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Production of esters by Staphylococci

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Abstract

The ability of resting cells and extracellular concentrates of Staphylococci to synthesize ethyl esters was studied in the presence of ethanol and short chain acids considered individually. All the strains synthesized ethyl esters, *S. warneri* was the highest producer and *S. carnosus* the lowest. Resting cells esterified preferentially butanoic acid, extracellular concentrates esterified butanoic, valeric and hexanoic acids. Acetic, decanoic and branched acids were poorly esterified. The activity of the extracellular concentrates with ethanol and butanoic acid was not modified by the pH (pH 5.5 or 7.0); but it was decreased at a temperature of 14°C compared to 24°C. For the resting cells it was the opposite, the activity was inhibited by acid pH and was not influenced by the temperatures. So the Staphylococci could produce esters during sausage manufacture.

Keywords: Staphylococci; Esters; Flavour

1. Introduction

Strains of *Staphylococcus* added as starter culture in fermented sausage participate in the development of typical flavour (Berdagué et al., 1993; Stahnke, 1995). They influence the level of many fragant compounds such as esters, which have low sensory threshold values and will impart fruity notes to the sausage flavour (Stahnke, 1994; Montel et al., 1996). Most of the esters in sausages are ethyl esters, their production depending on the presence of ethanol and different acids as well as on the esterase activities of the strains. In previous work, we have shown that Staphylococci have intracellular and extracellular esterases that hydrolyse esters with different chain length of acids (Talon and Montel, 1997). The hydrolysis was important between 15 and 25°C, whereas acidic conditions and low water activity reduced it. However, it is important to determine if the Staphylococcal esterases are able to esterify ethanol with acids.

Concerning esterification, most of the studies had been performed in non-aqueous media that favoured the synthesis of esters (Gatefield, 1986; Gillies et al., 1987; Bloomer et al., 1992; Talon et al., 1996). However, the production of esters by *Pseudomonas fragi* (Reddy et al., 1968, 1969; Cormier et al., 1991; Raymond et al., 1991; Morin et al., 1994), *Erwinia carotovora* subsp. *atroseptica* (Spinnler and Djian,

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1991) have been characterised in culture media. Resting cells of coryneform bacteria and *Micrococcaceae* (from cheeses) incubated in phosphate buffer with substrates produced thioesters (Cuer et al., 1979a,b; Lamberet et al., 1995, 1997a,b). Also, Hosono et al. (1974) and Hosono and Elliott (1974) reported that crude extract of intracellular enzymes of lactic acid bacteria and *Pseudomonas* produced ethyl butanoate and ethyl hexanoate.

The aim of this work was to investigate the potential of different *Staphylococcus* species to form flavour esters, in order to understand their role in sausage fermentation.

2. Materials and methods

2.1. Growth conditions of the strains and preparation of enzymes

The seven following Staphylococci were studied: *Staphylococcus carnosus* (833, 836), *Staphylococcus xylosus* (831, 873, 16) *Staphylococcus warneri* (863), *Staphylococcus saprophyticus* (852). They were grown as described by Talon and Montel (1997).

Resting cells (RCs): after 24 h of growth, the cells were harvested by centrifugation at 10 000 *g* for 30 min at 4°C. The pellet was washed with 20 mM phosphate buffer, pH 7.0, resuspended in this buffer (0.5 g of wet cells/ml) and kept frozen at −20°C.

Extracellular concentrates (ECs): the proteins of the culture supernatants were precipitated with ammonium sulfate (60% saturation). Then the precipitates were dissolved and dialysed in 20 mM phosphate buffer, pH 7.0. The extracellular concentrates were kept frozen at −20°C. Proteins were assayed by the method of Bradford (1976) with a Bio-Rad assay (Bio-Rad Labs., Germany).

2.2. Ester production

Production of esters by RCs and ECs was carried out with ethanol and different acids: acetic, butanoic, 2-methylbutanoic, 3-methylbutanoic, valeric, hexanoic and decanoic, incubated separately. The assay mixture contained 2 ml of substrate prepared in 0.1 M phosphate buffer, pH 7.0 (ethanol 25 μmol ml^{-1} and acid 12.5 μmol ml^{-1}), 1.5 ml of phosphate buffer and 1.0 ml of the different preparations (RCs or ECs). The mixtures were incubated at 30°C with shaking at 150 rpm for 4 h.

To study the effects of the different factors on the production of esters, a full factorial experimental design with four factors including the interactions of first- and second-order was set up. The factors and their levels were: factor 1: strains (7); factor 2: preparations (RCs, ECs); factor 3: pH (5.5, 7.0) and factor 4: temperature (14, 24°C). The same assay conditions as described above were used but only one substrate (ethanol and butanoic acid) was studied.

Three samples of 1.3 ml were taken and frozen at −20°C before analysis. Esters, expressed as nmol g^{-1} wet cells for the RCs and nmol mg^{-1} protein for ECs, were quantified from calibration curves established for each ester studied.

2.3. Analysis by solid-phase microextraction–gas chromatography

The esters were extracted by solid-phase microextraction (SPME) with polydimethylsiloxane coating phase (100 μm), liquid sampling with NaCl saturated solution for 15 min according to the protocol of Vergnais et al. (1998). They were analysed by gas chromatography (GC) on capillary Carbowax CW 20 M column (Supelco, 50 m × 0.32 mm I.D., phase thickness 1 μm) and quantified by flame ionisation detection (FID, 280°C). The GC oven temperature was programmed as follows: 50°C held for 1 min, increased to 220°C at a rate of 3°C/min. Helium was used as carrier gas. For thermal desorption the SPME fiber remained in the injector (200°C) for 5 min.

2.4. Data analysis

The data from the experimental design were studied by analysis of variance on STAT-ITCF (Gouet and Philippeau, 1992) according to the model $X = m + Si + Ej + pHk + Tl + SEij + SpHik + STil + EpHjk + ETjl + pHTkl + SEpHijk + SETijl + SpHTikl + e$ with X ester production, m constant term, S meaning effect of strains ($i = 7$), E meaning effect of preparation ($j = 2$), pH meaning effect of pH ($k = 2$), T meaning effect of temperature ($l = 2$), SE, SpH, ST, EpH, ET, pHT mean effect of first-order interactions, SEpH, SET, SpHT, pHT

mean effect of second-order interactions, and *e* residual variation. The percentage of the variance explained by experimental factors was calculated: % of variance = SSD of factor/SSD of the model × 100 (SSD, sum of the squares of the differences). Comparisons between means were made according to Newman–Keuls test ($\alpha = 5\%$).

3. Results

3.1. Effect of chain length and structure of acids on ethyl ester synthesis

The incubation time of 4 h had been chosen after preliminary experiments with resting cells and extracellular concentrate of *S. warneri* showed that the esterification rate increased until 4 h, and then after 6 h and 24 h the same level of ester or slightly inferior was detected (data not shown).

After 4 h of incubation, esterification was detected in resting cells and extracellular extracts of the different species of Staphylococci (Table 1). The level of esterification depended on the strains, *S. warneri* 863 produced the highest quantities of esters and the two strains of *S. carnosus* produced the lowest quantities.

Esterification specificity varied according to the length of the acid carbon and to the structure of acid for the resting cells and for the extracellular extracts of Staphylococci.

Resting cells of *S. warneri* 863, *S. xylosus* 873, 16 and *S. saprophyticus* 852 preferentially synthesized ethyl butanoate with a rate of esterification varying from 200 to 300 nmol g^{-1} of wet cells (Table 1). *S. warneri* and *S. xylosus* 16 esterified also at a high rate with valeric and hexanoic acids. *S xylosus* 831 had a low activity compared to the two other strains of *S. xylosus*. All these strains weakly esterified acetic, decanoic and branched acids. The cells of *S. carnosus* 833, 836 produced low amount of esters around 4 nmol g^{-1} of wet cells. They showed no apparent substrate specificity, they synthesized esters from butanoic to hexanoic acids with quite the same rate and also showed an important esterification of branched acids (2- and 3-methylbutanoic acids).

The activity of the extracellular concentrate of *S. warneri* was the highest with a maximum of value of 1000 nmol mg^{-1} of proteins for ethyl hexanoate (Table 1). Ethyl butanoate and ethyl valerate were

Table 1
Synthesis of esters with different chain length of acids by restings cells and extracellular concentrates of the Staphylococci

Ethyl esters	EC2	EC4	E2C4	E3C4	EC5	EC6	EC10	Total
Resting cells (nmol g^{-1} of wet cells)								
S. warneri 863	0.00	292.53±48.98	11.60±2.22	8.00±1.10	110.53±15.75	175.51±43.43	1.36±0.45	599.53
S. xylosus 16	18.27±13.17	270.06±20.66	7.81±0.49	26.06±6.45	120.29±31.03	78.56±11.68	9.10±0.07	530.15
S. xylosus 873	32.46±0.01	203.60±55.22	8.45±0.14	2.00±0.74	30.05±0.39	2.06±0.24	3.34±0.04	281.96
S. xylosus 831	0.00	20.44±3.65	4.58±0.70	0.60±0.05	35.45±3.43	4.87±1.13	4.15±2.74	70.09
S. saprophyticus 852	19.95±2.59	197.22±9.12	2.91±0.22	1.37±0.37	17.94±0.98	5.08±2.60	0.00	244.47
S. carnosus 833	0.00	3.98±1.16	2.97±0.082	0.86±0.48	2.08±0.10	2.54±0.52	0.00	12.43
S. carnosus 836	0.00	2.92±0.97	1.03±0.09	0.75±0.21	3.62±0.17	4.41±0.53	0.00	12.73
Extracellular concentrates (nmol mg^{-1} protein)								
S. warneri 863	110.40±10.30	450.00±45.13	8.20±1.25	39.38±12.5	412.17±40.14	1065.32±125.2	13.18±2.18	2098.65
S. xylosus 16	3.53±0.59	16.03±1.89	10.44±0.50	2.91±0.77	16.56±6.18	19.05±0.38	9.10±0.01	77.62
S. xylosus 873	0.00	20.04±1.84	2.69±0.39	0.51±0.05	5.29±1.30	8.63±1.18	1.77±0.11	38.93
S. xylosus 831	0.00	54.76±7.75	9.87±0.85	1.41±0.11	4.16±1.17	40.99±16.8	1.86±0.15	113.05
S. saprophyticus 852	0.00	81.18±16.17	6.45±0.34	0.51±0.10	7.65±1.07	18.17±0.70	1.30±0.15	115.26
S. carnosus 833	0.00	6.87±0.71	2.76±0.34	1.58±0.04	15.03±2.44	7.25±1.03	0.00	33.49
S. carnosus 836	0.00	5.61±0.56	1.11±0.08	0.83±0.11	19.02±2.21	4.51±0.06	0.30±0.15	31.38

EC2: Ethyl acetate, EC4: ethyl butanoate, E2C4: ethyl 2-methylbutanoate, E3C4: ethyl 3-methylbutanoate, EC5: ethyl valerate, EC6: ethyl hexanoate, EC10: ethyl decanoate.
Results are the means of three replicates, ±standard deviation.
Total: Sum of the esters EC2 to EC10.
Esterification was carried out at 30°C, pH 7.0 for 4 h.

the two other esters produced in high quantity. The enzymatic extracts of the other strains showed a total activity varying from 30 to 110 nmol mg^{-1} of protein. *S. saprophyticus* preferentially esterified butanoic acid. The three *S. xylosus* strains esterified butanoic, hexanoic then valeric acids. *S. carnosus* esterified valeric acid at a higher rate followed by butanoic and hexanoic acids. Short chain acid (acetic), long chain acid (decanoic) and branched acids, particularly 3-methylbutanoic acid, were poorly esterified.

3.2. Effect of strains, enzymatic extracts, pH and temperature on ethyl butanoate production

As butanoic acid was esterified by all the strains, the effect of different factors on its esterification was studied in detail. The results of the statistical analysis are summarized in Table 2.

The bacterial strain was the dominant factor, it explained 26% of the variance. There were also significant interactions between this factor and preparation, pH and temperature (Table 2). It is obvious that whatever the conditions, *S. warneri* 863 and *S. xylosus* 16, 873 produced the highest level of ethyl butanoate (Fig. 1), whilst, *S. carnosus* 833, 836 produced the lowest quantities (Fig. 1). For the strains *S. warneri* 863, *S. xylosus* 831, *S. carnosus* 833, 836, the extracellular concentrates were more active than the resting cells, whereas for the two *S. xylosus* strains 16, 873 it was the opposite (Fig. 1). The resting cells and the extracellular extract of the strain *S. saprophyticus* 852 produced ethyl butanoate at similar levels.

Table 2
Factors affecting ethyl butanoate production by different Staphylococci

Factors	1	2
Strains (S)	25.89	****
Preparations (E)	0.15	NS
pH	11.91	****
Temperature (T)	0.91	**
Main interactions		
S × E	19.76	****
S × pH	11.23	****
S × T	4.68	***
E × pH	11.31	****
EE × T	3.38	****
pH × T	0.48	**
S × E × pH	8.22	***
S × E × T	1.01	**
S × pH × T	0.74	NS
E × pH × T	0.11	NS
Residual factor	0.17	

1: Percentage of variance explained by the factors: (SSD of factor/SSD of total variation) × 100, where SSD is the sum of the square of the difference.
2: Significant levels: NS: not significant; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

The pH was also an important factor explaining 12% of the variance (Table 2). This factor interacted with the strains (11% of variance) and the enzymatic preparations (11%) (Table 2). The production of ester was higher at pH 7.0 than at pH 5.5 for the strains *S. warneri* 863, *S. xylosus* 16, 873 and *S. saprophyticus* 852 (Fig. 2A). For these strains, esterification was carried out essentially by resting cells which activities were drastically inhibited at pH 5.5 (Fig. 2B). For the two stains of *S. carnosus* 833, 836 and *S. xylosus* 831 the esterification was similar at both pH values (Fig. 2A). This was because esterification was done mainly by extracellular concentrates which activities were not modified by the pH (Fig. 2B).

The effect of temperature varied with the strains and the preparations (Table 2). We can consider two groups of strains. The first group included *S. warneri* 863, *S. carnosus* 833, 836 and *S. xylosus* 831, it was characterised by a high production of ester at 24°C compared to 14°C (Fig. 3A). For these strains, esterification was essentially done by the extracellular concentrates which activities were inhibited by low temperature (Fig. 3B). The second group included *S. xylosus* 16, 873 and *S. saprophyticus* 852 which produced the same quantity of ester at 24°C and 14°C (Fig. 3A). This can be explained by the fact that the resting cells mainly responsible for esterification were not affected by the temperatures used (Fig. 3B).

4. Discussion

The results on esterification agreed with those on hydrolysis of *p*-nitrophenyl esters by Staphylococci. The strains *S. warneri*, *S. xylosus* 16, 873 and *S. saprophyticus* with the highest hydrolysis activity (Talon and Montel, 1997) produced the highest level

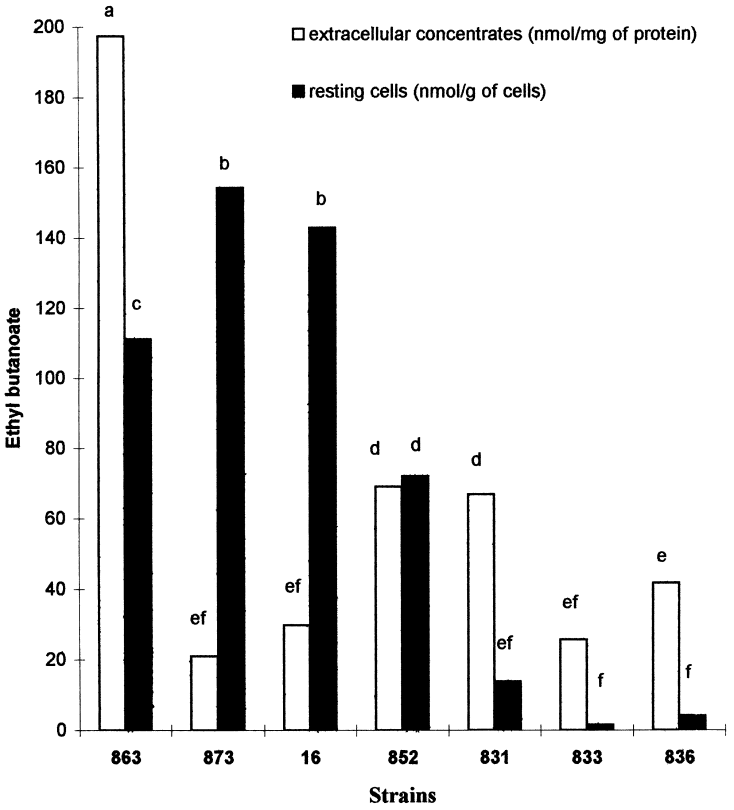

Fig. 1. Production of ethyl butanoate by the extracellular concentrates and the resting cells of *S. warneri* 863, *S. xylosus* 873, 16, 831, *S. saprophyticus* 852 and *S. carnosus* 833, 836 (a, b, c, d, e, f: statistical groups obtained with the Newman–Keuls test, $\alpha = 5\%$).

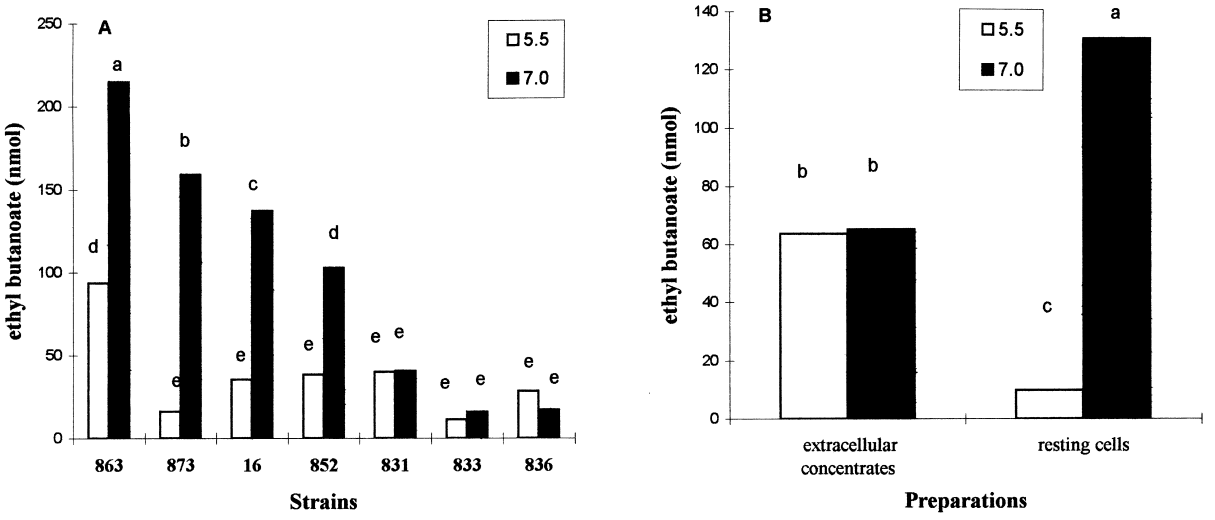

Fig. 2. Effects of factors strains–pH (A) and preparations–pH (B) on the production of ethyl butanoate. (Strains: *S. warneri* 863, *S. xylosus* 873, 16, 831, *S. saprophyticus* 852 and *S. carnosus* 833, 836; a, b, c, d, e: statistical groups obtained with the Newman–Keuls test, $\alpha = 5\%$).

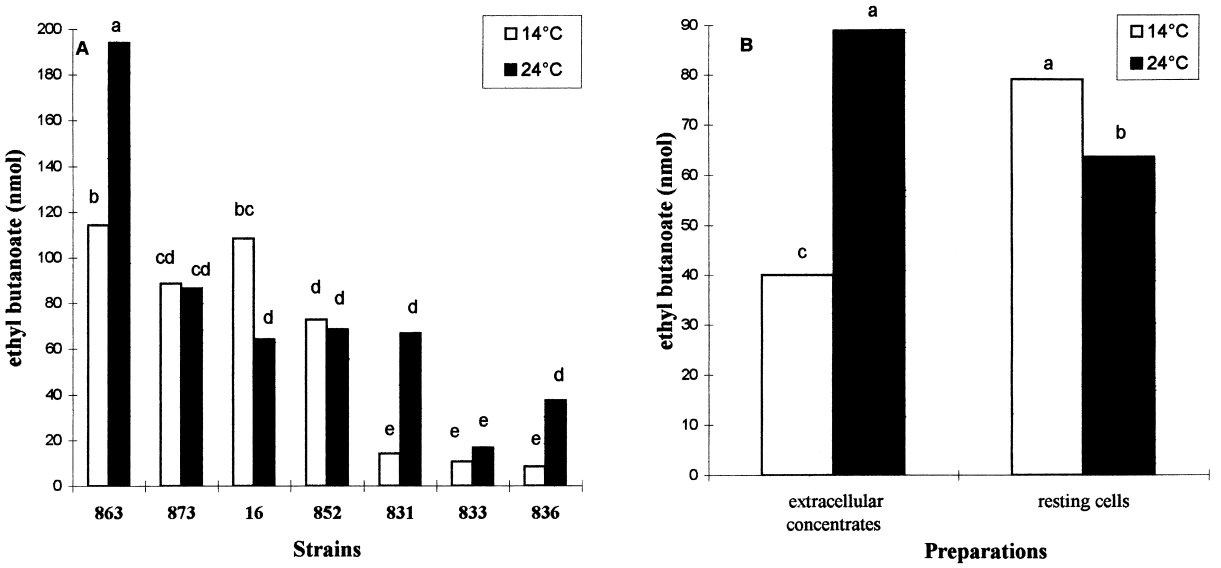

Fig. 3. Effects of factors strains–temperatures (A) and preparations–temperatures (B) on the production of ethyl butanoate. (Strains: *S. warneri* 863, *S. xylosus* 873, 16, 831, *S. saprophyticus* 852 and *S. carnosus* 833, 836; a, b, c, d, e: statistical groups obtained with the Newman–Keuls test, $\alpha = 5\%$).

of esters. In contrast, the strains *S. carnosus* with low hydrolysis activity produced low quantity of esters. It is difficult to compare the rate of hydrolysis and esterification because the substrates were different (ethanol and acids for esterification and *p*-nitrophenyl esters for hydrolysis). Also hydrolysis was carried out with cell free extract and esterification with the resting cells of the strains.

Our results on the level of ethyl esters produced by resting cells of Staphylococci, ranging from 3 to 300 nmol g^{-1} 4 h^{-1}, were comparable to those of thioesters produced by coryneform bacteria, *Micrococcaceae* and Lactococci (Lamberet et al., 1997a,b). In fact, the synthesis of *S*-methyl thioacetate varied from 7 to 240 nmol g^{-1} of wet cells. Intracellular esterases of *Pseudomonas* and lactic acid bacteria produce ethyl esters, those of *Pseudomonas* produce similar quantities of ethyl butanoate and ethyl hexanoate at a rate of 1 μmol 3 h^{-1} g^{-1} of wet cells (Hosono and Elliott, 1974; Hosono et al., 1974). Intracellular esterases of lactic acid bacteria produced mainly ethyl butanoate at a rate ranging from 0.1 to 0.4 μmol 3 h^{-1} g^{-1} of wet cells. In more recent studies, the growth conditions of *Pseudomonas fragi* were optimised to produced high quantity of esters (Raymond et al., 1990, 1991; Cormier et al., 1991). This biomass was used to produce ethyl valerate in phosphate buffer, and a high yield of 13 μmol g^{-1} of wet cells after 48 h of incubation was obtained (Morin et al., 1994).

Concerning the substrate specificity, very close profiles of synthesis and hydrolysis of esters were noticed between the resting cells and the cell free extracts and the extracellular extracts of the Staphylococci (Talon and Montel, 1997). It seems that the same esterases are involved in hydrolysis and esterification. Resting cells of Staphylococci synthesised preferentially ethyl butanoate, and their esterase activity was low with branched acids. For resting cells of coryneform bacteria, *Micrococcaceae* and Lactococci, *S*-methyl thioacetate was the dominant ester (Lamberet et al., 1997a,b). Also, branched acids were esterified to a less extent by some coryneform bacteria (*Brevibacterium linens*) and *Micrococcaceae* and were not esterified by other coryneforms and Lactococci (Lamberet et al., 1997a,b).

The synthesis of ethyl butanoate by resting cells of Staphylococci was inhibited by acid pH of 5.5. Similar inhibition was noticed for the resting cells of Lactococci incubated at pH 5.0 (Lamberet et al., 1997a,b). Also, Hosono and Elliott (1974) observed that acid pH limited the activity of intracellular

esterases of Lactococci, Lactobacilli and *Pseudomonas*. The decrease of pH during the growth of *Pseudomonas fragi* seemed also responsible for the limited production of esters (Raymond et al., 1990). However, the esterase activities of resting cells of coryneform bacteria and *Micrococcaceae* were not inhibited at pH 5.0 (Lamberet et al., 1997b). We also noticed that extracellular enzymes of Staphylococci were not inhibited by acid pH.

The synthesis of ethyl butanoate by resting cells of Staphylococci was higher at 14°C than at 24°C, whereas it was the opposite for the extracellular concentrates. The high potential of production of esters by *Ps. fragi* at low temperature (11°C) was also mentioned by Raymond et al. (1991). The optimal temperature of production of esters by intracellular esterases of *Pseudomonas*, Lactococci and Lactobacilli was 32°C (Hosono and Elliott, 1974). At 20°C, the activity of lactic bacteria enzymes was only 30 to 40% of that at 32°C, whereas those of *Pseudomonas* was still very important at 20°C.

In conclusion, Staphylococci had a high potential for esterification either by their resting cells or their extracellular concentrates. Their esterase activities will depend on the pH, on the temperature but also on the availability of the substrates (acids and alcohols) during sausage manufacturing.

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