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Study of the effects of temperature, pH, NaCl, and a_w on the proteolytic and lipolytic activities of cheese-related lactic acid bacteria by quadratic response surface methodology

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Abstract

The individual and interactive effects of temperature, pH, NaCl, and a_w on the proteolytic and lipolytic activities of *Lactobacillus delbrueckii* subsp. *bulgaricus* B397, *Lactococcus lactis* subsp. *lactis* T12, and *Lb. plantarum* 2739 were studied by quadratic response surface methodology. The effects on enzyme activities depended on the interactions among the independent variables, type of activity, substrate and, especially, species. The proteinase activity of strains B397 and T12 was affected differently by pH as individual or interactive terms depending on the type of substrate α_{s1}- or β-casein. The increase of NaCl concentration (2.5–7.5%) under cheese-like conditions had a negative effect on the proteinase activity of strain T12. The effect of NaCl was related to the corresponding decrease in a_w. Aminopeptidases N and A, iminopeptidase and endopeptidase of *Lc. lactis* subsp. *lactis* T12 were strongly inhibited by pH 5–6 and NaCl concentration higher than 3.75%. The negative effects of these independent variables was in several cases enhanced by their interaction and/or by the interaction with the lowest temperatures. In contrast, the same peptidases of *Lb. plantarum* 2739 retained a high activity under the very hostile environmental conditions. Iminopeptidase and especially endopeptidase activities of strain 2739 were stimulated slightly by NaCl at concentrations up to 5%. Lipase/esterase activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 was markedly inhibited under cheese-like conditions.

Keywords: Cheese ripening; Proteolytic enzymes; Lipolytic enzymes; Lactic acid bacteria; Non-starter lactic acid bacteria; Response surface methodology

1. Introduction

Proteolysis and lipolysis are the most significant biochemical events that occur during cheese ripening. Starter and nonstarter lactic acid bacteria (NSLAB) contribute markedly to the ripening of several cheeses. After primary proteolysis of caseins by residual coagulant and to a lesser extent by plasmin, microbial proteinases, and peptidases cause the secondary proteolysis which produces small peptides and amino acids. Amino acids not only contribute directly to the cheese flavor but also act as precursors of other important flavor and aroma components [1]. Owing to the low taste threshold of some fatty acids, the large number of weakly lipolytic micro-organisms, such as lactic acid bacteria, has also been shown to play a moderate role in lipolysis of long-ripened cheeses [2–3].

Proteolysis by dairy lactic acid bacteria has been studied by genetic, biochemical, and ultrastructural methods (for reviews see [1,4]). Esterases and lipases have been purified and characterized biochemically [2–3,5–6].

Cheese flavor may be improved by using selected strains which well adapt to the cheese environment. *Lactobacillus* spp., NSLAB, and dairy lactococcal strains isolated from commercial starters, curds, and fresh cheeses have been screened based on several biochemical and technological characters [7–8], and simple tests for predicting the lytic behavior and proteolytic activity of lactococcal strains in cheese have been proposed [9]. The influence of NaCl,

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$CaCl_2$, and reduced pH has been determined under simulated cheese-like conditions for aminopeptidase, lipase/esterase, and the methanethiol-producing capability of selected lactic acid bacteria and brevibacteria [10–11]. Although elevated ripening temperatures and the inclusion of exogenous enzymes, chemically or physically modified cells, genetically modified starters, adjunct cultures, and cheese slurries are proposed [12], the selection of starter strains based on the adaptability of their enzymes to the cheese environment could be the easiest and most productive way to accelerate cheese ripening.

To our knowledge, quadratic response surface methodology has been not applied to study the combined effects of cheese-like environment on enzymes of cheese-related bacteria. Study of the individual and interactive effects of temperature, pH, NaCl, and water activity (a_w) should be helpful to understanding the true enzyme potentialities and to differentiate the behavior of starter and NSLAB in cheese ripening.

Thermophilic lactobacilli (e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) are common starter bacteria used in hard Italian and Swiss cheeses, whereas lactococci are used to produce cheeses the curds of which are cooked at low temperature and are ripened for a medium period, e.g. cheddar. Mesophilic homofermentative lactobacilli (e.g. *Lb. casei* and *Lb. plantarum*) are the principal NSLAB in several cheeses. They either survive pasteurisation or enter the cheese milk or curd as postpasteurisation contaminants and may play an important role in flavor development during secondary ripening [13].

This communication describes the individual and interactive effects of temperature, pH, NaCl, and a_w on the proteolytic and lipolytic activities of *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lactococcus lactis* subsp. *lactis* T12 and *Lb. plantarum* 2739 by using the quadratic response surface methodology.

2. Materials and methods

2.1. Strains of lactic acid bacteria

Lb. delbrueckii subsp. *bulgaricus* B397, *Lc. lactis* subsp. *lactis* T12 (Culture Collection of the Institute of Dairy Microbiology, Perugia, Italy), and *Lb. plantarum* 2739 (Culture Collection of the University College Cork, Cork, Ireland) were cultivated twice in MRS broth (Oxoid, Basingstoke, UK) at 40 and 30°C, and in M17 broth (Oxoid) at 30°C for 18 h, respectively. Three liters of the 18-h cells were harvested by centrifugation at 10 000 × *g* for 10 min at 4°C, washed twice with 50 mM sodium acetate buffer, pH 6.4, and used for subcellular fractionation. No proteolytic activities were detected in the cell-free culture media.

2.2. Preparation of cell-wall lysate and cytoplasmic extract

Aliquots of washed cell pellets (0.3 g of dry weight) of each lactic acid bacteria species were resuspended in 50 mM Tris-HCl, pH 7.0, incubated at 30°C for 30 min and centrifuged at 13 000 × *g* for 10 min at 4°C to remove loosely associated cell-surface enzymes. The cell-wall lysate and the cytoplasmic extract were prepared by lysozyme treatment in 50 mM Tris-HCl, pH 7.5, buffer containing 24% sucrose, as described by Crow et al [14]. The only modification was that the sphaeroplasts were resuspended in isotonic buffer and sonicated for 40 s at 16 amps/s (Sony Prep, Model 150, Sanyo, UK). Cell-wall lysate and loosely associated cell surface enzyme fraction were pooled to give the cell-envelop-associated fraction.

Cytoplasmic extract and cell-wall-associated fractions were freeze-dried (Edwards MOD E1PTB, Edwards, Milan, Italy), concentrated 10-fold by resuspending in 5 mM Tris-HCl, pH 7, and dialysed for 24 h at 4°C.

The cell-envelop-associated fraction contained proteinase activity and no peptidase activities. The cytoplasmic extract was used to determine general aminopeptidase (PepN) and lipase/esterase (LE) activities.

2.3. Partial purification of peptidases

To partially purify glutamyl aminopeptidase (PepA), proline iminopeptidase (PIP), and endopeptidase (PepO), aliquots (50 mg) of the freeze-dried cytoplasmic extracts of each lactic acid bacteria were separately resuspended in 50 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl and applied to a Q-Sepharose HR 16/50 column (Pharmacia-LKB Biotechnology, Uppsala, Sweden), that had been equilibrated with 50 mM phosphate buffer, pH 7, containing 0.1 M NaCl. Proteins were eluted at a flow rate of 12 ml/h with a linear NaCl gradient from 0.1–0.5 M. Fractions with PepA, PIP, and PepO activities and without or very low other peptidase activities were pooled, dialyzed for 24 h at 4°C against 5 mM Tris-HCl, pH 7, concentrated 20-fold by freeze drying, and resuspended in the same buffer.

2.4. Protein determination

Protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as standard.

2.5. Enzyme assays

Cell envelope proteinase (CEP) activity was measured by the method of Twinning [15] using fluorescent α_{s1}- and β-caseins (CN) as substrates. One unit of CEP activity was expressed as an increase of 0.1 unit of fluorescens/10 min.

PepN, PepA, and PIP activities were determined by using Lys- and Ala-, Glu-, and Pro-*p*-nitroanilide (*p*-NA)

substrates (Sigma, St. Louis, MO, USA), respectively. The assay mixture contained 20 μl of 20 mM *p*-NA derivative, 130 μl of the appropriate buffer, 60 μl of NaN_3 solution (0.05% final concentration) and 50 μl of the whole or partially purified cytoplasmic extract. After incubation, the reaction was stopped by adding 0.6 ml of 10% acetic acid. Samples were centrifuged (12 000 × *g* for 5 min) and the absorbance measured at 410 nm. One unit of PepN, PepA, and PIP activities corresponded to an increase of absorbance of 0.01 per 1 and 5 min, respectively.

PepO activity was measured by the modified method of Orlowski and Wilk [16]. N-CBZ-Gly-Gly-Leu-Leu-*p*-NA was used as substrate in a coupled reaction with leucine aminopeptidase (Sigma). The incubation mixture contained 10 μl of 30 mM N-CBZ-Gly-Gly-Leu-Leu-*p*-NA, 10 μl of leucine aminopeptidase (0.015 units), 130 μl of the appropriate buffer, 60 μl of NaN_3 solution (0.05% final concentration), and 50 μl of the partially purified cytoplasmic extract. After incubation, the reaction was developed as above reported. One unit of PepO activity corresponded to an increase of absorbance of 0.01 per 5 min.

LE activity was measured by using β-naphthyl (β-NA) butyrate (C4) as substrate. The assay mixture was the same as for aminopeptidase activities except that tap water containing 200 μg/ml cloramphenicol was used instead of the NaN_3 solution. After incubation, color was developed by addition of 0.6 ml of Fast Garnet GBC (Sigma) preparation (5 mg/ml in 10% sodium dodecyl sulfate) and further incubation at room temperature for 15 min. The absorbance was measured at 560 nm. LE activity was expressed as 1 μmoles of β-naphthol released from β-NA butyrate per min.

Specific enzyme activities were expressed as the number of units (U) per mg protein. The pH of the assay mixtures was varied using a universal buffer composed of boric acid (57 mM), citric acid (33 mM), NaH_2PO_4 (33 mM), NaOH (1 M), and varying amounts of 0.1 M HCl. NaCl was first dissolved in the aliquot of buffer used in the assay mixture. The concentration of NaCl used was 2.5, 3.75, 5.0, 6.25, and 7.5%, which corresponded to an a_w of the assay mixture of ca. (±0.002) 0.990, 0.977, 0.965, 0.952, and 0.940, respectively. Both the NaCl concentration and correspondent a_w are the values frequently found in cheeses [17]. Glycerol at the concentrations of 5, 10, 15, 20, or 25% gave approximately the same a_w values in the assay mixture. After preliminary assays, this compound was used to reduce a_w because it had no effect on enzyme activities.

2.6. pH and temperatura optima

The pH optimum of the enzyme activity by lactic acid bacteria was examined in the pH range 5 to 10 by using the universal buffer. The temperature optimum was determined at pH 7, in the range 20 to 55°C.

Table 1
Composition of the various runs of the two Central Composite Design

Run	Temperature (°C)	pH (unit)	NaCl (%)	Glycerol (%)
1	7	5.5	3.75	10
2	7	6.5	3.75	10
3	7	5.5	6.25	20
4	7	6.5	6.25	20
5	13	5.5	3.75	10
6	13	6.5	3.75	10
7	13	5.5	6.25	20
8	13	6.5	6.25	20
9	10	6.0	5.0	15
10	10	6.0	5.0	15
11	10	5.0	5.0	15
12	10	7.0	5.0	15
13	10	6.0	2.5	5
14	10	6.0	7.5	25
15	4	6.0	5.0	15
16	16	6.0	5.0	15
17	10	6.0	5.0	15

Central Composite Design 1 included temperature, pH and NaCl as independent variables; Central Composite Design 2 substituted glycerol for NaCl.

2.7. Experimental design

The effects of pH, NaCl, and temperature on the enzyme activities of *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lc. lactis* subsp. *lactis* T12 and *Lb. plantarum* 2739 were determined by modulating the variables according to a three-factor, five-level Central Composite Design (CCD). The 17 combinations used for each enzyme activity are shown in Table 1. The central composite design reduces the number of possible combinations to a manageable size, because it uses only a fraction of the total number of factor combinations for experimentation. In statistical literature, this technique is known as confounding [18]. Two replicates of each combination were used.

In order to evaluate whether the effects of NaCl on enzyme activities was linked to its presence or to the reduction of a_w, another CCD which included glycerol instead NaCl was considered (Table 1).

2.8. Statistical analysis

Statistical analysis was aimed at describing the enzyme activities of *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lc. lactis* subsp. *lactis* T12 and *Lb. plantarum* 2739 as functions of the independent variables of the CCD. A software package (Statistica for Windows, Statsoft, Tulas, USA) was used to fit the second order model to the independent variables by using the following equation:

$$\gamma = \sum B_i \chi_i + \sum B_{ii} \chi_i^2 + \sum B_{ij} \chi_i \chi_j$$

where γ is the dependent variable (enzyme activity) to be modeled, B_i, B_{ii}, and B_{ij} are regression coefficients of the model, and χ_i and χ_j are the independent variables in coded

Table 2
Characteristics of the enzyme activities of *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lc. lactis* subsp. *cremoris* T12 and *Lb. plantarum* 2739

Enzyme activities	Substrate	Optimum pH	Optimum temperature (°C)	Specific activity
CEP[a] (B397)[b]	α_{s1}-CN	6.5	45	76
CEP (T12)		6.5–7.0	40	112
CEP (2739)		8.0	45	58
CEP (B397)	β-CN	6.5	45	80
CEP (T12)		6.5–7.0	40	412
CEP (2739)		8.0	45	48
PepN (B397)	Lys-*p*-NA	7.0	40	644
PepN (T12)		7.0	35–40	212
PepN (2739)		7.0	35–40	242
PepN (B397)	Ala-*p*-NA	7.0	40	388
PepN (T12)		7.0	35–40	187
PepN (2739)		6.5–7.0	35–40	342
PepA (B397)	Glu-*p*-NA	7.0	40	334
PepA (T12)		7.0	40–45	200
PepA (2739)		7.0	35	217
PIP (B397)	Pro-*p*-NA	7.0	40	75
PIP (T12)		8.0	40	88
PIP (2739)		35	80	
PepO (B397)	N-CBZ[c]	7.0	40	80
PepO (T12)		6.0–6.5	35	255
PepO (2739)		7.0	40	102
LE (B397)	β-NA-C4	7.0	40	86
LE (T12)		6.5–7.0	35–40	288
LE (2739)		7.0–7.5	35	514

[a] For abbreviations of enzymes, see Section 2.
[b] Strains are indicated with the collection number reference.
[c] N-CBZ-Gly-Gly-Leu-Leu-*p*-NA.

values. The variables with a significance lower than 95% ($p > 0.05$) were not included in the final models. This model permitted evaluation of the effects of linear, quadratic, and interactive terms of the independent variables on the chosen dependent variables. Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones.

Table 3
Experimental results (two replicates) of the CEP activity of *Lc. lactis* subsp. *cremoris* T12 on β-casein

Run	Specific activity	
1	63.2	61.8
2	79.6	77.0
3	33.8	32.6
4	42.6	40.72
5	80.2	78.12
6	105.0	103.2
7	49.6	50.4
8	71.0	70.68
9	75.5	74.5
10	70.5	71.5
11	31.3	30.34
12	73.1	72.7
13	74.0	76.0
14	48.5	51.5
15	0.0	0.0
16	160.5	157.5
17	71.5	72.5

3. Results

Maxima of the specific enzyme activities under optimum conditions are shown in Table 2. The pH and temperature optima were generally in agreement with those reported by other authors for the same strains (for reviews, see [1,4,19]). All the proteolytic and lipolytic activities were optimal in the pH range 6 to 8 and at 35–45°C. Although strains differed in the specific enzyme activities, the objective of the study was to compare the sensitivity of their enzymes to levels of temperature, pH, NaCl, and a_w, which are characteristic of the cheeses during ripening. Polynomial equations describing the main, interactive, and quadratic effects of independent variables on enzymes of *Lb. delbrueckii* subsp. *bulgaricus* B397, *Lc. lactis* subsp. *lactis* T12, and *Lb. plantarum* 2739 are reported in Tables 4–6, respectively.

The CCD applied and related equations produced 73 figures, only the most significant are shown. When one of the three independent variables was not shown in the graphs, a constant value (the central points of the considered interval) was imposed on it. Being the nominal region of the explanatory variables bigger than the three-dimensional domain enclosed in the experimental designs, the predictions at the extreme levels of the chosen factors may be extrapolations. However, what CCD makes possible is to investigate the shape of the response variable which identified with the goal of this study and which has a technological significance. To show the differences which characterize the raw data of our enzyme assay, experimental results of CEP activity of *Lc. lactis* subsp. *lactis* T12 on β-CN are listed, as an example, in Table 3.

3.1. Proteinase activity

According to Table 3, the CEP activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 on α_{s1}-CN was significantly affected only by the pH both as quadratic and interactive term with temperature. NaCl did not significantly affect the activity. Hydrolysis of α_{s1}-CN was not detected at 4°C, but was markedly enhanced by increasing the temperature from 7 to 16°C. Only at 16°C, a slight effect of pH was found (Fig. 1a). The proteinase of *Lb. plantarum* 2739 similarly behaved (Table 6). The hydrolysis of α_{s1}-CN by *Lc. lactis* subsp. *lactis* T12 was considerably higher than that by the other two strains (Table 2); it possesses a P_{III} type proteinase. The activity was affected by temperature as linear and interactive terms, and by pH as quadratic term (Table 5). Increasing the temperature always had a positive effect, but at pH 5.0–5.5, the hydrolysis of α_{s1}-CN was very low (ca. 12 U/mg at 16°C, which corresponded to 10% of maximum activity) compared to that at pH 7.0 (ca. 42 U/mg, 37% of

Table 4
Best-fit equations for the effects of the different variables on the enzyme activities of *Lb. delbrueckii* subsp. *bulgaricus* B397 (only terms with $p < 0.05$ were included)

Enzyme activity	Equation[a]	R^2[b]	F[c]	SE[d]
CEP[e] on α_{sl}	$0.0760[pH][T] - 0.065[pH]^2$	0.968	113.85	0.7403
CEP on β	$-0.953[pH] + 0.191[pH][T] - 0.031[T]^2$	0.973	82.54	0.774
CEP on β[f]	$-0.546[pH] + 0.093[pH][T]$	0.981	191.88	0.599
PepN	$2.538[pH][T] - 1.646[NaCl]^2$	0.985	253.73	21.92
PepN[f]	$-35.83[T] + 8.9722[pH][T] - 0.409[gly][pH]$	0.993	371.77	21.21
PepN[g]	$-25.817[pH] + 2.842[pH][T] + 2.935[pH]^2 - 0.816[NaCl]^2 - [0.46T]^2$	0.992	151.33	8.87
PepA	$2.876[pH][NaCl] - 2.814[NaCl][T] + 1.123[T]^2$	0.945	38.70	28.55
PIP	$0.349[T] - 0.0419[NaCl][T] + 0.046[pH]^2$	0.958	51.11	1.04
PepO	$-4.068[pH] + 0.795[pH]^2 + 0.0547[T]^2$	0.971	76,08	3.04
LE	$0.488[pH][T] - 0.295[NaCl][T]$	0.950	70.93	5.44
LE[f]	$-7.290[T] + 1.591[pH][T] - 0.0089[gly]^2$	0.994	420.13	2.65

[a] [T], temperature (°C); [pH], pH value; [NaCl], NaCl concentration (%); [gly], glycerol concentration (%).
[b] Regression coefficient.
[c] F-value.
[d] Standard error of residuals.
[e] For abbreviations of enzymes see Section 2.
[f] Enzyme activity determined at the same a_w value but with glycerol substituted for NaCl.
[g] Enzyme activity determined on Ala-*p*-NA as substrate.

maximum activity) (Fig. 1b and Table 2). As shown in Fig. 1c, the CEP activity of strain T12 was also reduced when the NaCl concentration was increased. The negative sign of the interaction between NaCl and temperature (Table 5) indicates that the negative effect of NaCl increased when temperature decreased; at 10°C and pH 6, activity was inhibited with a NaCl concentration ranging from 3.75 to 7.5%.

Like the hydrolysis of α_{sl}-CN, CEP activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 on β-CN was affected by the pH as an individual and interactive term with temperature, which also appeared as quadratic term (Table 4). Comparison of Figs. 1a and 2a shows that the activity on β-CN depended more on variations in pH. Also for *Lc. lactis* subsp. *lactis* T12 pH sensitivity varied with the CN fraction (Table 5). In this case, the negative effect of reducing the pH was lower than for α_{sl}-CN hydrolysis (Fig. 2b vs. 1b). Temperature was the independent variable that had the greatest effect: activity at pH 7 and 5% NaCl varied from ca. 40 to 140 U/mg (9 to 34% of the maximum) on increasing the temperature from 10 to 16°C (Fig. 2b and Table 2). The negative effect of the highest concentrations of NaCl on the

Table 5
Best-fit equations for the effects of the different variables on the enzyme activities of *Lc. lactis* subsp. *lactis* T12 (only terms with $p < 0.05$ were included)

Enzyme activity	Equation[a]	R^2[b]	F[c]	SE[d]
CEP[e] on α_{sl}	$-3.744[T] + 1.151[pH][T] - 0.167[NaCl][T] - 0.303[pH]^2$	0.970	51.50	4.23
CEP on β	$11.83[pH] - 9.903[NaCl] + 0.415[T]^2$	0.974	89.15	18.21
CEP on β[f]	$1.70[pH][T] - 0.22[gly][T]$	0.970	119.88	19.43
PepN	$-14.87[pH] - 0.413[pH][NaC]l + 2.82[pH]^2 + 0.103[T]^2$	0.959	37.49	5.35
PepN[f]	$-15.67[T] - 0.615[pH][gly] + 2.171[pH]^2 + 0.941[T]^2$	0.964	62.52	9.00
PepN[g]	n.d.[h]			
PepA	$0.541[pH][T] - 0387[NaCl][T]$	0.961	21.49	9.27
PIP	$-4.791[pH] + 0.783[T] - 0.193[pH][NaCl] + 0.985[pH]^2$	0.978	71.33	2.398
PepO	$-4.364[pH] + 1.492[T] - 0.112[NaCl][T] + 0.174[pH]^2$	0.976	65.76	2.47
Esterase	$1.52[pH][T] - 0.563[NaCl]^2 - 0.281[T]^2$	0.980	112.91	10.76
Esterase[f]	$1.138[pH][T] - 0.188[T]^2$	0.982	208.88	9.93

[a] [T], temperature (°C); [pH], pH value; [NaCl], NaCl concentration (%); [gly], glycerol concentration (%).
[b] Regression coefficient.
[c] F-value.
[d] Standard error of residuals.
[e] For abbreviations of enzymes see Section 2.
[f] Enzyme activity determined at the same a_w value but with glycerol substituted for NaCl.
[g] Enzyme activity determined on Ala-*p*-NA as substrate.
[h] n.d., activity not detected on this substrate.

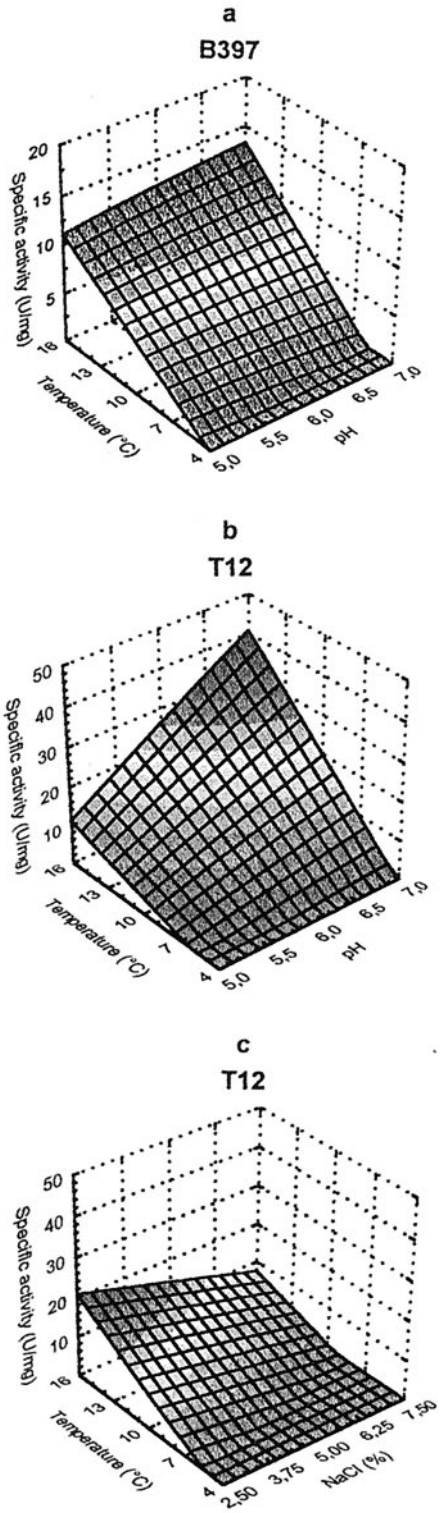

Fig. 1. CEP activity on α_{s1}-casein. Three dimensional plots of the interactions of temperature × pH (a) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × pH (b) and temperature × NaCl (c) on the activity of *Lc. lactis* subsp. *lactis* T12.

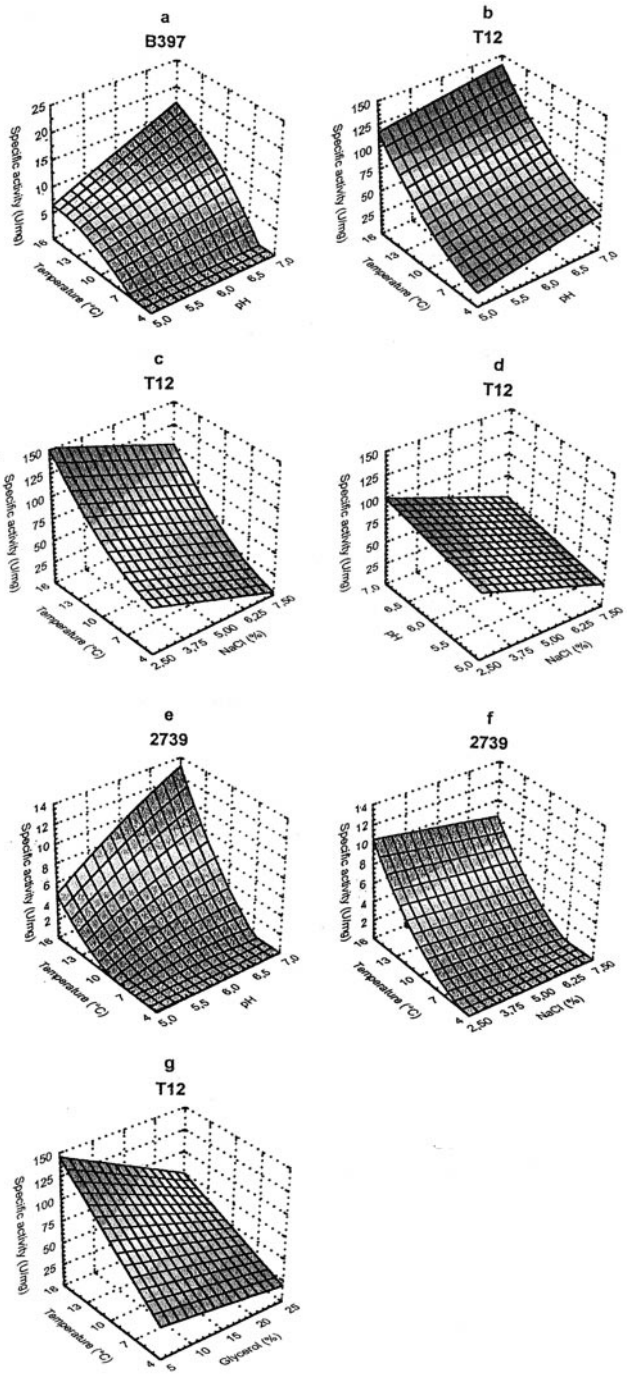

Fig. 2. CEP activity on β-casein. Three dimensional plots of the interactions of temperature × pH (a) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × pH (b), temperature × NaCl (c), pH × NaCl (d) and temperature × glycerol (g) on the activity of *Lc. lactis* subsp. *lactis* T12; temperature × pH (e) and temperature × NaCl (f) on the activity of *Lb. plantarum* 2739.

CEP activity of strain T12 was confirmed (Fig. 2c and d). The CEP activity of *Lb. plantarum* 2739 was influenced by all the independent variables of the CCD (Table 6). At 10°C, the hydrolysis of β-CN was almost totally inhibited, independently of the pH and NaCl concentration (Fig. 2e and f). At the highest values of temperature and pH, NaCl affected the activity only slightly (Fig. 2f).

Comparison of Fig. 2c and g, seems to suggest that elevated concentrations of NaCl negatively affected the

Table 6
Best-fit equations for the effects of the different variables on the enzyme activities of *Lb. plantarum* 2739 (only terms with $p < 0.05$ were included)

Enzyme activity	Equation[a]	R^2[b]	F[c]	SE[d]
CEP[e] on α_{s1}	$0.089[pH][T] - 0.082[pH]^2$	0.949	68.45	1.00
CEP on β	$1.46[pH] - 1.45[T] + 0.22[pH][T] - 0.014[NaCl][T] - 0.2397[pH]^2 + 0.0304[T]^2$	0.996	273.55	0.203
PepN	$2.129[pH][T] - 1.235[NaCl]^2$	0.983	218.67	20.45
PepN[f]	$1.987[pH][T]$	0.979	374.9	26.58
PepN[g]	$5.547[T]$	0.936	115.10	22.20
PepA	$1.161[pH][T] - 0.953[NaCl][T]$	0.976	119.77	14.09
PIP	$0.352[pH][NaCl] + 0.360[pH][T] - 0.325[NaCl]^2$	0.995	503.55	2.61
PepO	$-12.39[pH] + 6.987[NaCl] + 3.619[T] + 1.45[pH]^2 - 0.658[NaCl]^2 - 0.045[T]^2$	0.997	369.51	2.02
Esterase	$5.022[pH] + 0.604[T]^2$	0.968	112.48	28.08
Esterase[f]	$9.384[pH] - 9.250[T] + 0.567[T]^2$	0.965	63.96	8.01

[a] [T], temperature (°C); [pH], pH value; [NaCl], NaCl concentration (%); [gly], glycerol concentration (%).
[b] Regression coefficient.
[c] F-value.
[d] Standard error of residuals.
[e] For abbreviations of enzymes see Section 2.
[f] Enzyme activity determined at the same a_w value but with glycerol substituted for NaCl.
[g] Enzyme activity determined on Ala-*p*-NA as substrate.

activity of *Lc. lactis* subsp. *lactis* T12 CEP only via its effect on a_w, because reducing the a_w to the same values by adding increasing amounts of glycerol inhibited the hydrolysis of β-CN to about the same extent.

3.2. Activity of aminopeptidase N and A

Lb. delbrueckii subsp. *bulgaricus* B397 had the highest PepN and PepA activities under optimal conditions (Table 2). The equation relative to PepN activity showed an interactive effect between pH and temperature (Table 4). At pH 5 and 5% NaCl, the increase in activity due to the increase of temperature from 4 to 16°C was considerable (ca. 160 U/mg, 24% of the maximum activity), although the increase was smaller than those at pH 7.0 (ca. 245 U/mg, 38% of the maximum activity; Fig. 3a and Table 2). NaCl appeared in the equation as a quadratic term, it was optimal at the lowest percentage used and only partially inhibitory at the highest concentration (Fig. 3b and c). Except for a lower effect of NaCl, a similar response was observed for the PepN activity of *Lb. plantarum* 2739 (Table 6). The PepN activity of *Lc. lactis* subsp. *lactis* T12 differed from that of the other two species especially because of the greatest influence of pH which appeared in the equation of Table 4 both as an individual and interactive term with NaCl. Also at 16°C and 5.0% NaCl there was no activity on Lys-*p*-NA when the pH was 5 to 6 (Fig. 3d). Likewise, at pH 5–5.5 and 10°C, the activity was almost fully inhibited in the presence of all the NaCl concentrations studied (Fig. 3e).

As shown for CEP activity (Fig. 2), when glycerol was substituted for NaCl, the same a_w of 0.94 (corresponding to 25% glycerol) had about the same negative influence on the PepN activity of both strains B397 and T12 (data not shown).

When an aliphatic (Ala) instead of a basic (Lys) *p*-NA derivative was used as substrate, the sensitivity of PepN of *Lb. delbrueckii* subsp. *bulgaricus* B397 differed. The equation was more complex (Table 4) and the influence of the pH was greater (Fig. 3a vs. f). In our CCD, the cytoplasmic extract of *Lc. lactis* subsp. *lactis* T12 had detectable activity on Ala-*p*-NA only at 16°C, pH 6 and 5.0% NaCl (data not shown). PepN activity of *Lb. plantarum* 2739 on Ala-*p*-NA was not significantly affected by pH and NaCl (Table 6). At 13°C, pH 5 and 5–7.5% NaCl it retained 41% of the maximum activity (data not shown).

The equation describing the activity of PepA from B397 differed in part from that of PepN from the same strain (Table 4). Fig. 4a shows a greater influence of temperature because the hydrolysis of Glu-*p*-NA was very low at 10°C and 5.0% NaCl (ca. 20–40 U/mg, corresponding to 6–12% of the maximum activity) independent of the pH value. NaCl interacted with both temperature and pH, being considerably inhibitory only at a concentration of 7.5% (Figs. 4b and c). Although to a lesser extent than PepN, PepA activity of *Lc. lactis* subsp. *lactis* T12 was also sensitive to variations in pH (Fig. 4d and f vs. Fig. 3d and e). At 16°C, 5% NaCl and within the pH range considered, it varied from ca. 12–46 U/mg corresponding to 6–23% of the maximum activity. Sensitivity to NaCl was also confirmed for this type of peptidase; at 16°C, pH 6 and 5–7.5% NaCl the activity decreased drastically (Fig. 4e). Little differences were detected between the PepN and PepA activities of *Lb. plantarum* 2739, except for the interactive term NaCl and T instead of NaCl as a quadratic term (Table 6). At 13°C, pH 5 and 5–7.5% NaCl it maintained 37% of the maximum activity (data not shown).

3.3. Iminopeptidase and endopeptidase activities

Variations of temperature and pH moderately influenced the PIP activity of *Lb. delbrueckii* subsp. *bulgaricus* B397

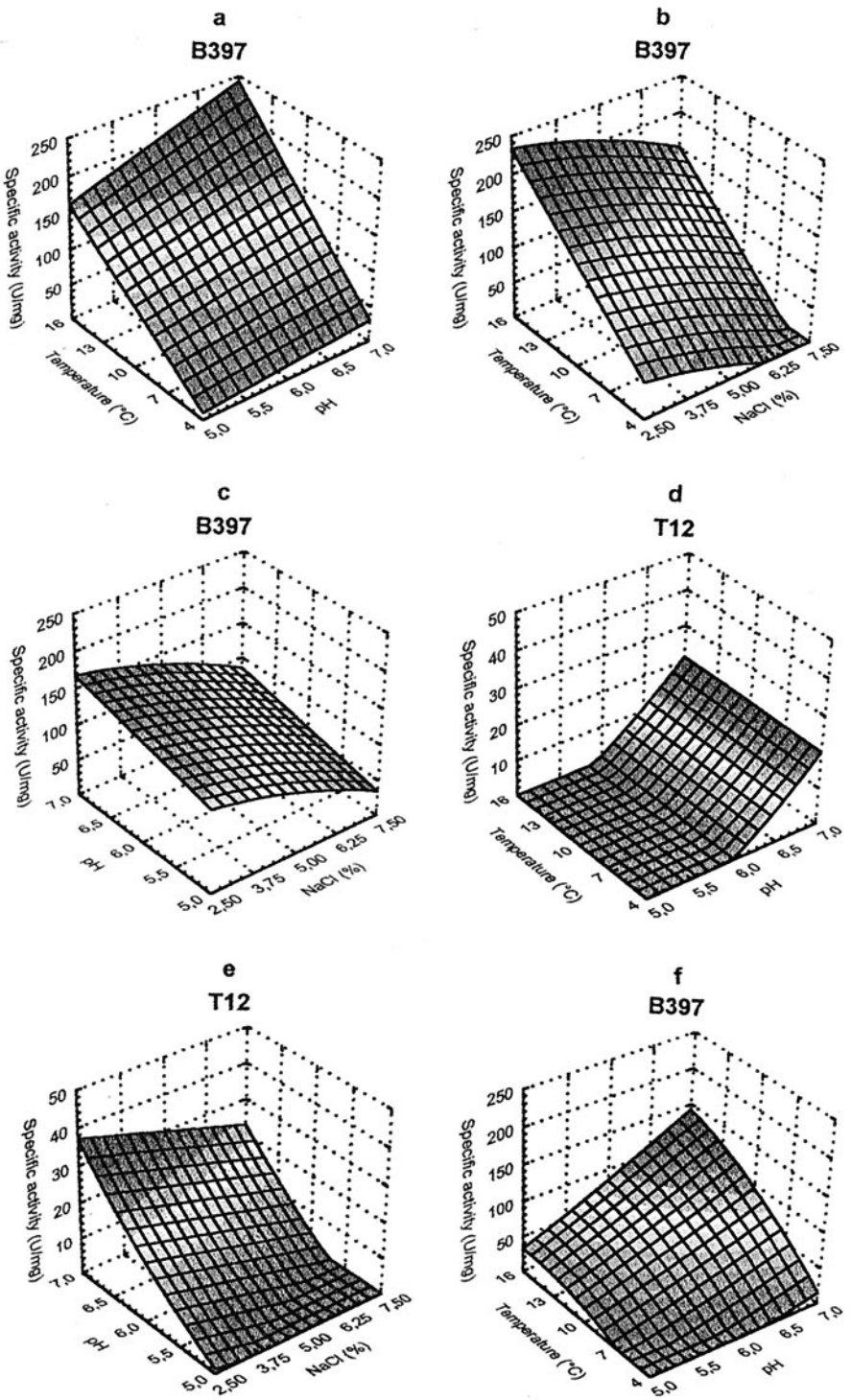

Fig. 3. PepN. Three-dimensional plots of the interactions of temperature × pH (a), temperature × NaCl (b), pH × NaCl (c) and temperature × pH (when Ala-*p*-NA was used as substrate) (f) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × pH (d) and pH × NaCl (e) on the activity of *Lc. lactis* subsp. *lactis* T12.

(Table 4 and Fig. 5a). NaCl had an interactive effect with temperature, and only at 7.5% did it almost offset the increase of the enzyme activity due to increasing temperature (Fig. 5b). A range of activity from 36 to 20% of the maximum was observed under all conditions assayed. Like the other peptidases, PIP activity of *Lc. lactis* subsp. *lactis* T12 was also quite sensitive to the lowest pH values (pH compared both as individual and quadratic terms in the equation of Table 5; Fig. 5c). At 10°C, pH 5–5.5 and 3.75–7.5% NaCl reduced the activity to value lower than

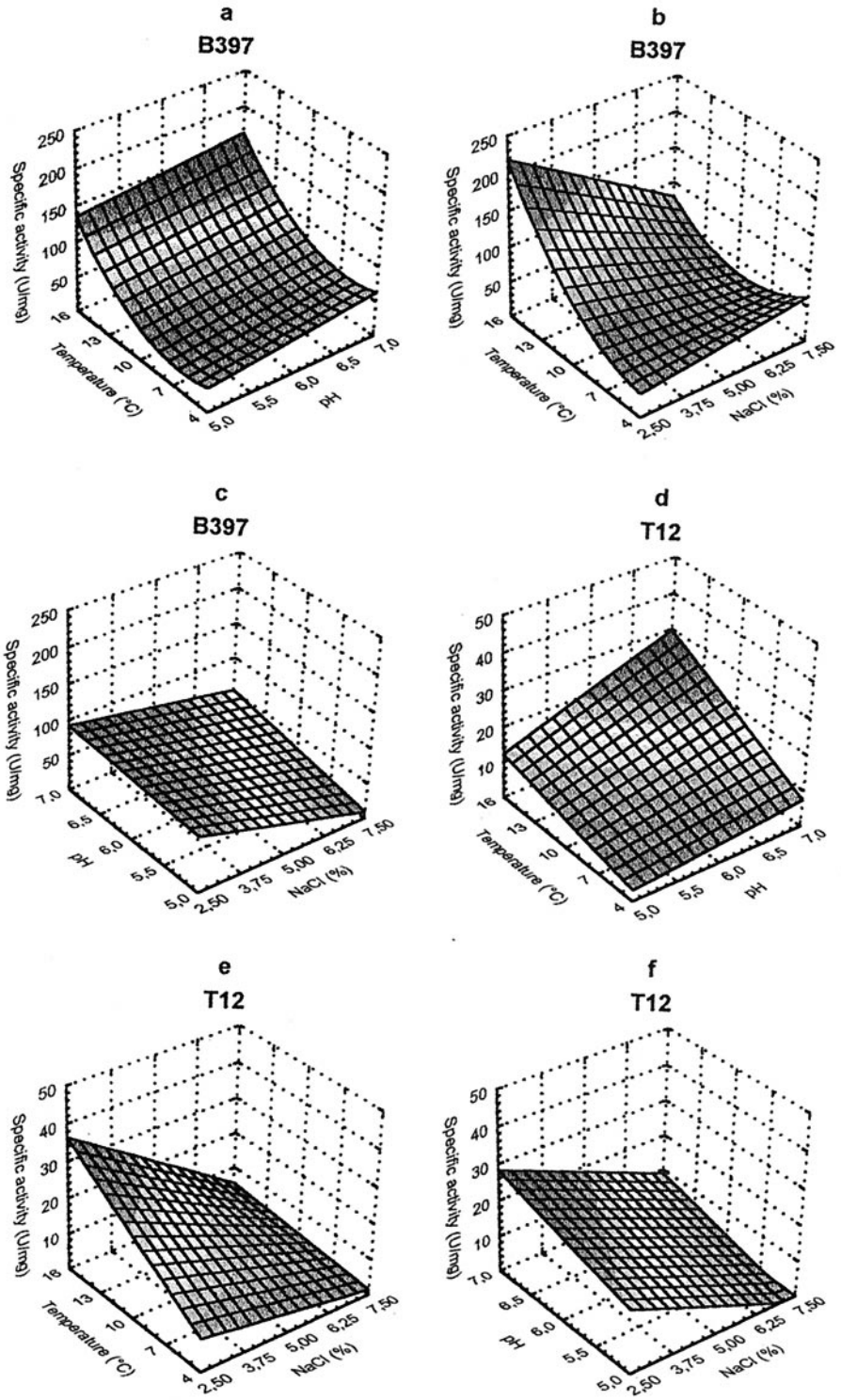

Fig. 4. PepA. Three-dimensional plots of the interactions of temperature × pH (a), temperature × NaCl (b) and pH × NaCl (c) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × pH (d), temperature × NaCl (e) and pH × NaCl (f) on the activity of *Lc. lactis* subsp. *lactis* T12.

10% of the maximum (Fig. 5d). At all pH and temperature values, the PIP activity of *Lb. plantarum* 2739 behaved optimally until the NaCl concentration of 5% was reached, then decreased very slightly (Fig. 5e and f). The pH was not included in the equation as an individual term (Table 6). Its slight effect was confirmed since at 5% NaCl the hydrolysis of Pro-*p*-NA was always higher than 20 U/mg corresponding to 22% of the maximum (Fig. 5f and Table 2), indepen-

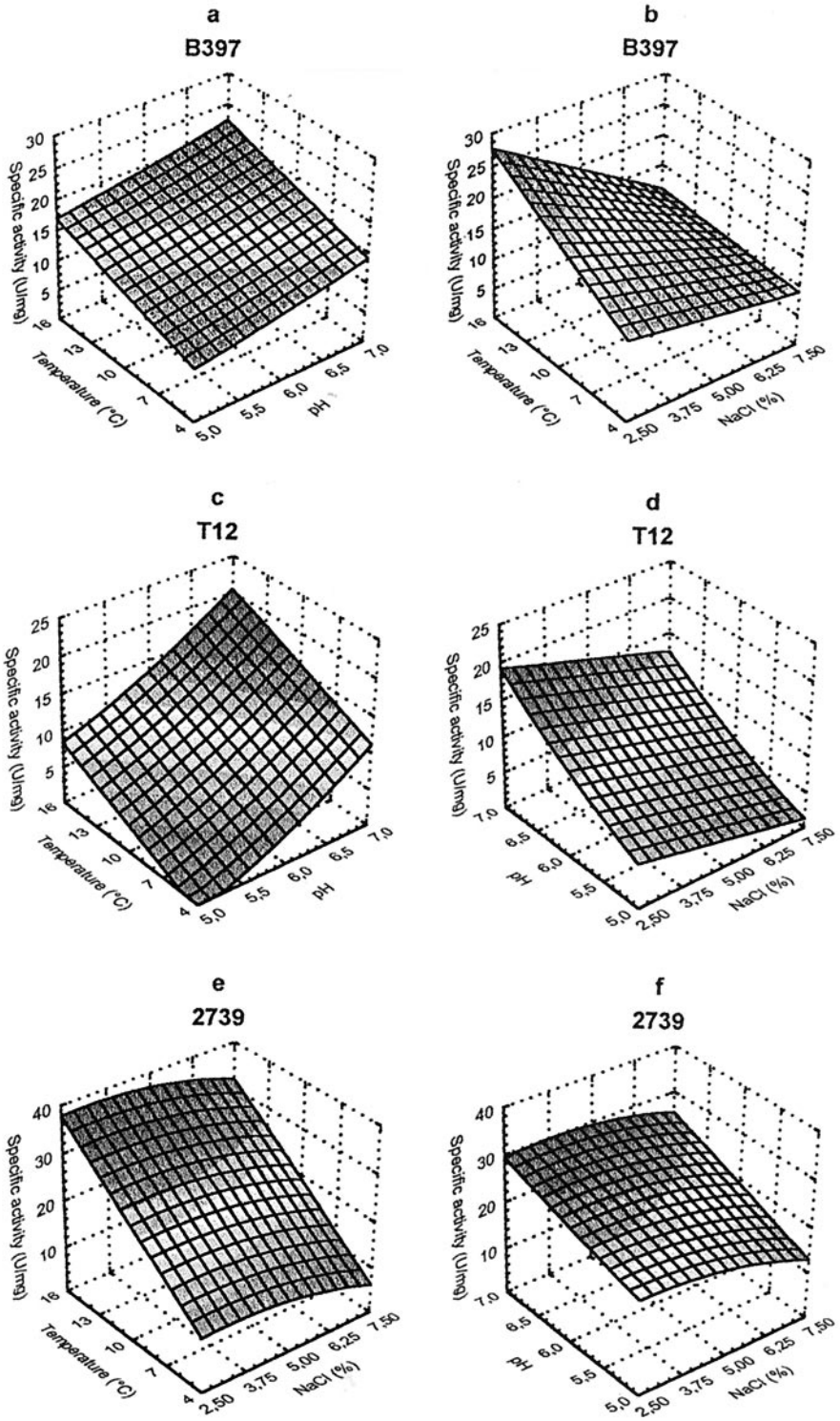

Fig. 5. PIP. Three dimensional plots of the interactions of temperature × pH (a) and temperature × NaCl (b) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × pH (c) and pH × NaCl (d) on the activity of *Lc. lactis* subsp. *lactis* T12; temperature × NaCl (e) and pH × NaCl (f) on the activity of *Lb. plantarum* 2739.

dently from the pH. PepO activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 was negatively affected especially by pH values below 6 and by the lowest temperature (Fig. 6a). NaCl did not appear in the relative equation (Table 4). PepO activity of *Lc. lactis* subsp. *lactis* T12 was the highest under optimum conditions (Table 2) and in the cheese-like conditions behaved similarly to strain B397, except for a negative influence of NaCl (Table 5 and Fig. 6b). A very

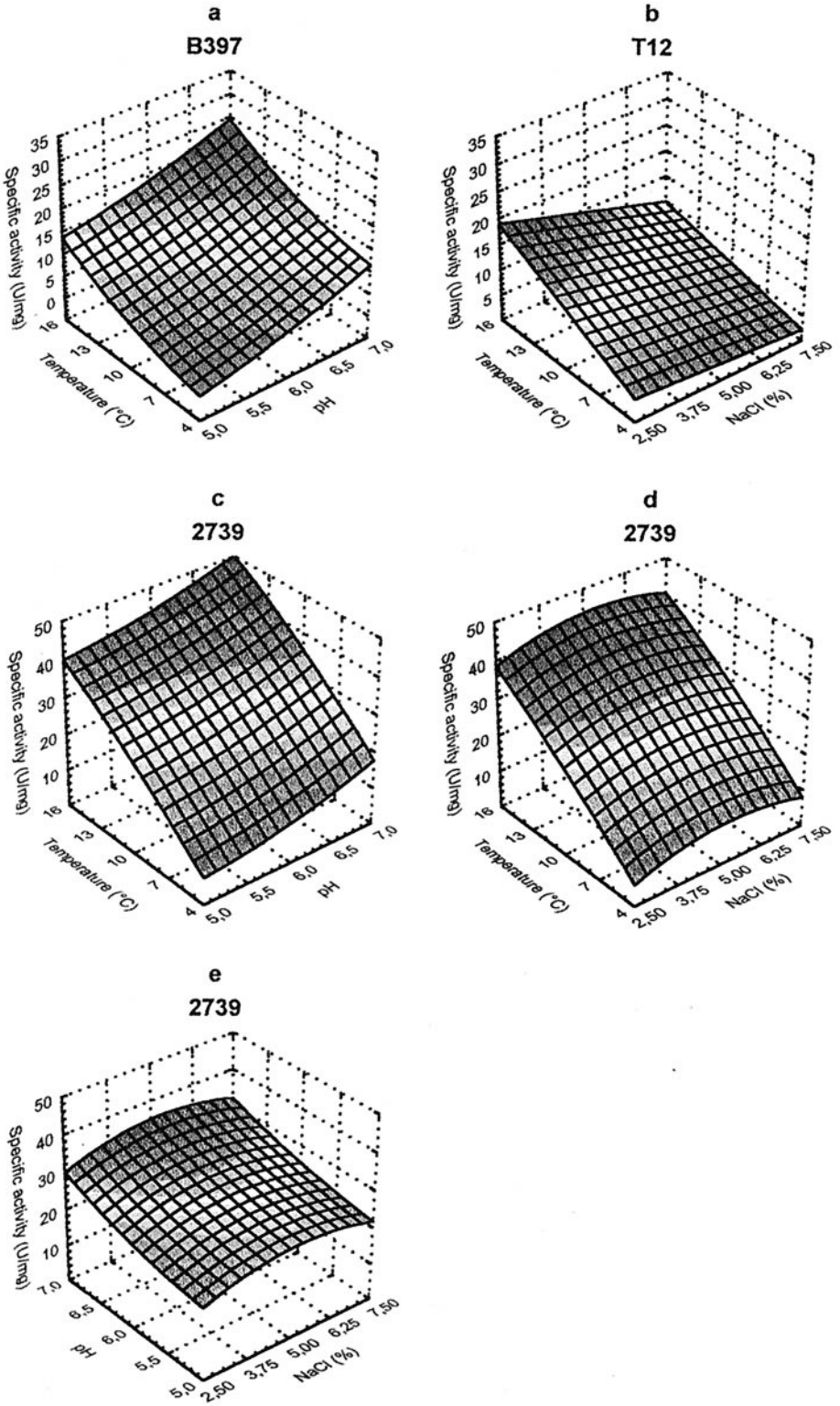

Fig. 6. PepO. Three dimensional plots of the interactions of temperature × pH (a) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × NaCl (b) on the activity of *Lc. lactis* subsp. *lactis* T12; temperature × pH (c), temperature × NaCl (d) and pH × NaCl (e) on the activity of *Lb. plantarum* 2739.

complex equation characterized the response of PepO activity of *Lb. plantarum* 2739 (Table 6). As shown by Fig. 6d and e, a NaCl concentration of ca. 5.0% seemed optimal for activity. A marked sensitivity to variations of temperature in the range 4 to 16°C was found and also in this case the influence of pH was limited (Fig. 6c and e).

3.4. Lipase/esterase activity

LE was one of the enzyme activities of *Lb. delbrueckii* subsp. *bulgaricus* B397 most influenced by the independent variables chosen. According to equation of Table 4, both pH and NaCl had an interactive effect with temperature, pH 5 as well as 5–7.5% NaCl markedly inhibited the activity, which at 16°C, NaCl 5% and pH 7.0 was ca. twice that at pH 5 (ca. 32 vs. 15 U/mg, 37 vs. 17% of the maximum) (Fig. 7a, b, and c, and Table 2). Compared to strain B397, the LE activity of *Lc. lactis* subsp. *lactis* T12 showed the same interactive effect between pH and temperature (Table 5) and was less sensitive to increased NaCl concentration (Fig. 7d). The LE activity of *Lb. plantarum* 2739 was the highest under optimum conditions (Table 2) and was not significantly affected by NaCl (Table 6). As shown in Fig. 7e, the pH also did not affect this LE activity.

Contrary to the effect on proteolytic enzymes, the lowest a_w values obtained by adding glycerol instead of NaCl only slightly affected the LE activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 (Fig. 7f vs. b).

4. Discussion

To our knowledge, this is the first paper that has applied the quadratic response surface methodology to study the influence of pH, temperature, NaCl, and a_w on several proteolytic and lipolytic activities of cheese-related bacteria. It has been shown that sensitivity to cheese-like conditions depended mainly on the interactions among the independent variables and varied with enzyme activities, substrates and, especially, the species of lactic acid bacteria.

All the enzyme activities were obviously influenced by the increase of temperature in the range 4 to 16°C; as a consequence the differences were mainly focused on the individual and interactive effects of pH, NaCl, and a_w at the low temperatures used in cheese ripening.

The hydrolysis of α_{s1}-CN by *Lc. lactis* subsp. *lactis* T12 was more influenced by pH and NaCl than that of *Lb. delbrueckii* subsp. *bulgaricus* B397 or *Lb. plantarum* 2739. The sensitivity of enzymes to cheese-like conditions was not strictly related to the pH and temperature optima: e.g. CEP activity of strain T12 on α_{s1}-CN had an optimum pH of 6.5–7 but was more influenced by the decrease in pH than the CEP activity of strain 2739, which had an optimum pH of 8 (Table 2). The pH sensitivity of CEP activity also varied with the CN-fraction. A decrease in pH had less effect on the hydrolysis of β- than α_{s1}-CN by CEP of *Lc. lactis* subsp. *lactis* T12. An opposite situation was found for strain B397. These differences may be explained in part through the interference of the ionic strength of the medium which varied with the CN-fraction and which may differently modify the available sites for enzyme hydrolysis [20–21]. Hydrolysis of β-CN by CEP of *Lb. plantarum* 2739 was very low compared to the lactococcal strain and more negatively influenced by the independent variables chosen. Mesophilic lactobacilli grow very poorly in milk due to the deficiency of small peptides [1] and to the very low proteinase activity and consequently do not contribute substantially to the proteinase activity during ripening [22]. Abdallah et al. [23] purified a serine proteinase from the same strain, 2739, which showed ca. 70% of maximum activity at ca. pH 7. As shown in this study, the relative activity may dramatically decreased when NaCl, pH, and temperature interacted at the values usually found in cheeses during ripening.

Aminopeptidase types N and A have been well characterized in lactobacilli and especially in lactococci. Their role in cheese ripening has been discussed and differentiated in part at strain level [4,10]. The PepN and PepA activities of *Lc. lactis* subsp. *lactis* T12 were markedly more sensitive to the interactions of pH and NaCl with temperature than aminopeptidases of lactobacilli and especially of *Lb. plantarum* 2739. Hydrolysis of Ala-*p*-NA by *Lc. lactis* subsp. *lactis* T12 was detected under the optimal conditions, but no activity was shown under cheese ripening conditions. Weimer et al. [11] have also shown that under cheese-like conditions, the PepN substrate hydrolysis pattern changed with respect to that determined in the optimal conditions used to characterize enzymes. Although the influence of pH and NaCl was not considered through the application of a mathematical model, other authors [30] have reported a considerable decrease of the lactococcal peptidase activities when assayed at pH 5.2 and 5% NaCl. Other results [10] confirmed the partial inhibition of the aminopeptidase activity of the mature cheddar at the pH 5.2 but also showed that lactococcal activity may be partially or fully restored by the addition of NaCl up to 2.5%, becoming inhibitory higher NaCl concentrations. This study considered concentrations of NaCl higher than 2.5% because it is more usual in various type of cheeses, especially Swiss and Italian cheeses [6,24], and because, after salting by immersion in brine, a gradient of NaCl in moisture of ca. 7 to 2.5% between the surface and core cheese may persist for a long time during ripening [25]. As shown in this study, lactococcal aminopeptidase activities under cheese-like conditions may be partially or fully inhibited in zones at elevated NaCl concentration or until the NaCl concentration equilibrates in the cheese. PepN and PepA activities higher than 35% of the maximum were maintained by *Lb. plantarum* 2739 under very hostile conditions of pH, temperature, and NaCl. A low sensitivity of *Lb. casei* aminopeptidase activity to the pH decrease has also been reported by other authors [10]. The use of mesophilic lactobacilli as adjunct starters and/or the characterization of their enzymes and of the factors that affect their growth is a recent topic in cheesemaking [8,12]. Mesophilic lactobacilli are the only microbial group that grows well in the hostile environment, such as the interior of the cheese, which has low pH (5), high salt content (4–6%), lacks a fermentable carbohydrate, is anaerobic, and may contain bacteriocins produced by starter bacteria [12]. In contrast to

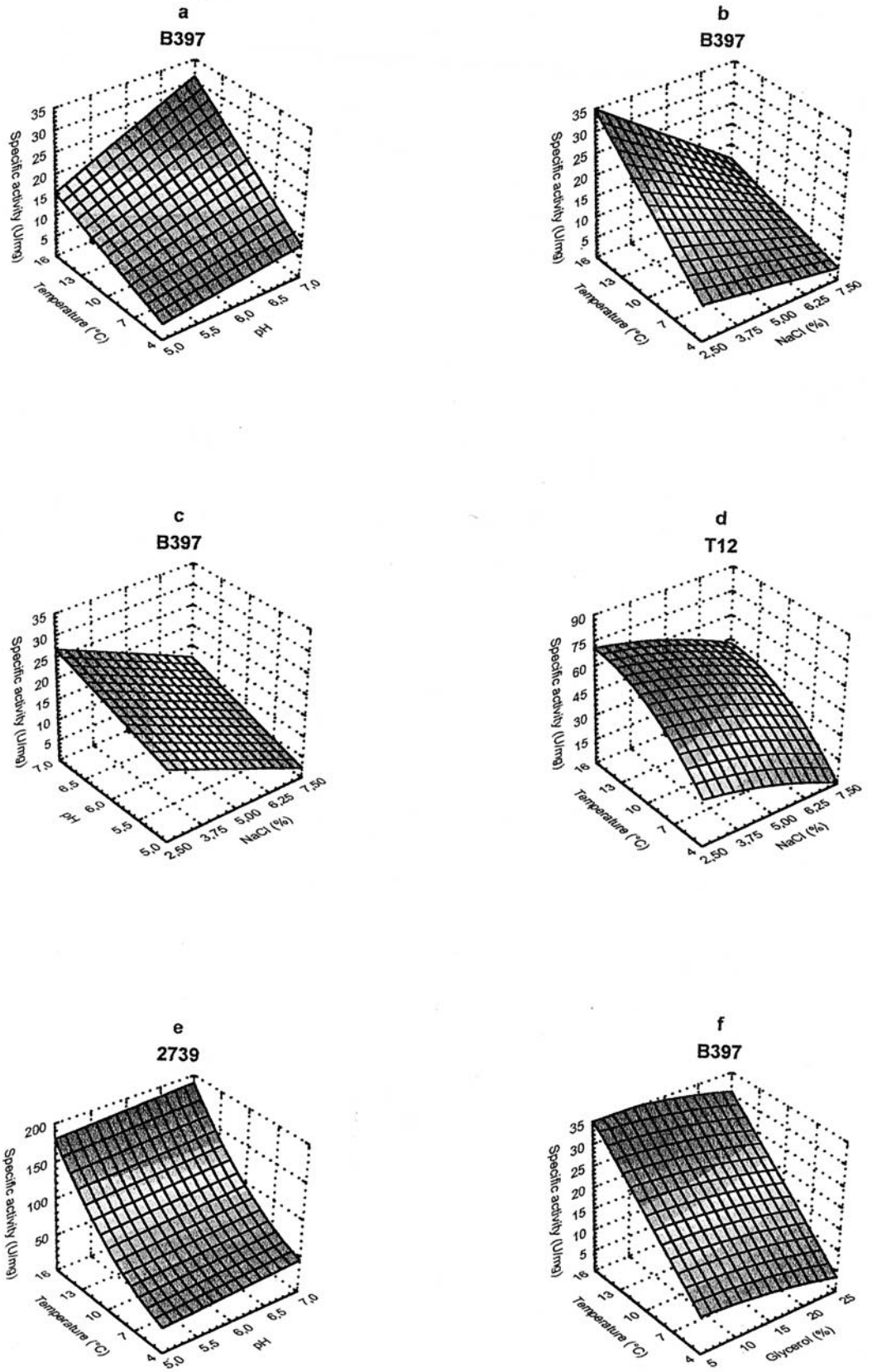

Fig. 7. LE. Three dimensional plots of the interactions of temperature × pH (a), temperature × NaCl (b), pH × NaCl (c) and temperature × glycerol (f) on the activity of *Lb. delbrueckii* subsp. *bulgaricus* B397; temperature × NaCl (d) on the activity of *Lc. lactis* subsp. *lactis* T12; temperature × pH (e) on the activity of *Lb. plantarum* 2739.

the starter bacteria, NSLAB grow from low numbers ($<10^1$ cfu/g) in fresh curd to dominate the microflora of mature cheese [26]. The role of NSLAB in ripening has yet to be resolved satisfactorily, although the inclusion of adjunct cultures of some strains of NSLAB with the starter lactococci or the use of raw milk in cheddar cheese manufacture indicated that they were involved in the release of amino acids and low-size peptides [13,27–28], leading to a more intense flavor. Besides, the flavor and texture of other semihard cheeses may be improved or ripening accelerated by using *Lb. plantarum* as adjunct starter [29–30]. The findings of this study showed that not only is the growth of NSLAB well adapted to the cheese environment but also that aminopeptidases retain a high level of relative activity compared to those of lactococcal strains.

Lactobacilli and lactococci are well equipped with enzymes for hydrolyzing peptides which contain Pro residues [4]. PIP is fundamental in cheese ripening because it prevents the accumulation of peptides with Pro as the N-terminal residue and allows secondary proteolysis to continue. Lactic acid bacteria also possess more than one type of PepO that specifically hydrolyzes peptides of ca. 4 to 20 amino acid residues [1]. With some exceptions, the results for aminopeptidases were confirmed for PIP and PepO activities. In addition, PIP as well PepO activity of *Lb. plantarum* 2739 showed a positive effect of NaCl until the optimal concentration of 5% was reached. Laan et al. [10] also found an optimum NaCl concentration of 3% for the peptidase activity of several strains of *Lb. casei*.

Esterase and lipase (LE) have been characterized in *Lc. lactis* subsp. *lactis* [5] and in the strain *Lb. plantarum* 2739 used in this study [2,6]. The use of β-NA butyrate as substrate do not permit a distinction between the two activities. LE activity of *Lb. delbrueckii* subsp. *bulgaricus* B397 and *Lc. lactis* subsp. *lactis* T12 behaved differently compared to their respective proteolytic enzymes. LE was the activity of strain B397 most influenced by the independent variables chosen. It seemed that NaCl had not only an indirect effect on LE activity through the a_w decrease but also a direct effect because the same a_w values produced by adding glycerol did not show the same inhibitory activity. It has been shown that the LE activity of other termophilic lactobacilli such as *Lb. helveticus* is very low under cheese-like conditions [11]. Although affected by variations in pH, LE activity of strain T12 was only moderately inhibited by NaCl and retained elevated relative activity also at low temperature. LE activity of *Lb. plantarum* 2739 was not significantly influenced by variations in pH and NaCl. Although lactic acid bacteria are weakly lipolytic, this result may confirm that some of them could be responsible for a moderate level of lipolysis during a long-time ripening of cheeses. It has been shown that *Lb. plantarum* 2739 possesses an intracellular lipase with somewhat different specificity from those of the porcine pancreatic lipase and rennet paste and that it probably may participate to the lipolysis during pecorino cheese ripening [2].

The application of a quadratic response surface methodology may be a practical and useful tool to determine the influence of the very complex and hostile cheese environment on microbial enzyme activity. Future progress will concern a validation of this model during cheese ripening and a large selection within mesophilic lactobacilli based on the mathematical model used.

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