles, and could therefore be related to optimum temperature. This was borne out by Baig and Hopton (1969) and Mohr and Krawiec (1980), although no such relationship was found by other authors (Shaw 1967, Hanus and Morita 1968, Baker 1974, Reichardt and Morita 1982). However, Hanus and Morita (1968) and Ward and Cockson (1972) point out that it is difficult to compare the work of different authors to resolve

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Table 1. Psychrotrophic food-spoilage isolates used during the study.

Organism	Supplied by	Collection number	Isolated from
Citrobacter freundii	Meat Research Institute	VR73	Hot deboned beef
Alcaligenes viscosus	National Collection of Type Cultures	3233	Ropy milk
Alteromonas putrefaciens[a]	Food Research Institute	EBF 44/148	Chicken
Serratia marcescens	American Type Culture Collection	4180	Milk
Pseudomonas sp. (non-pigmented)	Food Research Institute	EBT 2/167	Turkey
Pseudomonas sp. (pigmented)	Food Research Institute	MJT/F4/14(2)	Chicken
Moraxella sp.	National Collection of Industrial Bacteria	10763	Poultry
Acinetobacter sp.	Food Research Institute	MJT/F5/122	Chicken
Brochothrix thermosphacta	Ulster Curers' Association	M51	Spoiled fresh meat

[a] Deposited as *Pseudomonas putrefaciens* in the NCIB collection (NCIB 10761).

this as the organisms were grown under different conditions.

Ratkowsky et al. (1982) state that Arrhenius plots fit a curve rather than a straight line. They reported that the relationship between temperature and growth was better represented by the Square Root equation, which gave straight lines with high correlations for all the organisms tested. The authors also reported that the theoretical lowest temperature for growth, T_0 (derived from the Square Root equation), was related to the temperature range for growth of an organism. No other authors have confirmed these results for microbial growth.

The Arrhenius equation relates only the maximum growth rate of an organism with temperature. Since the Square Root plot relates the total time to achieve a given increase in colony count with temperature, the lag phase is taken into account. The lag phase, as well as eventual growth rate, is an important parameter in the shelf-life of foods.

The objective of the present study was to determine which (if either) of these relationships most appropriately describes the growth of psychrotrophic food-spoilage isolates at chill temperatures in culture media, both singly and in mixtures.

Materials and Methods

Organisms and growth conditions

The psychrotrophic food isolates used in this study are listed in Table 1. Organisms were maintained on agar slopes (All-purpose Tween (APT) broth (Difco) + 1·5% m V^{-1} agar (Oxoid)) at 5°C and subcultured approximately every 3 months.

The appropriate organisms were grown at 15°C for 48h in APT broth. One or two species of organisms were added to 1 l sterile APT broth to give a final concentration in the mother culture of approximately 5×10^3 cfu ml^{-1} for each organism. The mother culture was shaken thoroughly to ensure that the organisms were well dispersed and then dispensed aseptically in 20 ml quantities into sterile 100 ml sample pots (Medfor Products, Hampshire, UK).

Eight sample pots, with the lids tightly screwed on, were incubated at each of the following temperatures: 2°, 4°, 6°, 8°, 12° and 15°C.

Sampling and enumeration procedure

The original inoculum was enumerated from the mother culture. One sample pot was used on each subsequent sampling occasion. Samples were enumerated at different stages of the growth cycle. Each culture was thoroughly mixed to ensure dispersion and diluted as necessary in sterile quarter-strength Ringer solution (Oxoid) +0·1% mV^{-1} Bacteriological Peptone (Difco). Two dilutions were inoculated in duplicate on prepared and dried APT agar plates using the Spiral Plate Method (Jarvis et al. 1977). Plates were incubated at 15°C until colonies were visible (2 to 7 days, depending on the organism and temperature of incubation in broth). During studies on mixtures of organisms, both total colony count and the colony count for each component of the mixed flora were calculated.

Data analysis

A line of best fit was calculated for colony count data at each temperature using a computer program incorporating the Nelder-Mead Simplex Minimization Procedure. The program fits a sigmoidal curve to the data according to the equation:

$$y = \frac{A}{\left[1 + e^{-\frac{(\lambda + \kappa[x + 1])}{\theta}}\right]^{\theta}}$$

A, λ, κ and θ are parameters of the curve which must be estimated before the curve is fitted. More accurate numerical values for these parameters are then calculated when the sigmoidal curve is fitted to the data points. The program also calculates other information about the curve, i.e. maximum growth rate (k_{max}), time to k_{max} and the time to 1, 2, 3 and 4 $\log_{10}$ cycle increases in colony count.

Data were then analysed further using two procedures:

(1) Arrhenius plot (e.g. Ingraham 1958, Mohr and Krawiec 1980)

$$k = Ae^{-\mu/RT}$$

where k = specific growth rate (i.e. k_{max} × 2·303); A = constant; μ = temperature characteristic; R = Universal Gas Constant; T = absolute temperature.

$\log_e k$ is plotted against 1/T; μ can then be obtained from the gradient of the graph.

$$\text{gradient} = -\mu/R$$

Values of μ are obtained in units of cal mol^{-1}, but the meaning of the molar dimension in terms of bacterial growth is obscure (Reichardt and Morita 1982). Units have therefore not been given for μ in this paper.

(2) Square Root equation (Ratkowsky et al. 1982)

$$\sqrt{r} = b(T - T_o)$$

where b = slope of the regression line; T = absolute temperature; T_o = hypothetical lowest temperature of growth (i.e. if no changes in a_w occurred due to ice formation); r = reciprocal of a time, t, taken to achieve a specified increase in growth (e.g. time for a 1, 2, 3 or 4 $\log_{10}$ cycle increase or time to k_{max}).

A line of best fit was calculated for these relationships using linear least-squares regression. Values for T_o and μ were calculated, as were the correlation coefficients between growth rate and temperature parameters.

Results

The computer program fitted satisfactory curves for all organisms tested, both singly and in binary mixtures. High correlations between the fitted curves and the data points were obtained. Examples of fitted curves are shown in Fig. 1 for a non-pigmented *Pseudomonas* sp. grown at all six chill temperatures, and in Fig. 2 for a mixture of *Brochothrix thermosphacta* and a pigmented *Pseudomonas* sp. grown at 15°C.

Temperature/growth relationships were then studied using the Arrhenius and Square Root plots. For pure cultures, the results are summarized in Table 2. In all cases, the Square Root plot (using the time to reach a 2 $\log_{10}$ cycle increase in colony count) produced a better correlation between temperature and growth

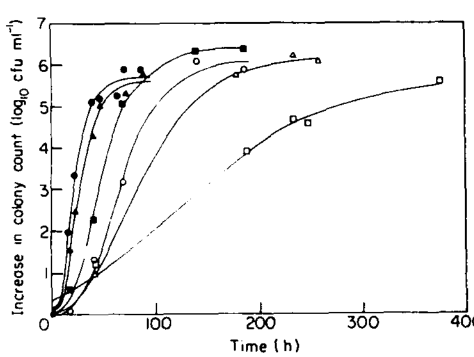

Fig. 1. Growth of a non-pigmented *Pseudomonas* sp. at various chill temperatures. ●, 15°C; ▲, 12°C; ■, 8°C; ○, 6°C; △, 4°C; □, 2°C; —, curves fitted by computer program.

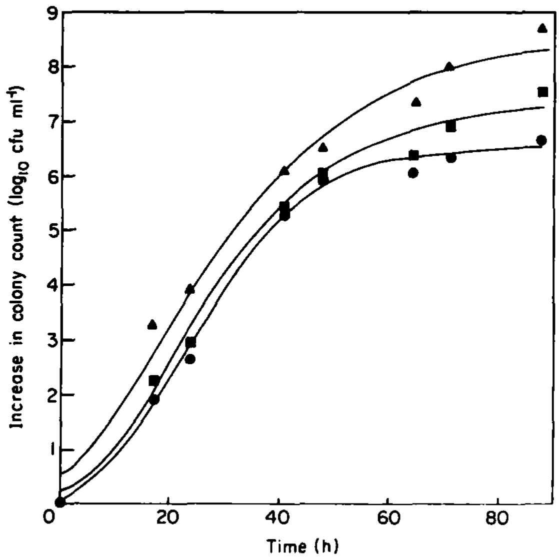

Fig. 2. Growth of a mixture of *Brochothrix thermosphacta* and a pigmented *Pseudomonas* sp. at 15°C. ●, *B. thermosphacta*; ▲, pigmented *Pseudomonas* sp.; ■, total colony count; —, curves fitted by computer program.

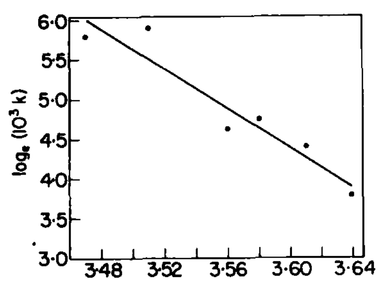

Fig. 3. Arrhenius plot for *Brochothrix thermosphacta*. ●, data points; —, regression line (correlation coefficient = −0·952).

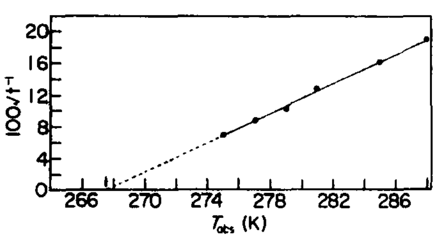

Fig. 4. Square Root plot for *Brochothrix thermosphacta*. ●, data points; —, regression line (correlation coefficient = 0·999); t = time (h) for count to increase by 2 $\log_{10}$ cycles.

rate parameters than did the Arrhenius plot. Examples of the two types of plots are shown for *B. thermosphacta* in Fig. 3 (Arrhenius plot) and Fig. 4 (Square Root plot).

T_0 values were considerably less variable between occasions than μ values for all the organisms tested (Table 2). The organisms in Table 2 have therefore been ranked according to their T_0 values. The μ values did not necessarily follow the same ranking except at either end of the range. However, they were so variable between occasions that listing the organisms in order of temperature characteristic would have very little real value.

Temperature/growth relationships were studied for mixtures using the Square Root plot. The results are summarized in Table 3 for the six mixtures. Data from pure cultures reported in Table 2 are included in Table 3 for comparison. Correlation coefficients for data from the components of the mixture and the total colony count were of the same order as those previously obtained

Table 2. Analysis of temperature/growth relationships using the Arrhenius and Square Root plots.

Organism	Number of data sets	Square Root plot (using time to a 2 $\log_{10}$ cycle increase): Mean[a] correlation coefficient[c]	Mean T_0 (K)	S.D.[a]	COV[b] (%)	Arrhenius plot: Mean[a] correlation coefficient[c]	Mean μ	S.D.[a]	COV[b] (%)
Pseudomonas sp. (non-pigmented)	2	0·989	265·2	1·7	0·64	−0·945	20,200	4,300	21·3
Pseudomonas sp. (pigmented)	3	0·978	265·2	2·7	1·02	−0·929	20,400	7,200	35·3
Brochothrix thermosphacta	4	0·980	268·0	2·1	0·78	−0·862	24,500	3,900	15·9
Alteromonas putrefaciens	3	0·978	268·0	2·6	0·97	−0·936	22,200	6,800	30·6
Acinetobacter sp.	3	0·941	268·4	0·4	0·15	−0·881	27,400	6,200	22·6
Alcaligenes viscosus	3	0·987	268·6	2·1	0·78	−0·884	21,000	6,800	32·4
Moraxella sp.	2	0·978	268·8	2·1	0·78	−0·811	22,000	3,200	14·5
Citrobacter freundii	2	0·991	271·2	0·7	0·26	−0·875	33,000	0	0
Serratia marcescens	3	0·966	273·3	2·5	0·91	−0·962	45,500	9,700	21·3

[a] Of the sets of data.
[b] Coefficient of variation (S.D./mean for T_0 or μ values).
[c] Between temperature and growth rate parameters.

Table 3. Analysis of growth/temperature relationships for mixtures of organisms and their components (using the Square Root plot).

	Individual results from pure culture[a]		Individual results from mixed culture		Total count from mixed culture	
Mixture components	Correlation coefficient[d]	T_0	Correlation coefficient[d]	T_0	Correlation coefficient[d]	T_0
1[e] *Brochothrix thermosphacta*	0·980	268·0	0·993	267·1	0·994	266·1
Pigmented *Pseudomonas* sp.	0·978	265·2	0·994	265·7		
2[e] *Brochothrix thermosphacta*	0·980	268·0	0·987	266·6	0·987	267·8
Citrobacter freundii	0·991	271·2	0·954	270·7		
3[c] *Brochthrix thermosphacta*	0·980	268·0	0·995	269·6	0·999	270·8
Serratia marcescens	0·966	273·3	—[b]	—[b]		
4[c] *Serratia marcescens*	0·966	273·3	1·000	275·6	0·982	271·3
Alcaligenes viscosus	0·987	268·6	0·970	270·0		
5[c] *Serratia marcescens*	0·966	273·3	0·945	268·9	0·988	268·3
Acinetobacter sp.	0·941	268·4	0·998	268·9		
6[c] Pigmented *Pseudomonas* sp.	0·978	265·2	0·994	267·9	0·994	267·7
Acinetobacter sp.	0·941	268·4	0·990	265·9		

[a] Data from Table 2.
[b] Two data points only.
[c] One occasion.
[d] Between temperature and growth-rate parameter.
[e] Mean of results from two occasions.

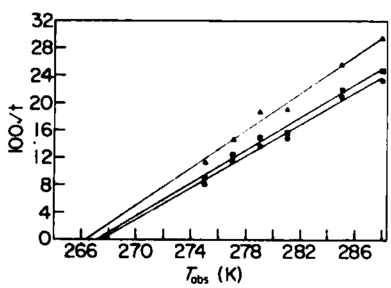

Fig. 5. Square Root plot for a mixture of a pigmented *Pseudomonas* sp. and *Brochothrix thermosphacta*. ●, *B. thermosphacta*; ▲, pigmented *Pseudomonas* sp.; ■, total colony count; —, regression lines (correlation coefficients 0·994, 0·993 and 0·994 respectively).

for pure cultures. T_0 values obtained for the components of the mixture were also similar to those obtained for the same organisms growing in pure culture (within the standard deviations of the values in Table 2). T_0 values for the total colony count fell between those for the two components of the mixture. Figure 5 shows examples of Square Root plots for one of the mixtures tested. The plot for total colony count lies between those for its component flora.

The Square Root plot could be calculated using the time to k_{max}, or time to 1, 2, 3, 4 or sometimes 5 $\log_{10}$ cycles increase in colony count. Time to 2 $\log_{10}$ cycles increase was chosen for the calculations in Tables 2 and 3 and Figs 4 and 5 because (i) it consistently produced a good correlation between temperature and growth rate parameters; (ii) all the organisms achieved the increase, and (iii) the 2 $\log_{10}$ cycles increase occurred during the exponential phase of growth.

Discussion

The objectives of the current study were to determine whether the Arrhenius or the Square Root plot is more appropriate in the description of microbial growth rate at chill temperatures. Colony counts were used rather than optical density or nephelometer readings to provide a comparison for later work with food samples. Differential counting of types of organism in a mixture was also possible.

One of the problems with growth rate studies, especially with limited numbers of data points, is the fitting of growth curves. In preparing data for analysis, the use of a computer program was an advantage in fitting the best growth curves and calculating useful parameters of the fitted curves. In all cases, the computer was able to fit curves which had a high correlation with the raw data (e.g. Figs 1 and 2).

Arrhenius plots derived from the fitted data gave consistently lower correlation coefficients between temperature and growth rate parameters than did the Square Root plots (Table 2). This study therefore confirms the suggestion of Ratkowsky et al. (1982) that the Square Root plot describes the relationship between temperature and microbial growth more accurately than does the Arrhenius plot. The Arrhenius equation decribes the relationship between temperature and the rate of first-order chemical reactions. Bacterial growth would be expected to be a far more complex system than this (Mohr and Krawiec 1980) and there is therefore no apparent reason why the Arrhenius equation should apply. However, there is also no known reason for the good correlations achieved with the Square Root plot, although it is interesting to note that the rate of nucleotide degradation in spoiling carp muscle follows a similar relationship (Ohta and Hirahara 1977), and the Square Root plot has been shown (Pooni and Mead 1984) to fit spoilage data better than does the relative spoilage rate equation of Olley and Ratkowsky (1973). The Square root equation may well prove valuable in the prediction of shelf-life, since the length of the lag phase is taken into account.

Values of T_0 for the organisms tested (Table 2) were within the range reported by Ratkowsky et al. (1982) and showed a similar variation. The cardinal (i.e. optimum, minimum, maximum) temperatures for growth were not established for the bacteria used in the present study. Nevertheless, ranking them by T_0 value (Table 2) suggests that T_0 and cardinal temperatures might be related, as those organisms generally regarded as the more mesophilic species (e.g. *Serratia marcescens, Citrobacter freundii*) had higher values of T_0. Values of μ obtained from Arrhenius plots were very variable between occasions (Table 2) and no firm conclusions can be drawn about the relationship between μ and cardinal growth temperatures. Reichardt and Morita (1982) have suggested that μ is more dependent on conditions and substrates for growth than on temperature ranges and optima; we cannot prove this here as a wide variety of results was obtained for organisms grown under comparable conditions.

With mixtures of organisms, T_0 values and correlation coefficients for the Square Root plot were similar to those obtained with pure cultures (Table 3). Since the curve-fitting program and the Square Root equation have been shown to be applicable to mixtures, it may be possible to extend these observations to the growth rate of the total bacterial flora on foods. The Square Root plot may then be used to predict growth rate at lower temperatures from data obtained at elevated temperatures.

In conclusion, it is apparent from this study that the Square Root equation is a better description of bacterial growth/ temperature relationships than is the Arrhenius equation. In addition, the theoretical lowest temperature for growth, T_0, may be useful in classification of organisms as psychrotrophs, mesophiles or thermophiles.

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