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Modelling the influence of temperature and carbon dioxide upon the growth of *Pseudomonas fluorescens*

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Pseudomonas fluorescens *was chosen as an indicator micro-organism for spoilage of ready-to-eat or Grade IV leafy vegetables. The effect of the extrinsic storage conditions (temperature and carbon dioxide concentration) upon the growth in a model system were studied. The bacterial growth curve was described with the logisitic and Gompertz equation. These sigmoidal functions were modified to give the lag time and maximum absolute growth rate for* P. fluorescens *under controlled storage conditions.*

In a temperature range of 4 to 12°C the lag time of P. fluorescens *showed an exponential dependence with temperature, whereas a linear relationship of the maximum absolute growth rate with temperature was observed. Carbon dioxide was shown to be effective against the proliferation of aerobic psychrotrophic Gram-negative bacteria by principally increasing the lag phase and to a lesser extent the generation time. The dependence of the lag time and the maximum absolute growth rate of* P. fluorescens *with the carbon dioxide concentration (0·03–15%, with an excess of oxygen) was described using an exponential function. Moreover, the effectiveness of the inhibition increased with decreasing temperatures.*

Introduction

Driven by consumer needs, the food industry is aggressively seeking preservation technologies which deliver convenience products which are 'fresh-like', 'chef-like' and have the image of invisible manufacturing (Mallki 1987). One of these 'new' technologies consists of refrigeration in combination with modified atmosphere packaging of minimally processed vegetables, commonly named 'ready to use' or 'Grade IV' products. The processing step consists of washing, cutting or peeling and packaging in a semi-permeable plastic film. This market is growing both in sales volume and assortments of products, e.g. vegetables such as endive, lettuce, carrots, peppers, onions, cabbage etc, both as single vegetables and in mixtures (Anon 1988, Saracino et al. 1991).

Grade IV products consist of living, respiring vegetable tissue, whereby the metabolic activities continue after harvesting and processing. Therefore, the oxygen in the package is consumed and carbon dioxide, water and heat are produced. The atmosphere is modified and depending on the permeability of the packaging material a dynamic atmosphere is created during storage. This depletion of oxygen and the acuumulation of carbon dioxide retards microbial

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proliferation and quality deterioration of Grade IV products. (Brecht 1980, Daniels et al. 1985, Ballantyne 1989, Kader et al. 1989). Carbon dioxide is shown to be effective against the proliferation of aerobic, psychrotrophic Gram-negative bacteria by increasing the lag phase and the generation time. The degree to which carbon dioxide is effective generally increases with concentration but is independent of the oxygen concentration in the gas mixture above 5% (King and Nagel 1967, Daniels et al. 1985, Lefevre 1990). The modification of the atmosphere inside the package can alter the general microbial profile and selects the microflora towards Gram-positive bacteria, and more specifically, lactic acid bacteria (Sinell 1980, Brackett 1987, Barriga et al. 1991).

However, the most critical factor for the extension of the shelf-life of these Grade IV products is temperature. The aim of cooling, as a preservation technique, is to retard microbial growth, metabolic processes (respiration) and chemical and/or enzymatic processes (oxidation, browning of cut surfaces). Temperature control during the entire cold chain is necessary and, as a consequence, the responsibility for the shelf-life and the quality-life of Grade IV products lies with the producer and the distributor as well as the consumer. In Belgium, these products must be kept at a refrigeration temperature of maximum 7°C during transport, storage and display (Anon 1982). However, in practice this regulation is violated frequently whereby temperatures of 12°C and higher are not unusual in the retail display cabinets as well as domestic refrigerator systems (own measurements, not shown). Similar results were reported by others (James and Evans 1990, Daniels 1991, Tolstoy 1991). These results demonstrate the necessity of a close control of temperature conditions and residence time in the distribution of refrigerated products of extended durability.

The traditional approach to evaluate the shelf-life of a food product has been to incubate it under conditions that are representative of those that would be encountered during distribution, storage and use, and then determine whether the microbial load exceeded acceptable levels (challenge test). The disadvantage of this type of storage trial is well known. If any changes in the formulation, packaging, storage or use of the product are made, the test has to be repeated. In addition, this trial does not provide information about the magnitude of influence of the controlling factors upon microbial growth. (Baird-Parker and Kilsby 1987, Cole 1991). In order to avoid these disadvantages of the traditional approach, predictive microbiology uses mathematical equations to estimate the growth, survival or death of indicator and/or index microorganisms as affected by extrinsic (processing and storage conditions) and intrinsic parameters of the food (e.g. salt concentration, pH or a_w). These models can be used to predict the microbial safety and shelf-life of the food product (Baird-Parker and Kilsby 1987, Gould 1989, Cole 1991).

The predominant microflora of fresh leafy vegetables are aerobic, psychrotrophic, Gram-negative rods, with *Pseudomonas* and *Erwinia* spp. being most numerous, with a count of approximately 10^5 cfu g^{-1}. However, during cold storage of Grade IV leafy vegetables pectinolytic strains of *Pseudomonas* are responsible for bacterial soft rot (Ceponis et al. 1970, Koek et al. 1983, Denis and Picoche 1986, Bartz and Eckert 1987, Brackett 1987, Brocklehurst et al. 1987, Liao and Wells 1987, Nguyen-The and Prunier 1989, Carlin et al. 1990, King et al. 1991, Snowdon 1991). Within

the genus Pseudomonas, *P. fluorescens* was identified as an indicator micro-organism for spoilage of these Grade IV leafy vegetables.

The objective of this study was to quantify the growth of this indicator micro-organism, *P. fluorescens* in a model system. Secondly, the influence of the controlled storage conditions of temperature and gas composition was investigated and the kinetic growth parameters (lag time and maximum absolute growth rate) were modelled as a function of temperature (4–12°C) and carbon dioxide concentration (0·03–15%). The oxygen concentration used in the gas mixtures was 20% volume.

Materials and Methods

Organism and growth medium

The organism used in this study was *P. flourescens*, obtained from the collection of the Laboratory of Industrial Microbiology, Faculty of Agricultural Sciences, Katholieke Universiteit Leuven, Belgium. The bacterial growth curve is measured under controlled storage conditions in a highly nutritious medium and as a consequence, the substrate is not a limiting factor to growth until the maximum population density is reached. The model system used was Tryptone soya medium (OXOID, UK). Tryptone soya agar (TSA) was used as counting medium, and Tryptone soya broth (TSB) for growth. The pH of the broth (pH = 7·3 ± 0·2) was slightly buffered (K_2HPO_4, 2·5 gl^{-1}). The pH was measured with a microprocessor pH meter (pH 535, Multical WTW, Germany). The pH at the end of the growth experiment was 7·8 (air); 7·3 (5·01% CO_2); 7·6 (10·1% CO_2); and 7·0 (15·0% CO_2).

Inoculum preparation and measurement of growth

In order to reduce the dependence of the lag phase on the history of the inoculum, the inoculum was taken from a stationary phase (72 h, 23°C) after equilibration overnight at the appropriate refrigeration temperatures. For the growth experiments 250 ml incubator flasks were used, each containing 100 ml TSB and inoculated with the test micro-organism to give an initial titer of 10^5 cfu ml^{-1}. The incubator flasks were flushed with the appropriate gas mixture (100 ml min^{-1}) entering the flask at the bottom. The concentrations of the components in the different gas mixtures (L'Air Liquide, Belgium) are shown in Table 1. The sterility of this gas stream was maintained by a 0·2 μm teflon membrane (Millex FG50, Millipore, France). This hydrophobic membrane permits the passage of the dry sterilized gas into the flask and protects against leaking of the medium. The volume reduction of the broth by the drying force of the gas was measured (1·05 ml day^{-1}) to account for the increase in bacteria number by the concentration of the medium. The experimental layout and the flow guarantee the necessary mixing of the growth medium.

The flasks were incubated in refrigerators at the desired temperatures and temperature was measured using an electronic temperature recorder with an accuracy of 0·1°C (0–80°C) and resolution of 0·1°C (Control One, VEL, Belgium). The flasks were incubated statically at different temperatures (in °C and s.d. in parenthesis): 1·1 (0·2); 4·6 (0·3); 5·9 (0·1); 6·2 (0·1); 7·2 (0·1); 7·6 (0·1); 11·4 (0·1) and 11·5 (0·2). At appropriate time intervals (depending on the refrigeration temperature) samples of 0·5 ml were taken and diluted in saline solution (NaCl, 0·7 g l^{-1}). The TSA plates were incubated at 23°C for 72 h before the colonies were counted. For the entire growth curve at least 15 data

Table 1. Gas mixtures and concentrations of components (volume %).

Good mixture	conc O_2(%)	conc O_2(%)	conc N_2(%)
Air	20·9	331 ± 9 ppm	78·1
5% CO_2	20·03 ± 0·3	5·01 ± 0·1	Q.S.[a]
10% CO_2	20·06 ± 0·3	10·05 ± 0·2	Q.S.
15% CO_2	20·01 ± 0·3	15·03 ± 0·3	Q.S.

[a]Quantum satis.

points were collected, evenly spread throughout each phase and sampling was ended when minimally three counts in the stationary phase were obtained.

Theoretical background on the growth models: the logistic and Gompertz equation

When plotting the bacterial population density [log(cfu ml^{-1})] against time for a set of controlled environmental conditions, the growth curve is sigmoidal and is characterized by several successive phases: the lag phase, the exponential phase, the stationary phase and finally the death phase. The death phase is not considered in this study. In literature the logistic (Verhulst, Eqn 1) and Gompertz equations (Eqn 2) are used extensively to describe the entire sigmoid bacterial growth curve (Gibson et al. 1987, Buchanan and Cygnarowicz 1990, Zwietering et al. 1990, Buchanan 1991, Garthright 1991):

$$Y(t) = A + C/[1 + \exp(-B.(t-M)] \quad (1)$$

where $Y(t)$: population density at time t : [log(cfu ml^{-1})]
t : time (h)
A : population density at time $\rightarrow \infty$
C : increase in population density at time $t \rightarrow +\infty$
M : time at which the absolute growth rate is maximal
B : relative growth rate at time $t = M$ (1/h)

$$Y(t) = A + C.\exp[-\exp(-B.(t-M))] \quad (2)$$

where the four parameters A, C, M and B are defined as in Eqn. 1.

By reparametrization the Gompertz and logistic equation can be modified in terms of the biological parameters which identify the growth curve (Zwietering et al. 1990, Garthright 1991): the estimated initial inoculum level (A), the lag time (λ), the maximum absolute growth rate (r_m) and the asymptotic increase in population density (C). The maximum absolute growth rate (r_m) is the slope of the tangent line at the inflection point (time $t = M$) and can be derived by calculating the first derivative of the Gompertz or logistic equations. The lag time (λ) is calculated as the intercept of this tangent with the initial population density. Table 2 shows the kinetic parameters derived from the Gompertz and logistic equation parameters. The population density is expressed as the logarithm of the number of organisms [log(cfu ml^{-1})]. In order to calculate directly the growth parameters, they are substituted for the mathematical parameters B and M. Moreover, Buchanan et al. (1989) showed that the lag time, growth rate and maximum population density are independent of the initial inoculum level for *Listeria monocytogenes*, whereas B and M are not. Analogous results were obtained for *Escherichia coli* by Jason (1983) and for *Salmonella* by Mackey and Kerridge (1988). Therefore these biological parameters are specific for a microorganism and its environment.

After substitution of the mathematical parameters with the biological growth parameters the modified logistic (3) and Gompertz equations (4) are:

$$\log(N) = A + C/[1 + \exp((4.r_{mL}/C).(\lambda_L - t) + 2)] \quad (3)$$

$$\log(N) = A + C.\exp[-\exp((e.r_{mG}/C).(\lambda_G - t) + 1)] \quad (4)$$

Data analysis

The modified Gompertz and logistic equations were fitted to the data of the growth experiments by non linear regression with a DUD algorithm (SAS 1982). The DUD algorithm is a derivative free algorithm for

Table 2. Expressions of lag time (λ) (h), maximum absolute growth rate (r_m) [log(cfu ml^{-1})/h], maximum population density (Y_m) [log(cfu ml^{-1})] and generation time (GT) h derived from logistic (L) and Gompertz (G) equation parameters. Population density (Y) expressed as [log(cfu ml^{-1})].

Growth parameters	Logistic equation	Gompertz equation
Lag time	$\lambda_L = M - (2/B)$	$\lambda_G = M - (1/B)$
Maximum absolute growth rate	r_{mL} = B.C/4	$r_{mG} = B\ .\ C/e$
Maximum population density	$Y_{mL} = A + C$	$Y_{mG} = A + C$
Generation time at $t = M$	GT_L = 4.log(2)/B.C	GT_G = e.log(2)/B.C

nonlinear least squares. This is an iterative search method to minimize the sum of squared differences between the predicted and the observed data points (Residual Sum of Square or RSS). The algorithm then provides the estimates for the lag time (λ), the maximum absolute growth rate (r_m), the initial inoculum level (A) and the asymptotic increase in population density (C). The same nonlinear regression technique was used for the global analysis of temperature and gas concentration upon the growth.

The selection of the best model consisted of the comparison of the variance or mean residual sum of squares (MRRS) on one hand and the analysis of the residual variation on the other hand. The mean residual sum of squares is calculated as the residual sum of squares (RSS) divided by the residual degrees of freedom which is equal to the number of experimental data points minus the number of regression parameters (Ratkowsky 1983, Wonnacott 1984).

Results and Discussion

Modelling the bacterial growth curve

In order to relate the behaviour of the indicator micro-organism to the extrinsic factors, the bacterial growth curve was measured and modelled under controlled storage conditions of temperature and carbon dioxide. Fig. 1. shows the growth curve of *P. fluorescens* (experimental data and best fitted modified logistic (Eqn 3) and Gompertz (Eqn 4). The logistic equation is symmetrical around the inflection point (M) whereas the Gompertz equation is not. Table 3 summarizes the calculated growth parameters for *P. fluorescens* at different temperatures and carbon dioxide concentrations. When using the mean residual sum of squares (MRSS) as the selection criterion between the logistic and Gompertz equation, it is impossible to obtain a straight answer. However, when comparing the asymptotic 95% confidence intervals for the lag time and maximum growth rate, the Gompertz equation has noticeable

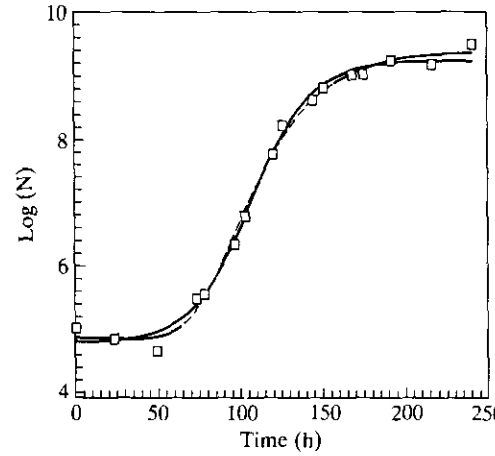

Fig. 1. Growth curve of *P. fluorescens* at 11·5°C and 15% CO_2 modelled with the logistic (—) and Gompertz (- - -) equations.

Table 3. Lag time (λ) (h), maximum absolute growth rate (r_m) [log(cfu ml^{-1})/h] and mean residual sum of squares (MRSS) derived from logistic (L) and Gompertz (G) equation for the experiments of *P. fluorescens* at different temperatures (°C) and carbon dioxide concentrations (vol %).

T	CO_2	r_{mL}	r_{mG}	λ_L	λ_G	$MRSS_L$	$MRSS_G$
4·6	0·03	0·0172	0·0162	83·8	85·4	0·0445	0·0518
5·9	0·03	0·0363	0·0351	70·7	68·8	0·0564	0·0641
6·2	0·03	0·0392	0·0388	54·4	56·4	0·0242	0·0223
7·2	0·03	0·0485	0·0510	47·1	50·6	0·0156	0·0199
7·6	0·03	0·0500	0·0517	12·1	22·4	0·0273	0·0234
11·5	0·03	0·0895	0·0920	15·4	17·4	0·0187	0·0215
11·5	5·01	0·0827	0·0856	32·5	33·9	0·0194	0·0176
11·5	10·01	0·0728	0·0689	49·0	46·9	0·0289	0·0339
11·5	15·00	0·0606	0·0590	70·3	68·8	0·0223	0·0117

smaller intervals. Note also the estimated lag time at 7·6°C in air. The logistic equation estimated a shorter lag time (12·1 h) as compared to 11·5°C (15·4 h).

A second useful measure of the goodness of fit is the analysis of the residual variation. A good (nonlinear) regression is characterized by a random distribution of the residuals around zero. As can be seen from Fig. 2 the residual variation of the data points modelled with the logistic (Fig. 2(a), and Gompertz equation (Fig. 2(b) showed a random distribution in each part of the growth curve. Analogous results were obtained for the other experimental growth curves.

Zwietering et al. (1990) proposed a modification by a reduction of the four parameter equations to a three parameter equation through the use of a relative population density. When dividing the population size by the initial inoculum size [$\ln(N/N_0)$ or $\log(N/N_0)$], parameter A is omitted in Eqns 3 and 4. However, this reduction in parameters of the model lead to a higher variance or mean residual sum of squares (20–40% in this case). A second drawback of the omission of the estimated initial population density (A) is that only the maximum population density ($Y_m = A + C$) is constant and independent of the initial inoculum level and not the increase in population density (C) (Buchanan et al. 1989).

The maximum population density ($Y_m = A + C$), calculated with the four parameter equations, showed little variation with temperature for the experiments with *P. fluorescens*. The 95% confidence intervals at each temperature includes the estimated maximum population density (Y_m) whereas the maximum population density decreases approximately linear with increasing carbon dioxide concentration as shown in Table 4 (calculations with the logistic equation).

It was concluded that the four parameter modified logistic and Gompertz equations are both useful for the estimation of the lag time, maximum absolute growth rate, initial inoculum level and maximum population density of *P. fluorescens* under controlled storage conditions in a liquid medium. The Gompertz equation was selected as the better model when comparing the 95% confidence intervals of the estimated

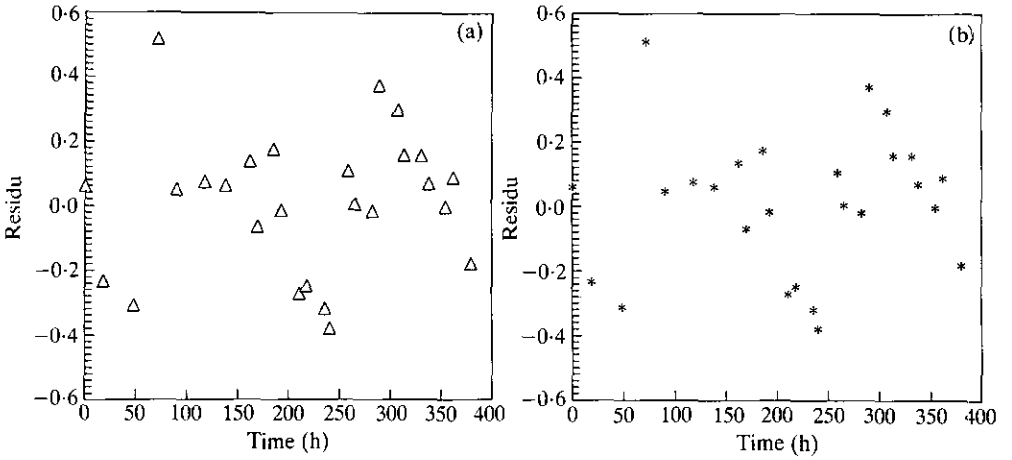

Fig. 2. (a) Residual variation of *P. fluorescens* growth data (at 4·6°C, in air) modelled with the logistic equation (Eqn 3). (b) Residual variation of *P. fluorescens* growth data (at 4·6°C, in air) modelled with the Gompertz equation (Eqn 4).

Table 4. Maximum population density ($Y_m = A + C$) [log(cfu ml^{-1})] and 95% confidence intervals estimated from logistic equation for the experiments of *P. fluorescens*. (a) Y_m for different temperatures [T in (°C)] in air and (b) for different carbon dioxide concentrations (CO_2 in [vol%]) at 11·5°C.

(a)			(b)		
T	Y_m	95% interval	CO_2	Y_m	95% interval
4·6	9·89	9·40 to 10·38	0·03	9·68	9·19 to 10·13
5·9	9·72	9·38 to 10·06	5·01	9·60	9·40 to 9·80
6·2	9·68	9·45 to 9·91	10·1	9·35	9·16 to 9·54
7·2	9·84	9·64 to 10·03	15·0	9·24	9·07 to 9·41
7·6	9·85	9·60 to 10·11			

growth parameters. From these relations, the estimated kinetic growth parameters of the indicator micro-organism were estimated and were related to extrinsic storage conditions of the Grade IV products: temperature and carbon dioxide concentrations (Gibson et al. 1988, Bratchell et al. 1989, Buchanan et al. 1989, Zaika et al. 1989, Buchanan and Phillips 1990, Yousef et al. 1991, Zwietering et al. 1991).

The effect of temperature

In order to predict the growth of *P. fluorescens* in air at different constant temperatures, the temperature dependence of the kinetic growth parameters was quantified. Various models have been proposed to describe the relationship between growth rate, lag time and temperature whereas the maximum population density is considered independent of temperature (Zwietering et al. 1991). In our discussion the temperature range is limited between the minimum and optimum temperature of growth of *P. fluorescens*: no growth was detected at 1·1°C after 320 h and 11·5°C was the highest temperature investigated. In the following paragraph the various models used to describe the temperature dependence of the growth rate are discussed. In order to compare the different models, no transformation of the growth rate (r_m or $1/\lambda$) was carried out.

Spencer and Baines (1964) proposed a linear relationship between the rate of microbial spoilage of wet white fish and temperature in the range −1 to 25°C:

$$r_T = r_0 . (1 + c.T), \qquad (5)$$

where r_T = rate of spoilage at temperature T
r_0 = rate of spoilage at temperature T = 0°C
c = constant for linear temperature response
T = temperature (°C).

In a limited temperature range, the Arrhenius equation satisfactorily describes the effect of temperature also:

$$r_m = A . \exp [-E_a/(R.T)], \qquad (6)$$

where r_m = maximum absolute growth rate
A = pre-exponential or frequency factor
E_a = activation energy (Jmol^{-1})
R = universal gas constant (8·314 Jmol^{-1} K^{-1})
T = absolute temperature (K).

Since microbial growth is a complex set of enzyme mediated biochemical reactions, microbial growth can be characterized by some overall activation energy (E_a), often called the temperature characteristic of the micro-organism. The higher the E_a the more sensitive the

growth rate is on temperature. E_a is obtained from the slope when plotting the natural logarithm of the growth rate (constant) versus the reciprocal of the absolute temperature. Table 5 shows the temperature characteristic of several *Pseudomonas* spp. In order to make a comparison between the growth rates, the reference temperature is set to 10°C [283·15 K]. The growth rate can then be calculated as:

$$r_m = r_{mref} \cdot \exp [E_a/R \cdot (1/T_{ref} - 1/T)] \quad (7)$$

where r_m = maximum absolute growth rate
r_{mref} = maximum growth rate at ref. temperature
T_{ref} = reference temperature T = 283·15 K
E_a = activation energy (J mol^{-1}).
R = universal gas constant (8·314 J mol^{-1} K^{-1}).
T = absolute temperature (K).

Beside the difference in micro-organisms, the specific growth rate and the temperature characteristic will depend on the determination method of the growth rate (constant) and the medium used. Given the statistical uncertainty in k itself, one could expect rather large confidence limits for E_a. Generally, the 95% confidence intervals on the temperature characteristic are not reported (Labuza and Riboh 1982).

Another model frequently used is the Square Root relationship of Ratkowsky et al. (1982). The model describes a linear relationship between the square root of the growth rate and temperature (between minimum and optimum temperature of growth):

$$r_m = [b \cdot (T - T_{min})]^2 \quad (8)$$

where r_m = maximum absolute growth rate
b = Ratkowsky regression parameter (1/K)
T = absolute temperature (K)
T_{min} = theoretical minimum temperature for growth (K).

T_{min} is the theoretical minimum temperature of growth of the organism obtained by extrapolation (r_m = 0) and can be different from the experimental minimal growth temperature (1·1°C for *P. fluorescens*).

All the previous models were traditionally applied to the growth rate (constant). Zwietering et al. (1991) proposed an hyperbolic equation to describe the

Table 5. Temperature characteristic (E_a) (kJ mol^{-1}) for *Pseudomonas* spp. (specific) growth rate (k_{ref}) at T = 283·15 K; temperature range [°C], medium, determination method for growth rate (constant) and reference of the experiment.

Micro-organism	E_a (kJ mol^{-1})	k_{ref} (1 h)	T Range (°C)	Medium	Method	Reference[a]
P. fluorescens	65·0	NR[b]	<37·9	Synthetic	Traditional	(1)
Pseudomonas strain 423a	63·0	0·248	10–30	Milk	Traditional	(2)
Pseudomonas fluorescens BL 78/34	83·0	0·197	0·2–20·8	Synthetic	Traditional	(3)
Pseudomonas strain BL 78/50	79·5	0·145	0·2–20·8	Synthetic	Traditional	(3)
Pseudomonas spp. (non-pigmented)	84·5	NR	2–15	Synthetic	Stannard	(4)
Pseudomonas spp. (pigmented)	85·4	NR	2–15	Synthetic	Stannard	(4)
Pseudomonas fragi (log phase)	74·0	0·264	2–22	Milk	Traditional	(5)
Pseudomonas fragi (lag phase)	76·3	0·083	2–22	Milk	Traditional	(5)

[a](1) Mohr and Krawiec (1980), (2) Langeveld and Cuperus (1980), (3) Brocklehurst and Lund (1981), (4) Stannard et al. (1985), (5) Fu et al. (1991).
[b]NR, not reported.

natural logarithm of the lag time behaviour with temperature:

$$\ln(\lambda) = p/(T - T_{min}), \qquad (9)$$

where λ = lag time (h)
p = hyperbolic regression parameter (K)
T = absolute temperature (K)
T_{min} = theoretical minimum temperature for growth (K).

T_{min} is the theoretical minimum temperature for growth at which the lag time is infinite. Through the combination of these models on both the lag time and the growth rate, and after introduction into the modified logistic and Gompertz equation, the parameters of the different models were estimated and the minimum residual sum of squares was used as selection criterion.

Table 6 shows the results of the global analysis of the relations describing the temperature dependence for the lag time and the maximum growth rate integrated in the modified logistic and Gompertz equation. The lag time dependence was restrained to be the hyperbolic relation Eqn (9). The growth rate dependence varied over three relations (eqns 5, 6 and 8) above. The linear relationship of Spencer and Baines (1964) (eqn 5) gave the lowest RSS. The global analysis favoured the logistic equation which is in contradiction to the individual analysis of the growth curves. This result indicated that both the logistic and Gompertz equation are useful models for the description of the bacterial growth curve. Figure 3. shows the final model describing the population density (logistic equation) at a given temperature (between 4·6 and 11·5°C) based on the hyperbolic relationship of the natural logarithm of the lag time with temperature and the linear relationship of the maximum growth rate with temperature (parameters in Table 6).

The temperature characteristic (Table 6) for the logistic and Gompertz equation was higher as compared to the values reported for other *Pseudomonas* spp. (Table 5) whereas the maximum growth rate at T_{ref} (r_{mref} = 0·0666 × 2·303 = 0·153) agreed fairly. The 95% confidence interval on the estimated E_a

Table 6. Estimated parameters and RSS of lag time and maximum growth rate temperature (K) dependence by the global analysis in the Gompertz (G) and logistic (L) equation. The temperature dependence of the lag time is modelled with the hyperbolic Eqn (9). The maximum absolute growth rate with the Spencer and Baines (5), Arrhenius (7) and square root (8) Eqn. The maximum population density (Y_m = A + C) is set independent of temperature. Lag time (h); Maximum growth rate [log (cfu ml^{-1}/h]; Temperature (K) and Maximum population density [log (cfu ml^{-1})].

Lag time – temperature	Maximum growth rate – temperature	Maximum population density (Y_m)	RSS
Hyperbolic Eqn (9)	Spencer and Baines (5)		
$\ln(\lambda_G) = 43{\cdot}37 / (T - 268{\cdot}4)$	$r_{mG} = -0{\cdot}030 \times [1 - 0{\cdot}353 \times (T - 273{\cdot}15)]$	9·872	7·711
$\ln(\lambda_L) = 40{\cdot}41 / (T - 268{\cdot}8)$	$r_{mL} = -0{\cdot}030 \times [1 - 0{\cdot}352 \times (T - 273{\cdot}15)]$	9·753	7·261
Hyperbolic Eqn (9)	Arrhenius (7) T_{ref}: 283·15K		
$\ln(\lambda_G) = 31{\cdot}26 / (T - 271{\cdot}4)$	$r_{mG} = 0{\cdot}0666 \times \exp[119201/8{\cdot}314 \times (1/283{\cdot}15 - 1/T)]$	9·845	11·034
$\ln(\lambda_L) = 28{\cdot}53 / (T - 271{\cdot}9)$	$r_{mL} = 0{\cdot}0668 \times \exp[121614/8{\cdot}314 \times (1/283{\cdot}15 - 1/T)]$	9·714	10·856
Hyperbolic Eqn (9)	Square Root (8)		
$\ln(\lambda_G) = 31{\cdot}47 / (T - 270{\cdot}9)$	$r_{mG} = [0{\cdot}0237 \times (T - 271{\cdot}6)]^2$	9·865	9·172
$\ln(\lambda_L) = 35{\cdot}95 / (T - 270{\cdot}2)$	$r_{mL} = [0{\cdot}0239 \times (T - 271{\cdot}8)]^2$	9·750	8·793

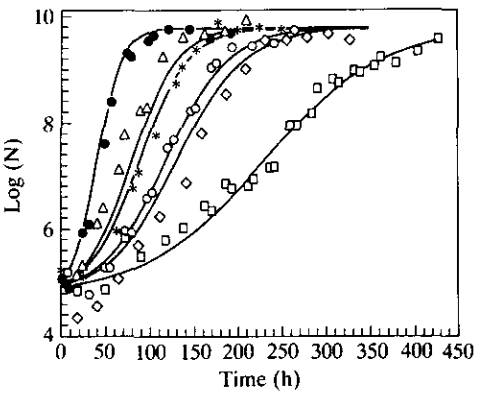

Fig. 3. Growth data of *P. fluorescens* (in temperature range 4·6–11·5°C, in air) and growth curves modelled with the logistic equation (Eqn 3) and the temperature dependence relations of lag time and maximum absolute growth rate. Model parameters in Table 6. Temperature (K). □, 277·75; ◇, 279·05; ○, 279·35; ∗, 280·35; △, 280·75; ●, 284·65.

was 99·7 to 138·7 kJ mol^{-1} for the Gompertz and 102·4 to 140·8 kJ mol^{-1} for the logistic equation. Probably this large confidence interval could be reduced by additional experiments in the temperature range. The extrapolation to the minimal theoretical temperature for growth (r_m = 0 or λ = infinite), resulted in temperatures lower than 0°C for both the hyperbolic and square root equation whereas experimentally no growth was detected at 1·1°C. Only the linear equation (eqn 5) extrapolated a positive theoretical minimal temperature of growth: T_{min} = 2·8°C for r_m = 0.

The effect of carbon dioxide concentration

At low concentration level, e.g. air (± 300 ppm), carbon dioxide (CO_2) stimulates many species, however, at higher concentration level, CO_2 becomes inhibiting. This bacteriostatic effect has been known for a long time and exploited for the preservation of packed food like meat, fish and poultry. The specific mechanism for this growth inhibitory effect of CO_2 is not known. (Daniels et al. 1985, Genigeorgis 1985, Dixon et al. 1987, Lefevre 1990). Carbon dioxide has been shown to be effective for foods whose spoilage is dominated by Gram-negative, aerobic, psychrotophic bacteria (Daniels et al. 1985, Lefevre 1990, Smith et al. 1990). The sensitivity to carbon dioxide varies according to the species. The overall effect of carbon dioxide is to increase both the lag phase and the generation time of spoilage micro-organisms but several factors influence this antimicrobial effect, specifically microbial load, gas concentration and temperature.

Enfors and Molin (1980) reported an increased CO_2 resistance in the order: *P. fragi, Bacillus cereus* to *Streptococcus cremoris*. As a consequence, preserving foods under high CO_2 concentrations selects the microflora towards lactic acid bacteria (Blickstad et al. 1981). Some investigators have reported a maximum partial pressure of carbon dioxide above which a further CO_2 pressure increase has but minor additional inhibitory effect. Gill and Tan (1979) indicate a maximum degree of inhibition of growth of *P. fluorescens* when an pCO_2 of 250 mmHg was attained. In contrast, other investigators (King and Nagel 1967, Enfors and Molin 1980) observed inhibitory effects which were approximately proportional to the partial pressure of CO_2 over the entire CO_2 pressure range. At low oxygen levels, the combined growth inhibitory effect of oxygen deficiency (lower then 1%) and of the presence of CO_2 seems to be purely additive (Dixon et al. 1987, Enfors and Molin 1980). No indication of a synergistic effect between CO_2 inhibition and oxygen limitation were noted.

In this study, different carbon dioxide

Table 7. Estimated parameters and RSS of lag time, maximum growth rate and maximum population density carbon dioxide dependence by the global analysis in the Gompertz (G) and logistic (L) equation (T = 11·5°C). The maximum population density ($Y_m = A + C$) showed a linear response with the CO_2 concentration. The CO_2 dependence of the lag time and the maximum absolute growth rate are modelled with an exponential and/or linear relation. Lag time (h); Maximum growth rate [log(cfu ml^{-1})/h]; CO_2 concentration (vol %) and maximum population density [log(cfu ml^{-1})].

Lag time – CO_2 concentration	Maximum growth rate – CO_2 concentration	Maximum population density – CO_2 concentration	RSS
Linear relationship	Exponential relationship	Linear relationship	
$\lambda_G = 17{\cdot}05 + 3{\cdot}244 \times CO_2$	$r_{mG} = \exp[-2{\cdot}307 - 0{\cdot}0379 \times CO_2]$	$Y_{mG} = 9{\cdot}73 - 0{\cdot}0210 \times CO_2$	1·755
$\lambda_L = 16{\cdot}76 + 3{\cdot}264 \times CO_2$	$r_{mL} = \exp[-2{\cdot}335 - 0{\cdot}0344 \times CO_2]$	$Y_{mL} = 9{\cdot}69 - 0{\cdot}0287 \times CO_2$	1·727
Exponential relationship	Linear relationship	Linear relationship	
$\lambda_G = [2{\cdot}986 + 0{\cdot}0855 \times CO_2]$	$r_{mG} = 0{\cdot}0954 - 0{\cdot}00247 \times CO_2$	$Y_{mG} = 9{\cdot}75 - 0{\cdot}0242 \times CO_2$	1·223
$\lambda_L = [2{\cdot}988 + 0{\cdot}0860 \times CO_2]$	$r_{mL} = 0{\cdot}0942 - 0{\cdot}00266 \times CO_2$	$Y_{mL} = 9{\cdot}70 - 0{\cdot}0304 \times CO_2$	1·127
Exponential relationship	Exponential relationship	Linear relationship	
$\lambda_G = [2{\cdot}977 + 0{\cdot}0860 \times CO_2]$	$r_{mG} = \exp[-2{\cdot}336 - 0{\cdot}0332 \times CO_2]$	$Y_{mG} = 9{\cdot}75 - 0{\cdot}0235 \times CO_2$	1·187
$\lambda_L = [2{\cdot}977 + 0{\cdot}0865 \times CO_2]$	$r_{mL} = \exp[-2{\cdot}352 - 0{\cdot}0302 \times CO_2]$	$Y_{mL} = 9{\cdot}70 - 0{\cdot}0300 \times CO_2$	1·092

concentrations in excess of O_2 (20% vol) were used (Table 2) to investigate this inhibitory effect upon the growth of *P. fluorescens* at a constant temperature (11·5°C). Table 7 and Fig. 4 shows the

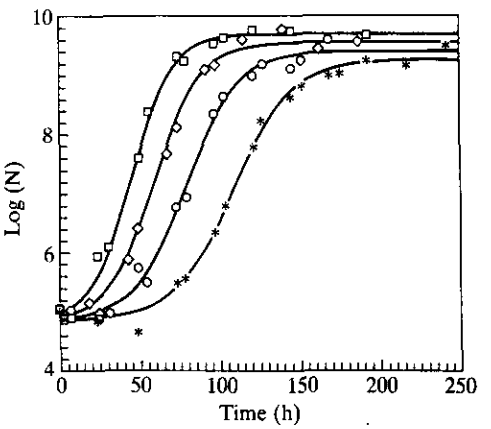

Fig. 4. Growth data of *P. fluorescens* (CO_2 range 0·03–15%, at 11·5°C) and growth curves modelled with the logistic equation (Eqn 3) and the CO_2-dependence relations of lag time, maximum absolute growth rate and maximum population density. Model parameters in Table 7. Carbon dioxide concentration in vol %. □, 0·03; ◇, 5·01; ○, 10·05; ∗, 15·03.

results of the global analysis of the CO_2 dependence relations. The maximum population density (Y_m) showed a linear response to CO_2 [Table 4(b)]. An exponential dependence of the lag time and the maximum growth rate with CO_2 gave the lowest RSS. However, in this limited CO_2 range, a combination of an exponential relation for the lag time and a linear relaction for r_m with CO_2 was satisfactory. Extrapolation to a zero growth rate (linear relation) lead to a concentration of 38% (Gompertz) and 35% CO_2 (logistic). This extrapolation would indicate that a CO_2 concentration above ± 40% with excess of O_2 in the package inhibits the growth of *P. fluorescens* completely at 11·5°C. This extrapolation will overshoot the reality, but nevertheless, the gas permeability of the packaging material of the Grade IV products will play an important role in the preservation. The permeability regulates the O_2 and CO_2 concentration within the package and therefore the inhibitory effect upon the growth of the

spoilage micro-organisms. Again the global analysis favoured the logistic equation as compared to the Gompertz equation in contradiction with the individual analysis of the growth curves (Table 3).

The combined effect of temperature and carbon dioxide

As can be seen from Table 3, an increase in CO_2 concentration from 0·03 to 15% results in the same prolongation of the lag phase as a decrease in temperature from 11·5 to 5·9°C. On the other hand, the influence of CO_2 upon the maximum absolute growth rate is less pronounced. These observations lead to the conclusion that the principal effect of CO_2 upon the growth of *P. fluorescens* is an increase in the lag phase duration. When comparing Fig. 3 and Fig. 4, temperature control is the best way to delay the bacterial proliferation through the combined effect of an extension of the lag phase and a lowering of the maximum growth rate. These observations reinforce the general agreement that temperature is the principal extrinsic factor controlling the rate of microbial growth.

Several authors have stated that the effectiveness of the CO_2-inhibition increase at decreasing temperatures (Gill and Tan 1979, 1980, Blickstad et al. 1981, Enfors and Molin 1981). The increased efficiency of CO_2 at lower temperature could be related to the increased solubility of CO_2 into the medium with lowering of temperature. (Genigeorgis 1985, Lefevre 1990). Two experiments were set up to verify this synergistic effect of CO_2 at low temperature. If there was no synergistic effect of the carbon dioxide concentration at lower temperatures, it must be possible to predict the growth curve based upon the temperature and carbon dioxide relations of lag time, maximum growth rate and maximum population density (Tables 6 and 7). Fig. 5 gives the observed (experimental) and calculated growth curves based on the estimated logistic parameters for the experiment at 7·6°C with 10% CO_2 and at 4·6°C with 15% CO_2. This figure shows the existence of the synergistic effect of CO_2 at lower temperatures upon both the lag time, the maximum growth rate and the maximum population density: the growth parameters were overestimated. Consequently, temperature abuse during distribution of the packaged foods, has a double effect on preservation: the microbial growth rate rises, on the one hand, because of the direct effect of temperature upon growth, and on the other hand, because of a poorer inhibiting effect of CO_2. As a consequence, temperature control during production and distribution of foods becomes more critical. We are aware that we only included some preliminary data on this synergistic effect and a detailed systematic study would be welcomed.

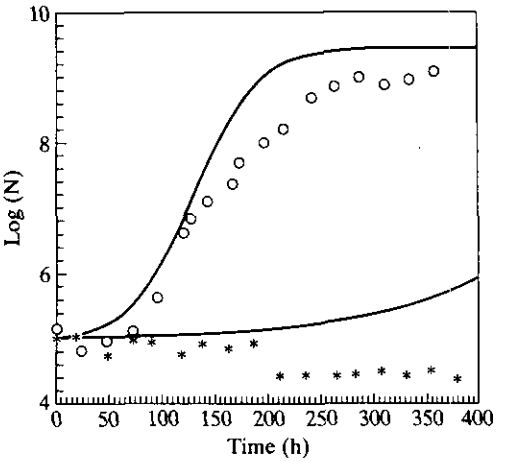

Fig. 5. Growth data of *P. fluorescens* at 7·6°C in 10% CO_2 (○) and 4·6°C in 15% CO_2 (*). and estimated growth curves modelled with the logistic equation (Eqn 3) and temperature and CO_2 dependence relations (Tables 6 and 7).

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