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Amino acid catabolism in cheese-related bacteria: selection and study of the effects of pH, temperature and NaCl by quadratic response surface methodology

Á.C. Curtin[1], M. De Angelis[2], M. Cipriani[2], M.R. Corbo[3], P.L.H. McSweeney[1] and M. Gobbetti[4]

[1]Department of Food Science, Food Technology and Nutrition (Food Chemistry Section), University College Cork, Ireland, [2]Dipartimento di Scienze degli Alimenti (Sezione di Microbiologia Agro-Alimentare), Università degli Studi di Perugia, Italy, [3]Instituto di Produzioni e Preparazioni Alimentari, Facoltà di Agraria di Foggia, Italy, [4]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Italy

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Aims: To screen the cystathionine lyase and L-methionine aminotransferase activities of cheese-related bacteria (lactococci, non-starter lactobacilli and smear bacteria) and to determine the individual and interactive effects of temperature, pH and NaCl concentration on selected enzyme activities.

Methods and Results: A subcellular fractionation protocol and specific enzyme assays were used, and a quadratic response surface methodology was applied. The majority of the strains, 21 of 33, had detectable cystathionine lyase activity which differed in the specificity. Aminotransferase activity on L-methionine was observed in only three strains. The cystathionine lyase activities of *Lactobacillus reuteri* DSM20016, *Lactococcus lactis* subsp. *cremoris* MG1363, *Brevibacterium linens* 10 and *Corynebacterium ammoniagenes* 8 and the L-methionine aminotransferase activity of *Lact. reuteri* DSM20016 had temperature and pH optima of 30–45°C, and 7·5–8·0, respectively. As shown by the quadratic response surface methodology these enzymes retained activities in the range of temperature, pH and NaCl concentration which characterized the cheeses from which the bacteria originated.

Conclusions: The enzyme activities may have a role in flavour development during cheese ripening.

Significance and Impact of the Study: The findings of this work contribute to the knowledge about the amino acid catabolic enzymes in order to improve cheese ripening.

INTRODUCTION

Cheese ripening is a complex process consisting of lipolysis, proteolysis and glycolysis. Proteolysis is believed widely to be the most important series of reactions (Fox 1989). The bacterial population of cheese is composed of starters, non-starter lactic acid bacteria (NSLAB), secondary or adjunct starters and adventitious bacteria, such as the strains from the surface of smear-ripened cheese, all of which contribute to proteolysis. The initial stages of proteolysis have been researched extensively; it is the later stage, where amino acids are catabolized, which is less well understood and yet may be more important from a flavour perspective (McSweeney and Sousa 2000). The contribution of each microbial group to amino acid catabolism is unclear. The majority of the work in this area has been performed on lactococci (Alting *et al.* 1995; Bruinenberg *et al.* 1997; Engels 1997; Yvon *et al.* 1997; Gummalla and Broadbent 1999; Dobric *et al.* 2000). In contrast, there has been little published on the contribution of other bacteria to amino acid catabolism during cheese ripening.

The amino acids produced by proteolysis can be metabolized further to flavour compounds or their precursors

Correspondence to: Marco Gobbetti, Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Via G. Amendola, 165/A, 70126 Bari, Italy (e-mail: gobbetti@unipg.it).

(Hemme *et al.* 1982; Weimer *et al.* 1999). The enzymes involved in the reactions include deaminases, transaminases and decarboxylases (Hemme *et al.* 1982). In particular, metabolism of sulphur-containing amino acids is considered important. The products of sulphur amino acid catabolism include flavour and aroma compounds such as methanethiol and *S*-methylthioesthers (Weimer *et al.* 1999). Cystathionine lyases are pyridoxal-5′-phosphate dependent enzymes which metabolize cystathionine to cysteine, α-ketobutyrate and ammonia (α,γ-elimination reaction) or homocysteine, pyruvate and ammonia (α,β-reaction). Cystathionine-β-lyases (CβL) have been purified from *L. lactis* subsp. *cremoris* B78 (Alting *et al.* 1995) and *Escherichia coli* (Laber *et al.* 1996), while cystathionine-γ-lyases (CγL) have been isolated from *L. lactis* subsp. *cremoris* SK11 (Bruinenberg *et al.* 1997) and *Lact. fermentum* DT41 (Smacchi and Gobbetti 1998). The role of these enzymes in the development of cheese flavour is understood poorly, but Weimer *et al.* (1999) suggested that the addition of these enzymes to the cheese matrix may have a benefical effect during ripening. Aminotransferases catalyse the transfer of the amino group from an α-amino acid to an α-keto acid. L-Methionine aminotransfease (L-Met AT) produces α-keto-γ-methylthiobutyric acid (KMBA), which can be converted to methanethiol (Engels 1997). Lactococcal aminotransferases have been purified by Engels (1997), Gao and Steele (1998) and Yvon *et al.* (1997).

The effect of cheese-ripening conditions on microbial proteolytic and lipolytic activities has been studied previously by Laan *et al.* (1998) and Gobbetti *et al.* (1999a), respectively. Gobbetti *et al.* (1999b) also studied the effects of cheese-ripening conditions on the peptidases of NSLAB. To our knowledge, the only such studies performed on amino acid catabolic enzymes involved methionine catabolism in lactococci, lactobacilli and brevibacteria (Dias and Weimer 1998a; Gao *et al.* 1998). Quadratic response surface methodology has not been applied to study the combined effects of a cheese-like environment on amino acid catabolic enzymes of cheese-related bacteria. A study of the individual and interactive effects of temperature, pH and NaCl should be helpful in understanding the true enzyme potentialities.

This paper screened 33 cheese-related bacteria for amino acid catabolic activities and applied a quadratic response surface methodology to study the individual and interactive effects of NaCl, pH and temperature on cystathionine lyases and L-methionine aminotransferase activities.

MATERIALS AND METHODS

Chemicals

L-Methionine was obtained from Carlo Erba Reagenti (Milan, Italy). EDTA and casein hydrolysate were purchased from BDH Limited (Poole, UK). Pyridoxal-5′-phosphate (PLP), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), α-ketoglutarate and ninhydrin were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). L-Cystathionine was from Fluka Biochemica (Switzerland). Skim milk powder was purchased from Difco Laboratories (Detroit, MI, USA). Peptone and yeast extract were obtained from Acumedia Manufacturers Inc. (Baltimore, MD, USA).

Bacterial strains

Lactobacilli and lactococci were from the Culture Collection of the Department of Food Science, Food Microbiology Section, University of Perugia, and originated mainly from Italian cheeses. The other strains were from the Culture Collection of Teagasc, Moorepark, Ireland, or from the Institute of Microbiology, Federal Dairy Research Institute, Kiel, Germany, and had been isolated from the surface of two smear-ripened cheeses, Tilsit and Gubeen. Some of the smear strains were not identified to species level and so were assigned simple numbers or letters as identification codes. All strains were incubated at 30°C overnight. *Lact. casei* 2736, 2766, 2756, 2752, *Lact. curvatus* 2768, 2770, 2771, *Lact. paracasei* 3982, 3970 and *Lact. plantarum* 4239 and 4232 were grown in MRS broth. The other strains (*Lact. brevis* AM8, O9, *Lact. alimentarius* O8, *Lact. fermentum* CC5, *Weissella confusa* 10XF1, *Lact. reuteri* DSM20016, *Lact. rhamnosus* 15H3 and *Lact. hilgardii* 51B) were cultivated in a modified MRS broth containing fresh yeast extract. M17 with added lactose (1% w/v) and glucose (1% w/v) was used as the growth medium for *L. lactis* subsp. *lactis* WG2 and *L. lactis* subsp. *cremoris* MG1363, respectively. The strains isolated from the smear-ripened cheeses included *Brevibacterium linens* (9 and 10), *Staphylococcus equorum* (6 and 14), *Brachybacterium* spp. (1 and 16), *Arthrobacter* subsp. (J and I) and *Corynebacterium* subsp. (B, E, 8 and V). These strains were incubated in shaking conditions (150 r.p.m.). A medium containing 5 g l^{-1} peptone, 2·5 g l^{-1} yeast extract, 30 g l^{-1} NaCl, 1 g l^{-1} skim milk powder, 1 g l^{-1} glucose and 10 g l^{-1} casein hydrolysate was used for these strains.

Subcellular fractionation

Cellular extracts [cell free supernatant (CFS), cell wall (CW) and cytoplasmic extract (CYT)] were obtained by the method of Coolbear *et al.* (1992), with several modifications. The extraction buffers contained EDTA (1 mmol l^{-1}) and PLP (0·01 mmol l^{-1}). After growth the cells were harvested by centrifugation (11 000 *g*, 10 min, 4°C) and the supernatant was recovered as CFS. The cells were washed with Tris-HCl (50 mmol l^{-1}), pH 7·5 containing $CaCl_2$ (0·1 mmol l^{-1}), and recentrifuged. Following resuspension

in warmed Tris-HCl (50 mmol l^{-1}, pH 7·5) and incubation at 30°C for 30 min, the samples were centrifuged at 14 000 *g* for 10 min at 4°C. The pellets were resuspended in Tris-HCl (50 mmol l^{-1}), pH 7·5, containing sucrose (24% w/v) and $MgCl_2$ (10 mmol l^{-1}). After incubation at 37°C for 30 min, lysozyme was added (1 mg ml^{-1} for the lactic acid bacteria, 10 mg ml^{-1} for smear strains). Following incubation with shaking at 150 r.p.m. at 37°C for 45 min to 1·5 h, the samples were centrifuged (21 000 *g*, 10 min, 20°C). The supernatant was recovered as the CW fraction. The precipitates were resuspended in Tris-HCl (20 mmol l^{-1}), pH 7·5 and treated ultrasonically for 10 cycles of 30 s. After the addition of 40 ml of more buffer the samples were centifuged at 25 000 *g* for 30 min. The supernatants were recovered as CYT. The CFS, CW and CYT were then dialysed against 50 times their volume of buffer (Tris-HCl, 20 mmol l^{-1}, pH 7·5) at 4°C for 24 h and frozen at −20°C until needed. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard protein.

Enzyme assays

Cystathionine lyase activities were assayed by measuring the amount of free thiols formed from cystathionine (Ferchichi *et al.* 1985). The method is based on the principle that DTNB reacts with the free thiols, producing a yellow colour detected at an absorbance of 412 nm (A_{412}). The reaction mixture, consisting of 2 μl DTNB (5 mmol l^{-1} in ethanol), 25 μl L-cystathionine (15 mmol l^{-1}), 2·5 μl sodium azide (final concentration 0·05%), 320 μl CFS, CW or CYT and 16 μl reaction buffer (Tris-HCl, 1 mmol l^{-1}, pH 8·0, or universal buffer, composed of boric acid (57 mmol l^{-1}), citric acid (53 mmol l^{-1}), NaH_2PO_4 (33 mmol l^{-1}) and NaOH (1 mol l^{-1}), was incubated at 30°C. Controls without substrate and without enzyme sample were included. After a suitable incubation time the reaction mixture was centrifuged at 25 000 *g* for 2 min, and A_{412} was read. One unit (U) of activity was defined as an increase in A_{412} of 0·01 in 10 min. Specific activity was expressed as number of units (U) per mg of protein.

The above method cannot distinguish between cystathionine-β-lyase (CβL) and cystathionine-γ-lyase (CγL). The presence of cysteine as detected by the ninhydrin method (Gaitonde 1967) was used to differentiate between the two enzyme activities. The ninhydrin reagent (consisting of 250 mg ninhydrin in a mixture of 6 ml of acetic acid and 4 ml of concentrated HCl) reacts with the cysteine in the sample, forming a pink colour which is detectable at an absorbance of 560 nm. The presence of a pink colour was taken to indicate γ-lyase activity, since cysteine is one of the products produced in CγL-catalysed reactions (Yamagata *et al.* 1993).

The rate of formation of L-glutamic acid was used to assay for L-methionine aminotransferase (L-Met AT) activity. The assay was based on the method of Yvon *et al.* (1997) with some modifications. The reaction mixture contained: 300 μl L-methionine (10 mmol l^{-1}), 50 μl PLP (1 mmol l^{-1}), 50 μl α-ketoglutarate (200 mmol l^{-1}) and universal buffer or Tris-HCl (0·2 mmol l^{-1}, pH 8·0), in a final volume of 750 μl. This mixture was preincubated at 37°C for 5 min. CFS, CW or CYT fraction (200 μl) was added and the mixture further incubated for 15 minutes. After the addition of 50 μl sulphosalicyclic acid (60%), the assay mixture was left at 0°C for 10 min followed by centrifugation at 17 000 *g* for 5 min. The glutamate produced during the reaction was determined by colourimetric assay using a kit supplied by Boerhringer Mannheim (Germany). One unit of activity (U) is the increase in c over 15 min, where c is the concentration of glutamic acid (g) produced per litre of sample solution. Specific activity was expressed as number of units (U) per mg of protein.

Data from enzyme activity are the means from triplicate reactions.

Temperature and pH optima

The optimum pH for the individual enzymes was determined at 30°C in the pH range 5–9 by using the universal buffer. The temperature optimum was determined between 20 and 55°C, at pH 7.

Experimental design

Following the initial screening, several strains were selected and the effects of three environmental parameters (NaCl, pH and temperature) on enzyme activity were studied by modulating the variables according to a three-factor, five-level, Central Composite Design (CCD). The 17 combinations of temperature, pH and salt concentration assayed are shown in Table 1. By using a technique known as confounding, CCD reduces the number of possible combinations to a manageable size (Gacula 1988). This is possible because it uses only a fraction of the total number of factor combinations for experimentation. Two replicates of each combination were considered.

Statistical analysis

The Statistica for Windows software (Statsoft, Tulas, USA) was used to describe the enzyme activities as functions of the independent variables of the CCD. A second-order model was fitted to the independent variables using the following equation:

$$\gamma = \Sigma\, B_i X_i + \Sigma\, B_{ii} X_i^2 + \Sigma\, B_{ij} X_i X_j$$

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

Run	Temperature (°C)	pH (units)	NaCl concentration (%)
1	14	5·0	2·5
2	14	7·0	2·5
3	14	5·0	7·5
4	14	7·0	7·5
5	28	5·0	2·5
6	28	7·0	2·5
7	28	5·0	7·5
8	28	7·0	7·5
9	21	6·0	5·0
10	21	6·0	5·0
11	21	4·0	5·0
12	21	8·0	5·0
13	21	6·0	0·0
14	21	6·0	10·0
15	7	6·0	5·0
16	35	6·0	5·0
17	21	6·0	5·0

where γ is the dependent variable (enzyme activity); B_i, B_{ii} and B_{ij} signify the regression coefficients of the model and X_i and X_j are the independent variables in coded values. Variables with a significance less than 95% ($P > 0{\cdot}05$) were not included in the final model.

The criteria for eliminating a variable from the full regression equation was based on R-value, standard error to estimate (SE) and significance F-test. Once the coefficients of the response surface were estimated, the non-significant terms were omitted one by one, using the procedure Stepwise Backwards, consequently only the terms significant at 95% were considered.

Using this model, it was possible to determine the effects of linear, quadratic and interactive terms of the independent variables on the dependent ones. Three-dimensional surface plots were produced to illustrate the main and interactive effects of the independent variables on the dependent variables.

RESULTS

Cell localization of the enzyme activities

After subcellular fractionation, the three bacterial cell fractions (CFS, CW and CYT) from the 33 cheese-related bacteria were screened for two of the enzyme activities responsible for the amino acid catabolism: cystathionine lyase and L-methionine aminotransferase. Activity was present only in the CYT, so this fraction was used subsequently for all enzyme assays.

Cystathionine lyase activities

The bacteria were first screened for cystathionine lyase activity at pH 8·0 and 30°C. Twenty-one of the 33 bacteria showed this activity under our assay conditions. The specific activities of these strains are shown in Table 2. All of the smear strains were active and *Coryne. ammoniagenes* 8 had the greatest activity within the strains studied (96·62 U mg^{-1}). Seven lactobacilli had detectable cystathionine lyase activity, with *Lact. reuteri* DSM20016 being the most active with a specific activity of 66·6 U mg^{-1}. Both lactococci were active on cystathionine, with *L. lactis* subsp. *cremoris* MG1363 having the greater activity (90·6 U mg^{-1}).

Strains with specific activities greater than 60 U mg^{-1} were assayed by the ninhydrin method to determine whether the reaction was catalysed by CβL or CγL. *Staph. equorum* 14, *B. linens* 9 and 10, and *Coryne. ammoniagenes* 8 all gave negative results, suggesting that they do not produce cysteine and so possess CβL activity only. The enzyme activities of *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363 produced positive ninhydrin results, so these strains possess CγL activity. According to the recent results of Dobric *et al.* (2000) on *L. lactis* subsp. *cremoris* MG1363, we did not exclude the presence of a CβL activity

Table 2 Cystathionine lyase activity on cheese-related strains

Strain	CγL/CβL* Specific activity (U mg^{-1})†
Lact. reuteri DSM20016	66·60 ± 0·20
Lact. brevis 09	1·85 ± 0·04
Lact. alimentarius 08	0·81 ± 0·03
Lact. confusus 10XF1	2·08 ± 0·40
Lact. hilgardi 51B	2·09 ± 0·30
Lact. casei 2752	1·09 ± 0·05
Lact. curvatus 2771	0·46 ± 0·01
L. lactis subsp. *cremoris* MG1363	90·60 ± 0·70
L. lactis subsp. *lactis* WG2	48·38 ± 0·80
S. equorum 14	60·65 ± 0·20
S. equorum 6	42·69 ± 0·20
B. linens 9	78·21 ± 0·50
B. linens 10	90·74 ± 0·20
Coryne. ammoniagenes 8	96·62 ± 1·00
Corynebacterium spp. B	54·34 ± 0·10
Corynebacterium spp. E	4·77 ± 0·30
Corynebacterium spp. V	11·11 ± 0·50
Arthrobacter spp. J	54·31 ± 1·00
Arthrobacter spp. I	30·98 ± 0·02
Brachybacterium spp. 1	30·29 ± 0·30
Brachybacterium spp. 16	42·88 ± 0·03

*CβL: cystathionine β-lyase; CγL: cystathionine γ-lyase. †Specific activity is expressed as units per mg of protein, where 1 unit is an increase on absorbance of 0·01 in 10 min.

in these latter two strains and we defined their enzyme activities as C$\beta\gamma$L.

L-Methionine aminotransferase activity

Under our assay conditions, the L-Met AT activity was not as widely distributed. Only three strains were found to be active: *Lact. reuteri* DSM20016 (51·27 U mg^{-1}), *S. equorum* 14 (21·81 U mg^{-1}) and *L. lactis* subsp. *lactis* WG2 (3·37 U mg^{-1}).

Temperature and pH optima

The C$\beta\gamma$L activities of *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363, the CβL activities of *B. linens* 10 and *Coryne. ammoniagenes* 8 and the L-Met AT activity of *Lact. reuteri* DSM20016 were selected for further studies. These enzyme activities were chosen because, in addition to being the highest, we believed that these strains were representative of the different cheese-related bacteria.

Optimal pH for the cystathionine lyase activities were in the range 7·5–8·0 for all the strains. Temperature optima varied widely from 30°C for *B. linens* 10 and *Coryne. ammoniagenes* 8–37°C for *Lact. reuteri* DSM20016 to 45°C for *L. lactis* subsp. *cremoris* MG1363. For *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363 the specific enzyme activities under these optimal conditions increased with respect to those reported in Table 2 and were 97·7 and 132·2 mg^{-1}, respectively. For both *B. linens* 10 and *Coryne. ammoniagenes* 8, the activity reported in Table 2 is the activity at the optimal pH and temperature conditions (pH 8 and 30°C).

The aminotransferase activity of *Lact. reuteri* DSM20016 was optimal (39·16 U mg^{-1}) at 35°C and pH 7·5.

Specific activities under the optimal conditions of pH and temperature were considered as maximum activities and compared to those determined by the application of quadratic response surface methodology.

Quadratic response surface methodology

To screen for cystathionine lyase and L-methionine aminotransferase activities under cheese-ripening conditions, the following intervals were chosen: pH 4·0–8·0, temperature 7–35°C and NaCl concentration 0–10%. These values represent those encountered most often during ripening of different cheese varieties. The pH of Cheddar cheese is 5·0–5·2 but it can be much higher (6·0–7·5) at the surface of smear-ripened cheese. The ripening temperatures of smear cheeses such as Pont l'Eveque and Trappist are much higher (18–20°C) than the ripening temperature (*ca* 12°C) of internally ripened cheeses, such as Cheddar (Rattray 1996). Fox and Guinee (1987) reported that in some Italian varieties which are brine salted, the salt content at the surface may be higher than 7%, while Cheddar can have a salt-in-moisture content of 4–5%.

The five bacterial enzymes were assayed at 17 combinations of pH, temperature and NaCl concentrations. The results were analysed statistically and the polynomial equations produced are shown in Table 3. These CCD equations describe the main interactive and quadratic effects of the independent variables on activity. Only the terms appearing in the equations had a significant effect on the activity of that enzyme. The graphs plotted are shown in Figs 1, 2 and 3. When one of the three independent variables is not shown in the graph, a constant value (the central point 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. In addition, since CCD may not necessarily be a good design to establish the location of optimum values for enzyme activity, they were determined formerly. However, what CCD makes possible is to investigate the shape of the response variable, which is identical with one of the goals of this study and which has technological significance.

Table 3 Best-fit equations for the effects of the different variables on the cystathionine lyase activities of *Lactobacillus reuteri* DSM20016, *Lactococcus lactis* subsp. *cremoris* MG1363, *Brevibacterium linens* 10 and *Corynebacterium ammoniagenes* 8, and on the L-methionine aminotransferase activity of *Lact. reuteri* DSM20016. (Only terms with $P < 0{\cdot}05$ were included)

Strain	Equation*	*R*†	F‡	SE§
Lact. reuteri DSM20016¶	1·2474[T] − 11·6564[pH] + 2·1596[pH]^2	0·993	337·83	5·1293
L. lactis subsp. *cremoris* MG1363	−3·8897[pH] + 0·3828[pH][T] + 0·6185 [pH]^2	0·9361	33·05	2·6019
B. linens 10	11·27[pH] − 4·393[T] + 0·9517[pH][T] − 1·875[pH]^2	0·9861	115·03	5·6229
Coryne. ammoniagnes 8	0·0506[T] − 0·3717[pH] − 0·0121[pH][NaCl] + 0·0670[pH]^2	0·9175	54·53	0·3023
Lact. reuteri DSM20016**	0·3338[T][pH] − 0·1610[NaCl]^2−0·0254[T]^2	0·986	161·51	5·0193

*[T], temperature (°C); [pH], pH value; [NaCl], NaCl concentration (%). †Regression coefficient ‡*F*-value §Standard error of residuals ¶Enzyme activity, cystathionine -β,γ -lyase (Cβ,γL) for *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363, cystathionine-β-lyase (CβL) activity for *B. linens* 10 and *Coryne. ammoniagenes* 8. **L-Methionine aminotransferase.

Temperature and pH both had linear effects on the C$\beta\gamma$L activity of *Lact. reuteri* DSM20016, with pH also displaying a quadratic effect (Table 3). The enzyme activity increased more than the other cystathionine lyases when the pH increased from 4 to 8, at 7°C and 5% NaCl (Fig. 1a). The enzyme-specific activity was relatively high at pH 6, 21°C and 5% NaCl (*ca* 40 U mg^{-1}) (Fig. 1a), corresponding to *ca* 41% of its maximum. A similar effect was found for the C$\beta\gamma$L activity of *L. lactis* subsp. *cremoris* MG1363 (Fig. 1b, Table 3). However, the increase in activity when pH was increased was less noticeable. For this enzyme, pH had linear and quadratic effects as well as an interactive effect with temperature (Table 3). A more complex equation described the effect of temperature and pH on the CβL activity of *B. linens* 10 (Table 3). As shown in Fig. 1c, the activity was completely inhibited when the pH was less than 5·0, irrespective of the temperature, as well as when the temperature was less than 14°C, irrespective of the pH values. Considerable activity (*ca* 30 U mg^{-1}) for *B. linens* 10 was found only at 21°C and pH 7·0–8·0, which corresponded at *ca* 25% of the maximum. All three variables significantly affected the CβL activity of *Coryne. ammoniagenes* 8 (Table 3). In the range 7–14°C, the activity was detected only when the pH was higher than 6·0 (Fig. 2a). At the combination 21°C, pH 7·5 and 5% NaCl the specific activity was *ca* 35 U mg^{-1}, corresponding to *ca* 36% of the maximum. The increase of the NaCl concentration had a negative effect on the enzyme activity (Fig. 2b,c).

Compared to the C$\beta\gamma$L activity, the L-methionine aminotransferase activity of *Lact. reuteri* DSM20016 seemed more negatively influenced by the interaction between pH and temperature (Fig. 3a, Table 3). The increase of the NaCl concentration slightly decreased the enzyme activity only in the presence of the lowest values of temperature and pH (Fig. 3b,c). At pH 6·0, 10% NaCl and 21°C, or at pH 7·0, 10% NaCl and 14°C, the L-Met AT activity of *Lact. reuteri* DSM20016 was *ca* 15 U mg^{-1}, which corresponded to *ca* 38% of its maximum.

DISCUSSION

Microbial catabolism of amino acids as nitrogen sources is believed to produce aroma compounds in cheese. These compounds can be significant in flavour development during cheese ripening. In particular, sulphur-containing compounds, such as methanethiol, are essential to aroma and flavour development.

The initial screening for cystathionine lyase and L-methionine aminotransferase activities in 33 cheese-related bacteria revealed the enzyme activities to be intracellularly located. There has been some debate about whether the bacteria producing amino acid catabolic enzymes lyse in the cheese and, if so, what the effect of

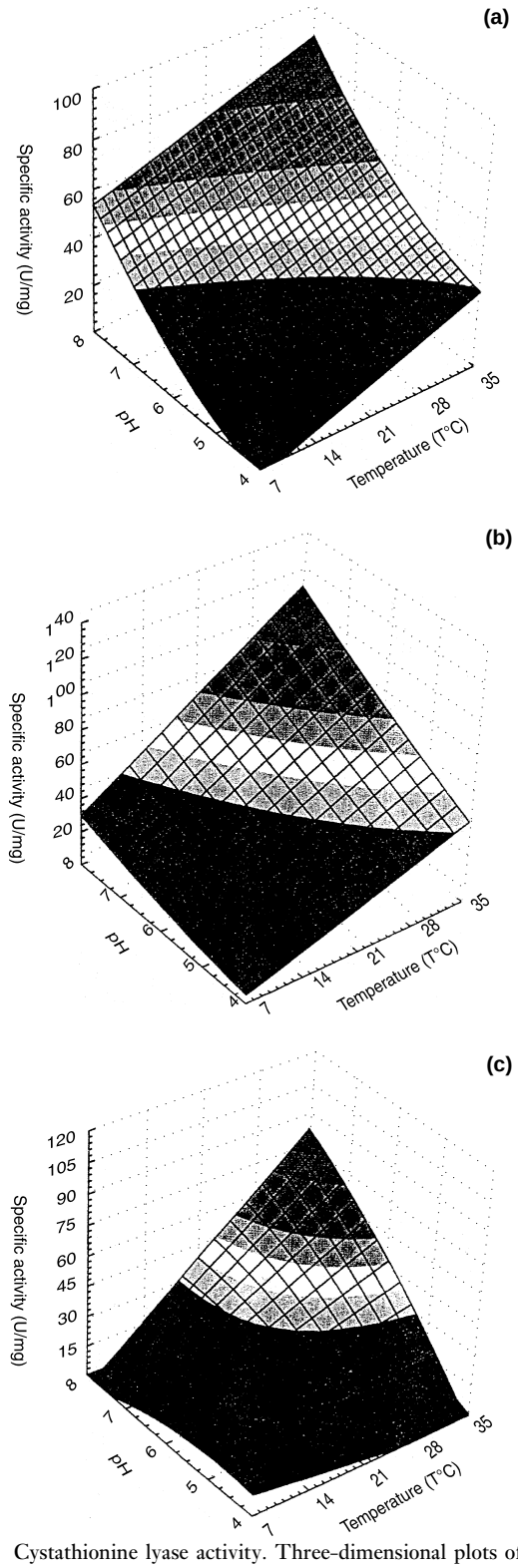

Fig. 1 Cystathionine lyase activity. Three-dimensional plots of the interaction of pH × T on the activity of *Lactobacillus reuteri* DSM20016 (a), *Lactococcus lactis* subsp. *cremoris* MG1363 (b) and *Brevibacterium linens* 10 (c)

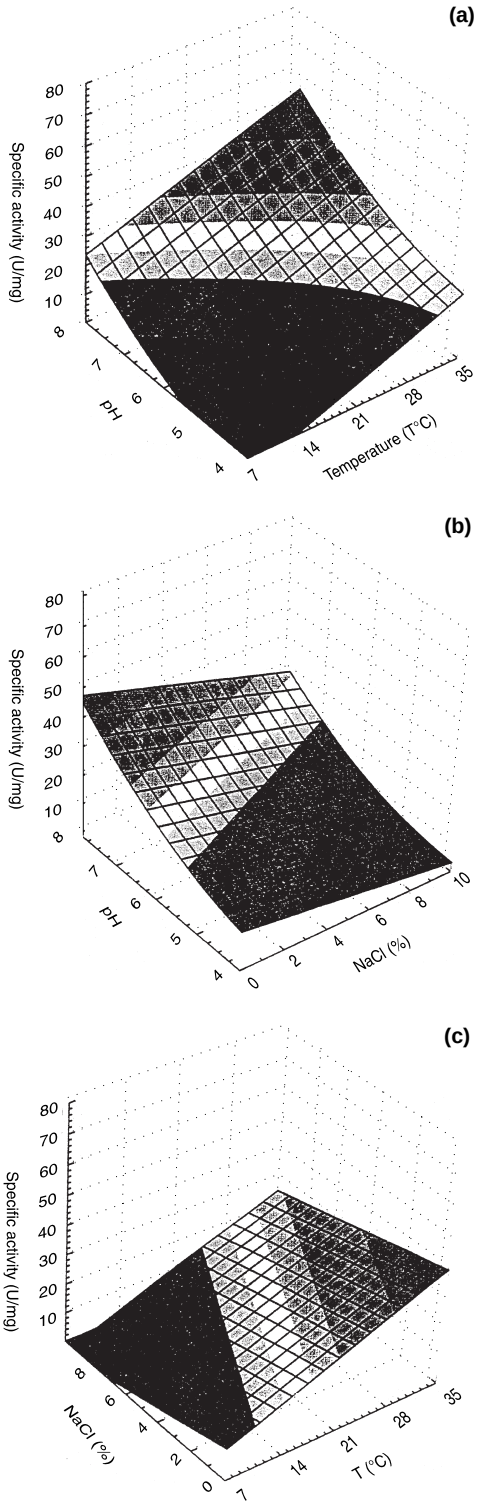

Fig. 2 Cystathionine lyase activity of *Corynebacterium ammoniagenes* 8. Three-dimensional plots of the interactions of pH × T (a), pH × NaCl (b) and NaCl × T (c)

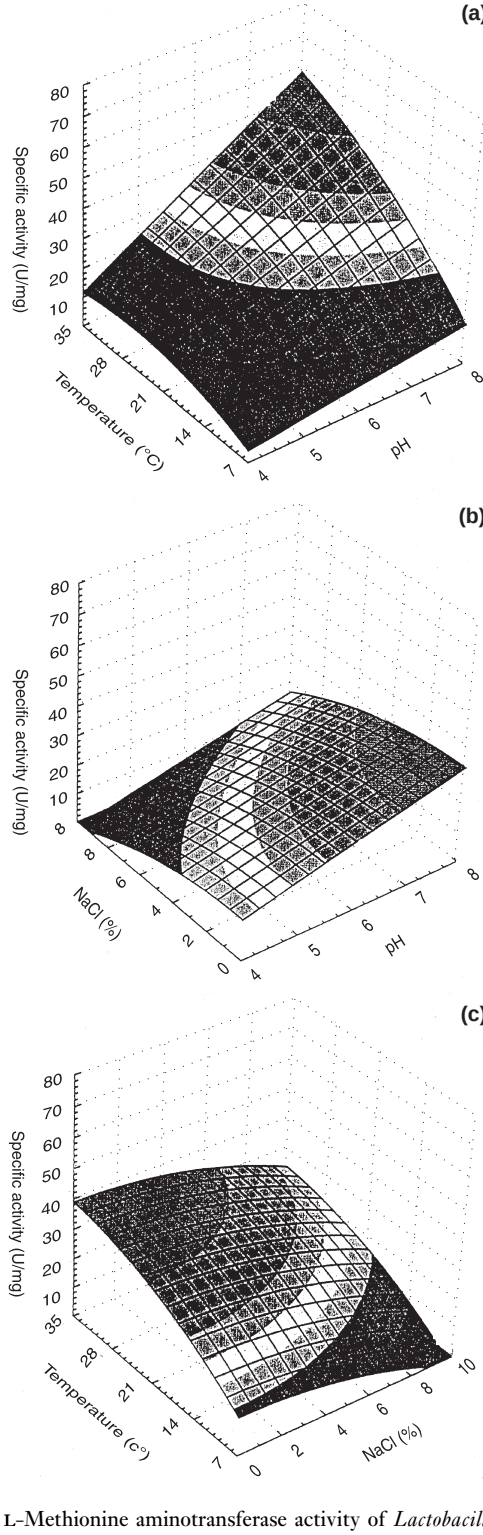

Fig. 3 L-Methionine aminotransferase activity of *Lactobacillus reuteri* DSM20016. Three-dimensional plots of the interactions of T × pH (a), NaCl × pH (b) and T × NaCl (c)

lysis is on activity. Alting *et al.* (1995) report that lysis of cells is necessary for optimum activity of a lactococcal cystathionine-β-lyase (CβL) during ripening. In contrast, Dias and Weimer (1998b) suggested that the cells of *B. linens* BL2 must remain metabolically active during cheese ripening for the methionine-γ-lyase to affect the cheese flavour. Interestingly, Gao *et al.* (1998) observed that both whole and lysed cells of lactococci are capable of methanethiol formation but these two types of cells use different pathways. If cell lysis does occur, the enzymes may be exposed to conditions which reduce their activities. However, since sulphur compounds have very low perception threshold values, e.g. 2 p.p.b. for methanethiol (Dias and Weimer 1999), even with reduced activity the enzymes can play a role in flavour development.

The first enzymes we screened were the PLP-dependent cystathionine lyases. CβL catalyses the conversion of cystathionine to homocysteine, with the release of ammonia and pyruvate, while cystathionine-γ-lyase (CγL) catalyses the α,γ elimination reaction which produces L-cysteine, α-ketobutyrate and ammonia. Although the main function of cystathionine lyase is to produce cysteine and homocysteine from cystathionine, Alting *et al.* (1995) and Bruinenberg *et al.* (1997) observed that the cystathionine lyases of *L. lactis* subsp. *cremoris* B78 and SK11 also produced methanethiol from methionine. As methanethiol is recognized as an important cheese flavour compound, the enzymes involved in its production in cheese could have vital roles in ripening. Weimer *et al.* (1999), in their review on sulphur metabolism in cheese-related bacteria, pointed out that the importance of CβL and CγL for the production of volatile sulphur compounds in cheese is unknown, but observed that the addition of these enzymes to the cheese matrix may have positive implications for ripening. The widespread distribution of cystathionine lyase activity in 21 of the 33 strains screened is not unexpected, as they also have a role in methionine biosynthesis (Laber *et al.* 1996), and methionine is known to play a central role in the interconversion of sulphur amino acids. However, under our assay conditions, the enzyme activity varied greatly in intensity. Apart from well-known lactococci strains, only *Lact. reuteri* DSM20016 had a considerable activity within the lactobacilli belonging to the group of non-starter lactic acid bacteria (NSLAB), while 10 of the 12 smear bacteria screened had high cystathionine lyase activity. The greatest activity was found in *Coryne. ammoniagenes* 8 and *L. lactis* subsp. *cremoris* MG1363. Based on the ninhydrin assay, all the smear strains tested seemed to have CβL activity while *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363 had positive ninhydrin results, suggesting the presence of CγL activity. However, the ninhydrin assay is limited in its ability to distinguish between CβL and CγL, in the sense that a negative result excludes CγL activity but a positive result does not exclude the presence of both CβL and CγL. A recent study on the cystathionine lyase of *L. lactis* subsp. *cremoris* MG1363 by Dobric *et al.* (2000) used HPLC to detect reaction products and the authors reported that the enzyme displayed both CβL and CγL activity on the same substrate. Therefore, we presumed that both activities were present in those strains. The pH (7·5–8·0) and temperature (30–45°C) optima determined for the cystathionine lyases were in agreement with those determined for other lyases from *L. lactis* subsp. *lactis* B78 (Alting *et al.* 1995), *E. coli* (Laber *et al.* 1996), *L. lactis* subsp. *cremoris* SK11 (Bruinenberg *et al.* 1997) and *Lact. fermentum* DT41 (Smacchi and Gobbetti 1998).

Transaminases or aminotransferases catalyse the transfer of the amino group from an α-amino acid to an α-keto acid. The enzymes have broad substrate specificity. Under our assay conditions, the distribution of L-methionine aminotransferase activity was much more limited than that of cystathionine lyase activity. Of the strains screened in this study, only four had L-methionine aminotransferase activity. The enzyme of *Lact. reuteri* DSM20016 was the most active. It is surprising to find such a low distribution of aminotransferase activity, as this enzyme has been reported previously in lactococci and lactobacilli. An aromatic aminotransferase isolated from *L. lactis* subsp. *cremoris* NCDO763 by Yvon *et al.* (1997), which catalysed the transformation of five amino acids (Leu, Phe, Trp, Tyr and Met), was reported to be active under cheese-ripening conditions, as have other aminotransferases isolated (Engels 1997; Dias and Weimer 1998a; Gao and Steele 1998). The aminotransferase of *Lact. reuteri* DSM20016 had pH and temperature optima of 7·5 and 35°C, respectively. The pH and temperature optimum are in agreement with previous reports; the temperature optimum is at the lower end of the optimum range (35–45°C) observed for a lactococcal aminotransferase isolated by Yvon *et al.* (1997). Other aminotransferases isolated from *L. lactis* subsp. *cremoris* B78 (Engels 1997) and *L. lactis* subsp. *lactis* S3 (Gao and Steele 1998) were also reported to have higher temperature optima.

To study effectively the effect of an enzyme on cheese flavour, the enzyme must be assayed in conditions similar to those present in ripening cheese. It must be remembered that it is not only the individual variables which can influence activity but also the interaction of these variables. For example, Laan *et al.* (1998) found that the aminopeptidase activities of lactic acid bacteria were reduced at the pH of cheese but that the addition of Na^+ and Ca^{2+} could at least partially restore activity. Gobbetti *et al.* (1999a, 1999b) previously used quadratic response surface methodology to study the effects of four variables (pH, temperature, a_w and salt concentration) on the lipolytic, peptidolytic and proteolytic activity of cheese related lactic acid bacteria, under simulated cheese-ripening conditions.

The sensitivity to cheese-ripening conditions varied with the enzyme, the substrate and the bacterial strain. In this study, we applied similar experimental design, to study the shape of the response variable on cystathionine lyase and L-methionine aminotransferase activities of selected strains under cheese-like conditions (pH, temperature and NaCl). While the C$\beta\gamma$L activities of *Lact. reuteri* DSM20016 and *L. lactis* subsp. *cremoris* MG1363 seemed to be more tolerant, when the lowest values of temperature and pH interacted the CβL activities of *B. linens* 10 and *Coryne. ammoniagenes* 8 were markedly inhibited. The activity of *B. linens* 10, the most typical smear bacteria, was not influenced by the variation of the NaCl concentration from 0 to 10%. These findings are not surprising, since the surface of the smear-ripened cheeses from which these strains are isolated have an elevated pH due to the lactic acid oxidation by yeasts and moulds, are frequently brined or rubbed with salt during ripening and the ripening temperature is unusually high (18–20°C) (Rattray 1996). The enzyme activities of the same strain differed in the sensitivity to the cheese-like conditions. Compared to the C$\beta\gamma$L enzyme, the L-methionine aminotransferase of *Lact. reuteri* DSM20016 showed a greater sensitivity to the interaction between the lowest values of pH and temperature and was negatively affected by an increase in the NaCl concentration. Amino acid catabolic enzymes from cheese-related bacteria which had been previously isolated and characterized were generally found to retain activity under pH, NaCl or temperature conditions found in ripening cheese; for example, the CγL from *Lact. fermentum* (Smacchi and Gobbetti 1998). The activity of CγL from *L. lactis* subsp. *cremoris* SK11 was reduced twofold by 5% NaCl, while the addition of 4% NaCl had only a slight effect on the CβL of *L. lactis* subsp. *cremoris* B78 (Alting *et al.* 1995). This later enzyme was also reported to have 10–15% of its maximum activity at pH 5·0–5·5. Aminotransferases from lactococci have been found to be active at pH of 5–5·5 (Engels 1997; Yvon *et al.* 1997; Gao and Steele 1998).

Overall, it can be said that all five enzymes examined were active over the temperature, pH and NaCl values which are generally used in the different types of cheeses from which they are often isolated. Since the reactions they catalyse produce flavour compounds or their precursors, the strains may be important in cheese ripening and so may be considered for use as flavour adjuncts. This screening study has been preliminary work for the isolation and characterization of a cystathionine lyase of *Lact. reuteri* DSM20016.

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