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Influence of controlled pH and temperature on the growth and acidification of pure cultures of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398

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Summary. The effect of pH and temperature on the growth and acidification characteristics of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398 were studied in order to optimize starter production. A quadratic two-variable model for each of these parameters is proposed. Optimal growth conditions were found at pH 6.5 and 40° C for *S. thermophilus* and pH 5.8 and 44° C for *L. bulgaricus*. Maximum acidification was obtained at pH values and temperatures higher than the optimal growth conditions. In addition, the two strains were generally more sensitive to pH effect.

Introduction

Thermophilic lactic acid bacteria are widely used in the dairy industry for manufacturing cheeses (emmental and gruyere) and fermented milk products such as yoghurts (Martley 1983).

Industrial starters are currently produced in pure cultures. Mixtures of a variable number of strains are then prepared to furnish the mixed starters regularly used (Stadhouders and Leenders 1984). In order to optimize starter production in fermentors, it is important to determine the effects of operating conditions on yields and kinetic parameters of the cultures. According to Tayeb et al. (1984), pH and temperature are important operating factors for *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, strains used to manufacture yoghurt. Several authors (Accolas et al. 1977; Martley 1983; Radke-Mitchell and Sandine 1986; Robinson 1988) have defined optimal growth and/or acidification temperatures of these strains in tests generally carried out in milk and without pH control. These conditions are somewhat different from those used in starter production, which requires automatic pH control. The optimal temperatures proposed (between 35° and 46° C for *S. thermophilus* and between 42° and 50° C for *L. bulgaricus*) correspond to wide ranges which cannot be explained by behaviour differences between the strains. Little work has been done on the optimum pH for the growth of these two bacteria although Tayeb et al. (1984) recommended pH values of 6.5 and 5.5 for *S. thermophilus* and *L. bulgaricus* respectively.

The present work was carried out with pure cultures at controlled pH and temperature. It defines the influence of these two parameters on the growth characteristics of *S. thermophilus* 404 and *L. bulgaricus* 398, as well as on the production of the resulting lactic acid. An experimental design enabled the respective effects of each of the two parameters to be detected.

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Materials and methods

Strains and culture medium. The strains used were *S. thermophilus* 404 and *L. bulgaricus* 398, from the collection of the Centre National de Recherches Zootechniques (CNRZ), Jouy-en-Josas, France.

Inocula were prepared from a pure culture at controlled pH and temperature pH = 6.5, $T = 40°$ C for *S. thermophilus* and pH = 5.8, $T = 44°$ C for *L. bulgaricus*). They were harvested at the end of exponential growth, frozen and stored at −75° C. They were thawed at 40° C 1 h before inoculation which was then carried out at a concentration of about 10 bacterial/ml.

The culture medium was composed of mild whey (Bel Industrie, Paris, France) at 60 g/l, lactose (Prolabo, Paris, France) at 40 g/l, Bactopeptone (Difco, Detroit, Mich, USA) at 5 g/l, yeast extract (Difco) at 5 g/l and antifoam (Rhodorsil 426 R, Prolabo) at 1 ml/l. It was sterilized in the fermentor at 110° C for 20 min.

Table 1. Experimental conditions and growth and acidification characteristics of *Streptococcus thermophilus* 404 cultures; statistical analysis of experimental centre points

Test no.	Design matrix		Work matrix		Growth and acidification characteristics				
	pH_c	T_c	pH	T (°C)	X_M	μ_M	Y_M	AL_M	VA_M
S1	$-\alpha$	0	5.5	40.0	$5.90\ 10^7$	0.48	$1.31\ 10^9$	22.37	4.86
S2	α	0	7.5	40.0	$8.20\ 10^8$	1.68	$1.60\ 10^{10}$	24.77	5.94
S3	0	$-\alpha$	6.5	34.3	$6.50\ 10^8$	1.56	$1.65\ 10^{10}$	28.51	5.82
S4	0	$+\alpha$	6.5	45.7	$4.19\ 10^8$	1.56	$6.61\ 10^9$	34.13	11.40
S5	−1	−1	5.8	36.0	$9.59\ 10^7$	0.72	$1.70\ 10^{10}$	26.53	5.70
S6	−1	1	5.8	44.0	$2.98\ 10^8$	1.74	$6.00\ 10^{10}$	29.30	7.74
S7	1	−1	7.2	36.0	$2.40\ 10^9$	1.74	$4.20\ 10^{10}$	30.85	7.62
S8	1	1	7.2	44.0	$1.62\ 10^9$	1.62	$2.54\ 10^{10}$	35.12	10.86
S9-S13	0	0	6.5	40.0	$5.11\ 10^9$	1.88	$7.73\ 10^{10}$	33.53	9.22
	Standard deviation (σ)				$1.09\ 10^9$	9.11	$2.30\ 10^{10}$	1.84	0.49
	Coefficient of variation (σ/m)				0.21	0.06	0.29	0.055	0.05

Symbols: pH_c and T_c = coded values of pH and temperature; X_M = maximal bacterial population (cells/ml); μ_M = maximum growth rate (h^{-1}); Y_M = maximum growth yield on lactose (cells/g); AL_M = final lactic acid concentration (g/l); VA_M = maximal rate of lactic acid produciton (g/l per hour)

Cultures. Batch cultures were prepared in a 7-l fermentor (Sétric Génie Industriel, Toulouse, France), stirred at 200 rpm. Temperature and pH were respectively controlled from 34.7° to 49.7° C and from 4.8 to 7.5, depending on the experiment. The pH was controlled by adding 10 *N* sodium hydroxide, which was continuously weighed (Mettler balance, Viroflay, France) in order to follow changes in the process. Peristaltic pumps (Watson Marlow, Prolabo) were used to control pH and to remove samples.

Analytical methods. Growth was observed by microscopic counting according to the technique recommended by Oner and Erickson (1986). For each sample, the reported value is the mean of 60 fields.

Lactose, galactose and lactic acid were analysed by HPLC after precipitating proteins in the sample with trichloroacetic acid (12% v/v final) and centrifuging for 30 min at 2000 *g* at 4° C. The HPLC system (Waters Associates, Milford, Mass, USA) was composed of an automatic injector (710 B), a pump (510), an oven thermostatted at 35° C, a differential refractometer detector (410) and an integrator (740). The mobile phase was 0.01 *N* H_2SO_4. Analysis was made on a cation exchange column (Aminex Ion Exclusion HPX-87 H (300 × 7.8 mm), BioRad, Richmond, Calif, USA) operating at a flow-rate of 0.6 ml/min. Propionic acid (1%) was used as internal standard.

Glucose was assayed by the PAP enzymatic method (Biomérieux, Charbonnières les Bains, France).

Experimental design. For different types of microorganisms, several authors (Andreyeva and Biryukov 1973; Famelart et al. 1987; Lallai et al. 1988; Eroshin et al. 1976) have shown that specific growth rates and yields, as well as acidification rates, can be represented by second degree polynomial equations specific for the parameters studied (temperature, pH, dissolved oxygen, etc.).

The experimental design used here was the Central Composite Rotatable Design (CCRD) defined by Cochran and

Table 2. Experimental conditions and growth and acidification characteristics of *Lactobacillus bulgaricus* 398 cultures; statistical analysis of experimental centre points

Test no.	Design matrix		Work matrix		Growth and acidificaiton characteristics				
	pH_c	T_c	pH	T (°C)	X_M	μ_M	Y_M	AL_M	VA_M
L1	$-\alpha$	0	4.8	44.0	$8.40\ 10^7$	0.97	$5.70\ 10^9$	16.06	2.94
L2	α	0	6.8	44.0	$1.61\ 10^8$	0.78	$3.40\ 10^9$	19.38	4.14
L3	0	$-\alpha$	5.8	38.3	$4.60\ 10^8$	1.68	$1.12\ 10^{10}$	22.65	5.76
L4	0	$+\alpha$	5.8	49.7	$3.90\ 10^8$	1.56	$1.14\ 10^{10}$	26.24	5.82
L5	−1	−1	5.1	40.0	$4.80\ 10^8$	0.96	$3.05\ 10^{10}$	14.53	3.30
L6	−1	1	5.1	48.0	$3.40\ 10^8$	1.14	$1.36\ 10^{10}$	14.92	3.48
L7	1	−1	6.5	40.0	$1.80\ 10^9$	1.68	$4.50\ 10^9$	20.66	5.16
L8	1	1	6.5	48.0	$1.13\ 10^9$	2.10	$2.67\ 10^{10}$	23.07	4.62
L9-L13	0	0	5.8	44.0	$1.82\ 10^9$	2.15	$3.91\ 10^{10}$	28.22	6.28
	Standard deviation (σ)				$5.27\ 10^8$	0.19	$8.02\ 10^9$	2.29	0.57
	Coefficient of variation (σ/m)				0.21	0.09	0.205	0.08	0.09

Symbols as in Table 1

Cox (1957). It enables each explained variable to be represented by a linear quadratic model including pH and temperature, and to considerably reduce the number of experiments.

A multivariable second order linear regression, carried for each explained variable (Z) as a function of pH and of temperature (T) enabled polynomials of the following form to be established:

$$Z = K + a_1 \cdot \mathrm{pH} + a_2 \cdot T + a_{11} \cdot \mathrm{pH}^2 + a_{22} \cdot \mathrm{pH}^2 + a_{12} \cdot \mathrm{pH} \cdot T \quad (1)$$

The coefficients of each polynomial (K, a_1, a_2, a_{11}, a_{22}, a_{12}) were determined by the least squares method of fitting. The quality of the multivariable linear regression was estimated by the multiple correlation coefficient (R_M):

$$R_M = \frac{\sum (\hat{Z}_i - \bar{Z})^2}{\sum (Z_i - \bar{Z})^2} \quad (2)$$

where Z_i = experimental value of Z; $\bar{Z}$ = mean value of Z_i; $\hat{Z}_i$ = estimated value of Z_i calculated from Eq. 1; R_M represents the fraction of variability explained by Eq. 1.

A statistical analysis bearing on the coefficients of each polynomial was used to define their confidence level. Thus, only coefficients with a confidence level higher than 95% were retained.

The design used, shown on the left of Tables 1 and 2, includes five centre points of coded value (0, 0) enabling the experimental variance to be studied, and eight experimental points located on a circle of radius $\alpha = \sqrt{2}$. The values of each parameter assigned to the centre points were chosen from published data cited above, by situating near the presumed optima: $T_0 = 40°$ C and $\mathrm{pH}_0 = 6.5$ for *S. thermophilus* and $T_0 = 44°$ C and $\mathrm{pH}_0 = 5.8$ for *L. bulgaricus*. These centre points enabled the factorial points and extremes to be calculated with a step of 0.7 for pH and 4° C for temperature, using the equations:

$$\mathrm{pH} = \mathrm{pH}_0 \pm 0.7 \cdot \mathrm{pH}_c$$
$$T = T_0 \pm 4 \cdot T_c$$

with pH_c and T_c representing the coded values of pH and temperature. The 13 tests were randomized at the beginning of the experiment.

Definition and determination of growth and acidification characteristics. In order to characterize the growth of each strain and its acidifying activity as a function of pH and temperature, five explained variables were adopted: (1) maximal bacterial population X_M (cells/ml); (2) maximum growth rate μ_M (h^{-1}); (3) maximum growth yield on lactose Y_M (cells/g); (4) final lactic acid concentration AL_M (g/l); (5) maximal rate of lactic acid production VA_M (g/l per hour).

The bacterial population (X) determined by microscopic counting had a substantial dispersion, leading to a smoothing of the experimental points. The smoothing method chosen involved a linear regression according to the Weibull distribution (Lebreton and Millier 1982), applied to changes in the natural logarithm of the population ($\mathrm{Ln}\,X$). It enabled the fitted curve of $\ln X$ versus time to be plotted and to determine the value corresponding to the maximal population (X_M). The growth rate (μ) was determined by the derivative of the equation of the change in $\ln X$ versus time. The Y_M was calculated by considering the lactose concentration defined when the bacterial population was maximal (X_M). The variables concerning acidification (AL_M and VA_M) were determined after a similar smoothing of the experimental values.

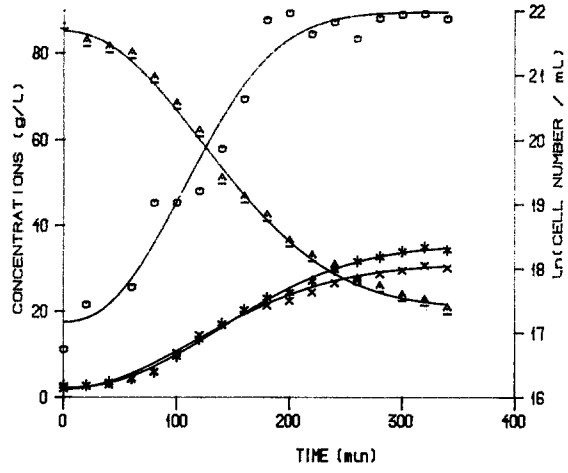

Fig. 1. Changes in carbohydrate and lactic acid concentrations associated with *Streptococcus thermophilus* 404 growth (pH = 6.5, T = 40° C): O, ln (cell number/ml); *, lactic acid concentration (g/l); △, lactose concentration (g/l); X, galactose concentration (g/l)

Results and discussion

Analysis of growth and acidification characteristics

Typical changes in the main parameters measured in pure cultures of *S. thermophilus* 404 and *L. bulgaricus* 398 are shown in Figs. 1 and 2. They show a more or less pronounced partial uncoupling between growth and acidification: the latter continued after growth ceased for *L. bulgaricus*. Furthermore, the accumulation of galactose not meta-

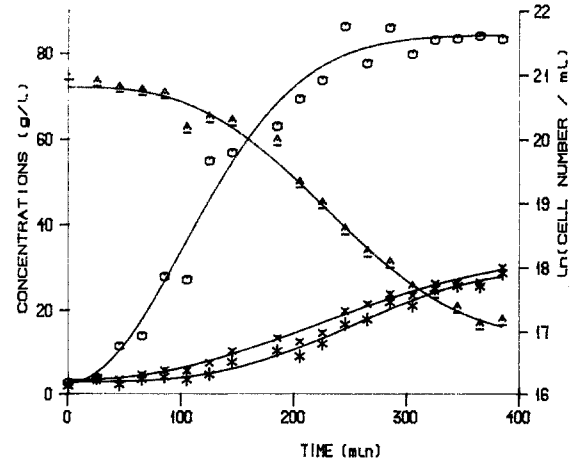

Fig. 2. Changes in carbohydrate and lactic acid concentrations associated with *Lactobacillus bulgaricus* 398 growth (pH = 5.8, T = 44° C): O, ln (cell number/ml); *, lactic acid concentration (g/l); △, lactose concentration (g/l); X, galactose concentration (g/l)

bolized by the bacteria was observed (O'Leary and Woychik 1976). Glucose concentrations were lower than 2 g/l throughout the culture, due to the rate of consumption being equal to that of its formation.

Tables 1 and 2 (right side) show the experimental values of the five explained variables of the experimental design, as well as the statistical analysis of the centre points (tests S9 to S13 and L9 to L13). They show that the growth characteristics were somewhat higher when the experimental conditions corresponded to the centre points. This observation is valid for the acidification characteristics (AL_M and VA_M) of *L. bulgaricus*; in the case of *S. thermophilus* these characteristics increased with temperature. These results agree with those of Radke-Mitchell and Sandine (1986).

The corresponding coefficients of variation for μ_M, AL_M and VA_M were satisfying, since they were lower than 10%. Those of X_M and Y_M, on the other hand, were much higher, 20%–30%. This effect was attributed to the inherent variability of the microscopic count. The comparison of coefficients of variation within each strain showed that variability was higher in *L. bulgaricus*, which can be explained by differences in morphology and polymorphism of the two strains (Accolas et al. 1980). The latter characteristic, particularly accentuated in lactobacilli, could explain the higher uncertainty observed.

Considering the mean values of the tests at the centre points, certain data can be noted: (1) growth stopped at around 2.10^9 to 5.10^9 cells/ml, i.e. at concentrations 2–3 times lower than those reported by Tayeb et al. (1984); this difference can be explained by the use of whey instead of milk; (2) maximum growth rates of the order of $2\ h^{-1}$, similar in both strains, agree with those reported by Tayeb et al. (1984); (3) maximum populations and yields in *S. thermophilus* were generally double those in *L. bulgaricus*. The acidification characteristics (AL_M and VA_M) of *S. thermophilus* were also higher (20%–50%).

Table 3. Coefficients of the polynomial describing growth and acidification characteristics of *S. thermophilus* 404 and coefficient of multiple correlation (R_M)

Polynomial parameters	Growth characteristics			Acidification characteristics	
	X_M	μ_M	Y_M	AL_M	VA_M
K	$5.11\ 10^9$	1.795	$8.22\ 10^{10}$	33.283	9.175
a_1	NS	NS	NS	1.692	0.821
a_2	NS	NS	NS	1.874	1.647
a_{11}	$-2.18\ 10^9$	−0.352	$-3.36\ 10^{10}$	−4.183	−1.657
a_{22}	$-2.13\ 10^9$	NS	$-3.22\ 10^{10}$	NS	NS
a_{12}	NS	NS	NS	NS	NS
R_M	0.92	0.83	0.91	0.90	0.94

Symbols as in Table 1; NS = not significant

Table 4. Coefficients of the polynomial describing growth and acidification characteristics of *L. bulgaricus* 398 and coefficient of multiple correlation (R_M)

Polynomial parameters	Growth characteristics			Acidification characteristics	
	X_M	μ_M	Y_M	AL_M	VA_M
K	$1.82\ 10^9$	2.156	$3.91\ 10^{10}$	28.224	6.016
a_1	NS	NS	NS	2.372	0.587
a_2	NS	NS	NS	NS	NS
a_{11}	$-5.78\ 10^8$	−0.579	$-1.44\ 10^{10}$	−5.950	−1.451
a_{22}	$-5.32\ 10^8$	−0.213[a]	$-1.11\ 10^{10}$	−2.587	NS
a_{12}	NS	NS	$9.77\ 10^9$	NS	NS
R_M	0.76	0.83	0.90	0.91	0.91

[a] Significance level 90%
Symbols as in Table 1; NS = not significant

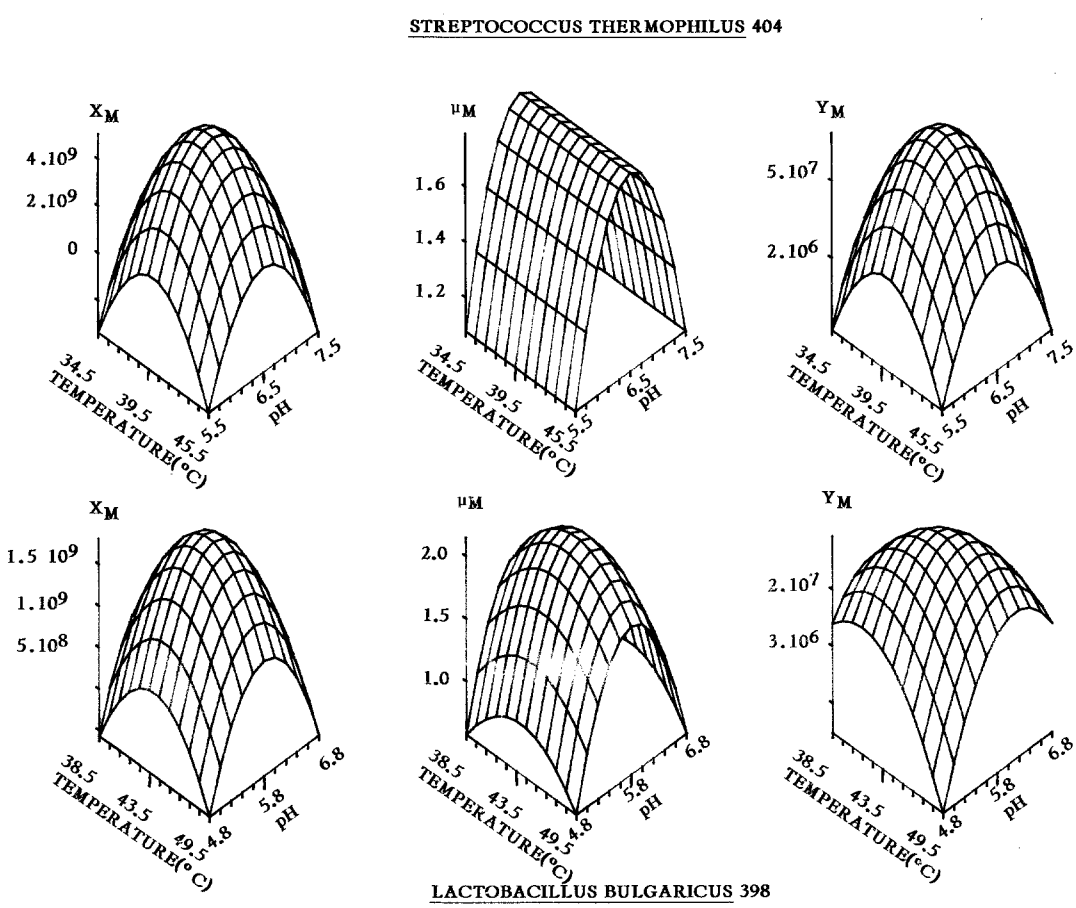

Fig. 3. Response surface representation of the effect of controlled temperature and pH on the growth characteristics of *S. thermophilus* 404 and *L. bulgaricus* 398: X_m = maximal bacterial population (cells/ml); μ_M = maximum growth rate (h^{-1}); Y_M = maximum growth yield on lactose (cells/g)

Effect of pH and temperature on growth and acidification characteristics

The coefficients of the polynomials (K, a_1, a_2, a_{11}, a_{22} and a_{12}) describing the five explained variables (growth and acidification characteristics) are listed in Tables 3 and 4. The values of these coefficients generate the following observations: (1) for the variables characterizing growth, only the coefficients of the quadratic terms were significant at the 5% threshold; the other terms were not significant (NS); (2) the statistical analysis of the coefficients assigned to the acidification characteristics for *S. thermophilus* show a clear influence of the linear terms for pH and T and of the quadratic term for pH; for *L. bulgaricus*, the effect of pH was similar, but that of temperature was quadratic for AL_M and null for VA_M; (3) except for one case, there was no interaction of the form $pH \cdot T$ between temperature and pH (coefficients not significant); (4) the multiple correlation coefficients (R_M) were acceptable (always higher than 0.83, except for X_M in the case of *L. bulgaricus*).

The three-dimensional representation of these polynomials within the experimental domain enables visualization of the response surfaces, which show the conjugated effect of pH and temperature. The response surfaces concerning the characteristics of growth and acidification are shown in Figs. 3 and 4. The maximum growth rate μ_M (Fig. 3) of both strains was much more sensitive to pH than to temperature. This is especially marked for *L. bulgaricus*. On the other hand, the sensitivity of the two strains for X_M and Y_M was equivalent.

The response surfaces characteristic of acidification (Fig. 4) show a slight shift in pH optima in relation to the centre point of the experimental designs. The effect of temperature on *L. bulgaricus* was less clear-cut than on *S. thermophilus*.

The weak effect of temperature on the explained variables does not mean that they are not

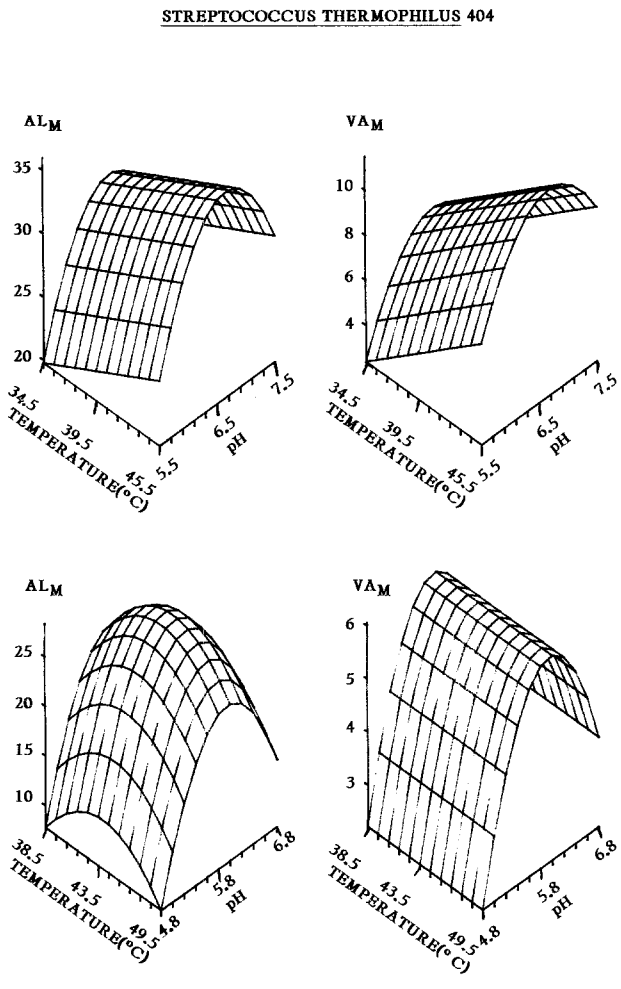

Fig. 4. Response surface representation of the effect of controlled temperature and pH on the acidification characteristics of *S. thermophilus* 404 and *L. bulgaricus* 398: AL_M = final lactic acid concentration (g/l); VA_M = maximal rate of lactic acid production (g/l per hour)

Table 5. Optimum pH and temperature of *S. thermophilus* 404 and *L. bulgaricus* 398 cultures

Growth and acidification characteristics	*S. thermophilus* 404		*L. bulgaricus* 398	
	T (°C)	pH	T (°C)	pH
X_M	40.0	6.50	44.00	5.80
μ_M	—	6.50	44.00[a]	5.80
Y_M	40.0	6.50	43.95	5.78
AL_M	45.7[b]	6.64	44.00	5.94
VA_M	45.7[b]	6.67	—	5.94

[a] Significance level 90%
[b] Extreme value of the experimental design
Symbols as in Table 1

subjected to the effect of this parameter. Rather, this means that in the experimental domain chosen, the effect of temperature is not very visible, since it is overshadowed by the measurement variability or is impossible to detect in statistical analysis. Only a broader temperature range would have enabled the effect of this parameter to be quantified. This confirms and justifies a posteriori the relative broadness of the temperature ranges proposed by the authors cited above.

Determination of pH and temperature optima of S. thermophilus and L. bulgaricus

For each explained variable, the coordinates of the pH and temperature optima were determined by annulling the partial derivatives of the polynomial in relation to each of these factors. The results are listed in Table 5. It is seen that the optima of the growth characteristics (X_M, μ_M and Y_M) are practically identical, coinciding with the centre points of both experimental designs. In comparison with *L. bulgaricus*, the optimal temperature of *S. thermophilus* is 4° C lower and the pH optimum is 0.7 unit higher.

The pH and temperature optima of the acidification characteristics differ from those of growth: they are consistently higher by 0.1 to 0.2 pH units and by about 5° C, except in the case of *L. bulgaricus*, for which the optimal temperature is in all cases equal to 44° C.

The pH and temperature optima resulted directly from the polynomials determined previously and so are subjected to their variability. As a result, the calculated optimal values, even though defined in an exact manner, would be better reflected by pH or temperature intervals within which the optimal conditions of growth and acidification would be reached. These intervals are reasonably of the order of 1°–3° C and 0.1–0.2 pH units.

Finally, the differences observed between the optimal pH and temperature of each strain will pose a problem for mixed cultures. In the case of the production of mixed starters, the choice of the culture temperature and pH will favour one population over the other.

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