ELSEVIER

International Journal of Food Microbiology 36 (1997) 207–214

International Journal of Food Microbiology

Hydrolysis of esters by staphylococci

R. Talon*, M.C. Montel

Station de Recherches sur la Viande, INRA, Theix, 63122 Saint-Genès Champanelle, France

Received 5 December 1996; revised 14 February 1997; accepted 17 March 1997

Abstract

The objective of this work was to characterize the hydrolysis of esters by staphylococci in order to understand if they could contribute to the release of free fatty acids in sausage. Cell-free extracts and extracellular concentrates of staphylococci were examined for esterase activities against *p*-nitrophenyl esters and for lipolytic activities against triolein. Staphylococci showed intracellular and extracellular esterase activities with different esterase electrophoretic patterns. Cell-free extracts of *S. xylosus*, *S. warneri* and *S. saprophyticus* preferentially hydrolysed *p*-nitrophenyl butyrate whereas their extracellular concentrates were mainly active against *p*-nitrophenyl butyrate, *p*-nitrophenyl caproate and *p*-nitrophenyl caprylate. In addition their extracellular concentrates hydrolysed triolein. The two strains of *S. carnosus* differed as they did not show pronounced *p*-nitrophenyl substrate specificity and did not hydrolyse triolein. Staphylococci hydrolysed esters at a high rate between 15 and 25°C; acidic conditions inhibited the hydrolysis. The hydrolysis was also reduced when the water activity was decreased by addition of polyethylene glycol or glycerol.

Keywords: Staphylococci; Esterase; Lipase; Starter cultures

1. Introduction

Staphylococci are found as natural flora of fermented meat products, including *Staphylococcus xylosus*, *Staphylococcus carnosus*, *Staphylococcus saprophyticus* and *Staphylococcus warneri*, frequently identified in dry sausages (Seager et al., 1986; Montel et al., 1992). Only two species, *S. carnosus* and *S. xylosus*, are used as starter cultures.

Staphylococci have been shown to contribute to the development of typical flavour of sausage by modulating the level of volatile compounds (Berdagué et al., 1993; Stahnke, 1995; Montel et al., 1996). Among these compounds, esters and carboxylic acids with low threshold odors seem important.

Esterases and lipases can be involved either in the synthesis or in the hydrolysis of esters. Data in the literature deals mainly with the hydrolysis of triglycerides by the lipases of *Staphylococcus aureus* (Rollof et al., 1987), *Staphylococcus hyicus* (Lechner et al., 1988; Van Oort et al., 1989), *S. warneri* (Talon et al., 1995) and *S. xylosus* (Sorensen and Samuelsen, 1996; Sorensen and Jakobsen, 1996). Concerning esterases, only data on the esterase electrophoretic polymorphism of different strains of

*Corresponding author.

0168-1605/97/$17.00
PII S0168-1605(97)00042-1

S. aureus (Branger and Goulet, 1987) and on species of *Staphylococcus* isolated from mammalian skin (Zimmerman and Kloos, 1976) are available. Ester synthesis is favoured if water is removed from the medium, so in many studies esterification had been carried out in non-aqueous media (Gatfield, 1986; Talon et al., 1996). In sausage, the drying process can also increase the esterification. But sausage is a complex medium in which the availability of substrates (esters, acids, alcohols), pH, water activities change during manufacturing. All these factors affect either hydrolysis or synthesis of esters by staphylococci.

The purpose of this study was to characterize the ability of different staphylococci to hydrolyse esters in order to understand their role in sausage fermentations.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following strains of staphylococci were studied: *Staphylococcus carnosus* (833, 836), *Staphylococcus xylosus* (831, 873, 16), *Staphylococcus warneri* (863), *Staphylococcus saprophyticus* (852). All the strains were isolated from dry sausages (Montel et al., 1992) except *S. xylosus* (16) isolated from cheese. They were grown in 200 ml peptone, yeast extract and salt media (Lechner et al., 1988) for 24 h in 500 ml Erlenmeyer flasks at 30°C and 150 rpm. After 24 h, the cells in stationary growth phase, were harvested by centrifugation at 10 000 × *g* for 30 min at 4°C, washed with phosphate buffer 20 mM pH 7.0, resuspended in this buffer (0.25 g of wet cells/ml) and kept frozen at − 20°C.

2.2. Cell-free extracts (CFE)

The bacterial cells in suspension in the phosphate buffer were disrupted by ultrasonication (Bioblock Scientific Vibracell, France). Cells were treated eight times for 60 s with 60 s pauses in an ice bath, 30% pulse cycle (20 kHz, 40 W). After treatment, the samples were centrifuged at 15 000 × *g* for 30 min at 4°C. The supernatants (CFE) were kept frozen at − 20°C.

2.3. Extracellular concentrates (EC)

The proteins of the culture supernatants corresponding to the bacterial cells were precipitated with ammonium sulfate (60% saturation) at 4°C during 4 h. After centrifugation at 15 000 × *g* for 30 min at 4°C, the precipitates were dissolved in phosphate buffer 20 mM pH 7.0 and dialysed against the same buffer during 16 h at 4°C. The dialysates were centrifuged at 100 000 × *g*, 4°C for 1 h and the supernatants (EC) were kept frozen at − 20°C.

2.4. Protein determination

Proteins were assayed by the method of Bradford (1976) with the Bio Rad assay (Bio Rad laboratories GmbH, Germany) according to the manufacturer's instructions.

2.5. Esterase assay

Esterase activities of CFE and EC were determined using *p*-nitrophenyl esters (PN) of acetic (C2), butyric (C4), caproic (C6), caprylic (C8), capric (C10) and lauric (C12) acids (Sigma Chemical Co., USA). The PN substrates were prepared in acetone at a concentration of 10 mM, they were then diluted in phosphate buffer 0.1 M pH 7.0 to reach a final concentration of 0.16 mM.

Esterase activities were measured in microplates using the incubator of a Bioscreen C (Labsystem, Finland). The assay mixture contained 340 μl of PN substrate at pH 7.0 and 10 μl of the different enzyme extracts (CFE, EC). The assays were incubated at 25°C with shaking for 2 h. The release of *p*-nitrophenol was measured directly by its absorption at 405 nm. The standard curve was prepared by using *p*-nitrophenol. Esterase activity is expressed as nmol of *p*-nitrophenol/min/mg of protein. All assays were run in triplicate.

2.6. Lipase assay

Lipase activity was measured on CFE and EC at 334 nm and 25°C by a turbidimetric method with triolein as substrate as described by Talon et al. (1995). The method was calibrated with a commercially available lipase from *Pseudomonas* (Boeh-

ringer). The results are expressed as μmol of fatty acid released per min per mg of protein.

2.7. Effect of pH, temperatures, water activities on the p-nitrophenyl butyrate hydrolysis

Esterase activity was measured with *p*-nitrophenyl butyrate on CFE and EC as described with the Bioscreen C apparatus. Assays were carried out with substrate prepared in KH_2PO_4, Na_2HPO_4 buffer 0.3 M at different pH: 5.0, 5.5, 6.0, 6.5 and 7.0. Incubation was at 25°C during 2 h with shaking. When the temperatures 15 and 20°C were studied, the Bioscreen C apparatus was placed in a cool room to regulate the temperature at 15°C and 20°C. Incubation was at pH 7.0 for 2 h with shaking.

The water activity of the assay mixture was modified by preparing the substrate in the following different buffers (Chandler and McMeekin, 1989; Kets et al., 1996): phosphate buffer 0.3 M, pH 7.0 (a_w = 1.0); phosphate buffer 0.3 M, pH 7.0 + glycerol 20% v/v (a_w = 0.95); phosphate buffer 0.3 M, pH 7.0 + glycerol 50% v/v(a_w = 0.85); phosphate buffer 0.3 M, pH 7.0 + polyethylene glycol 50% v/v (a_w = 0.75); phosphate buffer 0.3 M, pH 7.0 + NaCl 10.5% w/v (a_w = 0.93); phosphate buffer 0.3 M, pH 7.0 + NaCl 22% w/v (a_w = 0.815). Incubation was at 25°C during 2 h under shaking. All the reactions were determined in triplicate.

2.8. Effect of pH and water activities on esterase stability

The CFE and EC extracts were diluted in the following different buffers: phosphate buffer 0.3 M, pH 7.0, 6.5, 6.0, 5.5, 5.0; phosphate buffer 0.3 M, pH 7.0 + glycerol 50% v/v; phosphate buffer 0.3 M, pH 7.0 + polyethylene glycol 50% v/v; phosphate buffer 0.3 M, pH 7.0 + NaCl 22% w/v.

The mixtures were kept over night at 4°C and the remaining activity was measured on *p*-nitrophenyl butyrate at pH 7.0, 25°C, for 2 h with shaking with the Bioscreen C apparatus. The activity is expressed as a percentage of the activity measured in phosphate buffer pH 7.0 (a_w = 1.0). The samples were run in duplicate.

2.9. Esterase patterns

Esterase patterns of CFE and EC were determined after fractionation by polyacrylamide gel electrophoresis under non denaturing conditions as described by Hames (1990). The gel (12 by 17 cm) was made with a 5% stacking gel and a 7.5% running gel. Migration was conducted at 20 mA/gel under refrigerated conditions.

Esterase activity was visualized by staining in a solution of 2% (w/v) α-naphtyl acetate or butyrate and 500 ppm fast red (Sigma Chemical Co., USA) as described by Harper et al. (1980). Tributyrin activity was visualized as described by Talon et al. (1995).

Protein bands with enzymatic activity were characterized by their relative mobility (R_f) values calculated as the ratio between the migration length of the unknown band and the migration length of bromophenol measured from the beginning of the resolving gel.

3. Results and discussion

Esterase activities were detected in cell-free and extracellular extracts of the different species of staphylococci (Table 1). Hydrolysis specificity varied according to the length of the acid carbon for the cell-free and the extracellular extracts of staphylococci.

The cell-free extracts of *S. saprophyticus* (852), *S. xylosus* (873, 16, 831) and *S. warneri* (863) preferentially hydrolysed *p*-nitrophenyl butyrate with a rate of hydrolysis varying from 24 to 230 nmol/min/mg of protein. By comparison, the activity of the cell-free extracts of the two strains of *S. carnosus* (833, 836) was lower (Table 1).

The activity of the extracellular concentrate of *S. warneri* (863) was the highest with a maximum value of almost 1600 nmol/min/mg of protein. The activities of the strains of *S. carnosus* (833, 836) were showing the lowest values of 10–30 nmol/min/mg of protein (Table 1). The other strains showed activities varying from 85 to 140 nmol/min/mg. The extracellular enzymes of *S. carnosus* (833, 836) showed no apparent substrate specificity whereas those of the other staphylococci hydrolysed preferentially PN-butyrate, PN-caproate and PN-caprylate.

Table 1
Hydrolysis of *p*-nitrophenyl esters with different chain length of acids by cell-free extracts and extracellular concentrates of the staphylococci

p-Nitrophenyl esters	Acetic	Butyric	Caproic	Caprylic	Capric	Lauric
Cell-free extracts (nmol/min/mg of protein)						
S. carnosus 833	9.18	10.20	11.36	8.4	10.00	0.00
S. carnosus 836	5.90	8.26	8.13	7.83	0.00	0.00
S. saprophyticus 852	21.56	39.57	23.61	24.08	0.00	0.00
S. xylosus 873	33.32	78.80	67.31	34.43	0.00	0.00
S. xylosus 16	24.99	77.00	62.50	61.60	15.91	0.00
S. xylosus 831	9.95	23.70	21.71	14.69	0.00	0.00
S. warneri 863	61.53	230.05	124.98	109.50	94.50	47.30
Extracellular concentrates (nmol/min/mg of protein)						
S. carnosus 833	9.91	10.13	7.34	6.91	8.51	2.70
S. carnosus 836	16.13	20.36	27.65	28.20	29.21	17.23
S. saprophyticus 852	22.10	96.56	63.10	59.52	30.94	17.15
S. xylosus 873	17.57	35.59	83.91	71.04	30.29	9.69
S. xylosus 16	23.48	69.29	138.99	114.70	64.10	16.53
S. xylosus 831	12.56	82.91	103.70	87.37	36.86	14.75
S. warneri 863	607.31	1502.85	1588.93	1250.00	725.99	551.42

Extracellular enzymes of *S. saprophyticus* (852), *S. xylosus* (873, 16, 831) and *S. carnosus* (833, 836) hydrolysed PN-laurate whereas cell-free extracts did not. For *S. warneri* (863), both cell-free and extracellular extracts hydrolysed PN-laurate, but the activity of the cell-free was lower (Table 1).

Zimmerman and Kloos (1976), and Branger and Goulet (1987) have assayed esterases in the intracellular fraction of different species of staphylococci, similarly to this study, and several other authors reported that esterase activities are mainly intracellular (El Soda et al., 1986; Khalid et al., 1990; Lee and Lee, 1990). Nevertheless extracellular activities were found in *Halomonas* and *Vibrio* (Hinrichsen et al., 1994) and in *Propionibacterium* (Dupuis et al., 1993). Concerning the specificity, the esterase activity of staphylococci has been studied only on short chain esters (acetate and butyrate) (Zimmerman and Kloos, 1976; Branger and Goulet 1987). Most papers in the literature report on lactic acid bacteria from dairy products. Similarly to our study, lactobacilli, lactococci, streptococci and *Propionibacterium* hydrolysed esters of C4, C6 and C8 whereas esters of C10 and C12 were poor substrates (Khalid et al., 1990; Dupuis et al., 1993; Gobetti et al., 1996).

In sausages, ethyl esters from acetate to decanoate had been identified (Stahnke, 1995; Montel et al., 1996). The staphylococci investigated will be able to hydrolyse these esters: their cell-free extracts will be more active on short chain esters whereas extracellular extracts will be active on medium chain esters (C6 to C10).

As shown in Table 2, *Staphylococcus warneri* had the highest lipolytic activity associated with the

Table 2
Hydrolysis of triolein by cell-free extracts and extracellular concentrates of staphylococci

Bacterial strains	Cell-free extract	Extracellular concentrate
	μmol/mg of protein/min	μmol/mg of protein/min
S. carnosus 833	0	0
S. carnosus 836	0	0
S. saprophyticus 852	0.05	3.65
S. warneri 863	2.82	787.00
S. xylosus 873	0.07	3.54
S. xylosus 16	0.32	35.43
S. xylosus 831	0.065	4.50

extracellular concentrate, confirming published data (Talon et al., 1995). At the opposite extreme the two strains of *S. carnosus* did not hydrolyse triolein. The extracellular enzymes of *S. saprophyticus* and *S. xylosus* strains also hydrolysed triolein but at a lower rate than *S. warneri*. These results corroborated results obtained on the screening of lipase producers in our laboratory (Talon et al., 1992).

The two *S. carnosus* strains (833, 836) had the same esterase profile, whereas all strains of the other species had patterns that differed both in number of bands and in their migration distances (Table 3). Moreover, for each strain, the esterase patterns of cell-free extracts (CFE) and extracellular concentrates (EC) were different showing that some enzymes were secreted in the supernatant.

Table 3
Esterase electrophoretic patterns of cell-free extracts (CFE) and extracellular concentrates (EC) of the different strains of staphylococci

Bacterial strain	Extract	Esterase band	R_f	Substrate[a]		
				α-Naphtylacetate	α-Naphtyl butyrate	Tributyrin
S. warneri 863	CFE	E1	0.01	(+)	+	(+)
		E2	0.28	−	+	(+)
		E3	0.80	+	+	−
	EC	E1	0.01	(+)	+	+ + +
S. carnosus 833, 836	CFE	E1	0.01	+	(+)	(+)
		E4	0.31	−	+	−
		E5	0.49	+ +	−	−
	EC	E1	0.02	+ +	+ +	(+)
		E6	0.16	+	−	−
S. xylosus 831	CFE	E1	0.01	−	+	(+)
		E5	0.49	+ +	−	−
	EC	E1	0.01	−	+	+ +
		E6	0.17	+ +	+ +	+
		E4	0.30	+	+	−
		E7	0.33	+	+	+
S. xylosus 873	CFE	E1	0.01	−	+	(+)
		E6	0.16	+ +	(+)	−
	EC	E1	0.01	−	(+)	+ +
		E8	0.04	+	−	−
		E9	0.11	+ +	+ +	−
S. xylosus 16	CFE	E1	0.01	−	+	(+)
		E9	0.11	+	+	(+)
		E10	0.57	+ +	−	−
	EC	E1	0.01	(+)	(+)	+ + +
		E6	0.16	+ +	+ +	(+)
		E10	0.55	+	−	−
S. saprophyticus 852	CFE	E1	0.01	−	+	(+)
		E6	0.17	+	+	−
		E2	0.27	−	−	(+)
		E7	0.34	+	−	−
	EC	E9	0.11	+ +	+ +	+ +
		E11	0.14	+	+	−
		E6	0.17	(+)	(+)	−
		E12	0.21	+	+	−
		E4	0.30	(+)	(+)	−
		E7	0.34	(+)	−	−

[a] (+) to + + + weak to strong reaction, − no reaction.
R_f Relative mobility of protein.

One band E1 (R_f 0.01) was common to all strains; it was detected in CFE and EC (except in *S. saprophyticus* EC). This enzyme was active on the three substrates in most samples; its activity was weaker in CFE than in EC. The activity of this enzyme on tributyrin was very high in EC of *S. warneri* (863) and *S. xylosus* (831, 873, 16). As the activity on tributyrin can reflect lipase activity (Castberg et al., 1975), this enzyme may be responsible for the hydrolysis of triolein (Table 2). The extracellular concentrate of *S. saprophyticus* also had a lipase active on tributyrin but with a different mobility (E9, R_f 0.11).

The two *S. carnosus* strains had 3 esterases bands in the CFE and 2 in the EC. Besides the enzyme E1 with a broad activity, two enzymes were specific for naphtyl-acetate (E5 R_f 0.49, E6 R_f 0.16) and one for naphtyl-butyrate (E4 R_f 0.31). The CFE of *S. warneri* had 3 esterases bands without pronounced substrate specificity. The EC only had the lipase E1 that hydrolysed tributyrin strongly and the two other substrates weakly. For *S. xylosus* (831, 873, 16), the CFE had 2 or 3 active bands and the EC 3 to 4 bands. One band (E5 R_f 0.49) from the CFE of *S. xylosus* 831 and one (E10 R_f 0.57) from the CFE of *S. xylosus* (16) were specific for hydrolysis of naphtyl-acetate, whereas all the other esterases had no clear specificity (Table 3). *S. saprophyticus* (852) had the greatest diversity of esterases both in CFE (4 bands) and EC (6 bands). Most of the enzymes hydrolysed both naphtyl-acetate and naphtyl-butyrate (Table 3).

The diversity of the esterase patterns observed for the staphylococci used in our study was also reported for different strains of *S. aureus* (Branger and Goulet, 1987) and for 18 *Staphylococcus* species isolated from mammalian skin (Zimmerman and Kloos, 1976). The diversity was also frequently reported for many other species of bacteria and is often used in taxonomy (Khalid et al., 1990; Dupuis et al., 1993; Gobetti et al., 1996).

The esterases of the staphylococci were active on *p*-nitrophenyl butyrate at the 3 temperatures tested (Table 4). The cell-free extracts of the staphylococci had a maximal activity at 25°C and the activity at 15°C represented around 50 to 65% of the activity measured at 25°C. The extracellular concentrate of *S. saprophyticus* (852) had a maximal activity at 20°C. Those of *S. xylosus* (16) and *S. warneri* (863) had quite similar activity at the 3 temperatures. For the other extracellular concentrates (833, 836, 873, 831), the activity at 15°C represented around 50 to 55% of the activity at 25°C. Gobetti et al. (1996) reported that mesophilic and thermophilic lactobacilli had an optimum temperature at 37°C and at 10°C, the esterase activity represented 75% of the maximum value.

Table 4
Effects of temperature on the hydrolysis of *p*-nitrophenyl butyrate by cell-free extracts and extracellular concentrates of staphylococci

Temperatures	15°C	20°C	25°C
Cell-free extracts (nmol/min/mg of protein)			
S. carnosus 833	5.91	7.54	10.20
S. carnosus 836	4.13	6.27	8.26
S. saprophyticus 852	17.38	31.60	39.57
S. xylosus 873	49.64	66.14	78.80
S. xylosus 16	42.35	60.60	77.00
S. xylosus 831	14.22	17.70	23.70
S. warneri 863	138.00	179.40	230.00
Extracellular concentrates (nmol/min/mg of protein)			
S. carnosus 833	4.82	6.64	10.13
S. carnosus 836	11.10	14.60	20.36
S. saprophyticus 852	111.47	184.61	96.56
S. xylosus 873	20.76	27.48	35.59
S. xylosus 16	69.15	73.03	69.29
S. xylosus 831	44.66	67.04	82.91
S. warneri 863	1426.06	1562.25	1502.85

Acid pH had a drastic effect on the esterase activity of the cell-free extracts and extracellular concentrates of the staphylococci (results not shown). The activity at pH 5.0 was strongly inhibited, corresponding to only 2–3% of the maximum activity measured at pH 7.0. Even at pH 6.5, the inhibitory effect was pronounced.

The inhibition of the activity of the esterases in acidic conditions is not likely to be explained by the denaturation of the enzymes as after one night at pH 5.0 and 4°C, the enzymes had retained at least 60% of the activity expressed as a percentage of activity measured at pH 7.0 (results not shown).

Optimum pH values in the range 6.5–7.5 were observed in several studies on the esterase activity of lactic acid bacteria (Khalid et al., 1990; Gobetti et al., 1996). Contrary to the staphylococci, the inhibition of lactic acid bacteria at pH 5.0 was not so drastic, some lactobacilli retained 60% of the activity

measured at pH 7.0, whereas others retained 20% of the activity measured at pH 7.0 (Gobetti et al., 1996).

The hydrolysis of *p*-nitrophenyl butyrate decreased slightly with the decrease of water activity controlled by glycerol or polyethylene glycol (Fig. 1). With sodium chloride in the media, the esterase activities of the cell-free extracts and the extracellular concentrates of staphylococci were activated (Fig. 1). This could be explained by the fact that ions (Na +) can form complexes with ionized acids changing their solubility, so the complex acid is removed from the active center of the enzyme (Sugiura, 1984).

All the enzyme extracts were stable in the buffers examined, retaining between 80 to 100% of the activity in phosphate buffer after one night at 5°C (results not shown).

In conclusion, the staphylococci investigated synthesized esterases with broad substrate specificity. These enzymes hydrolysed esters over a range of temperatures (15–25°C) and water activities (0.98–

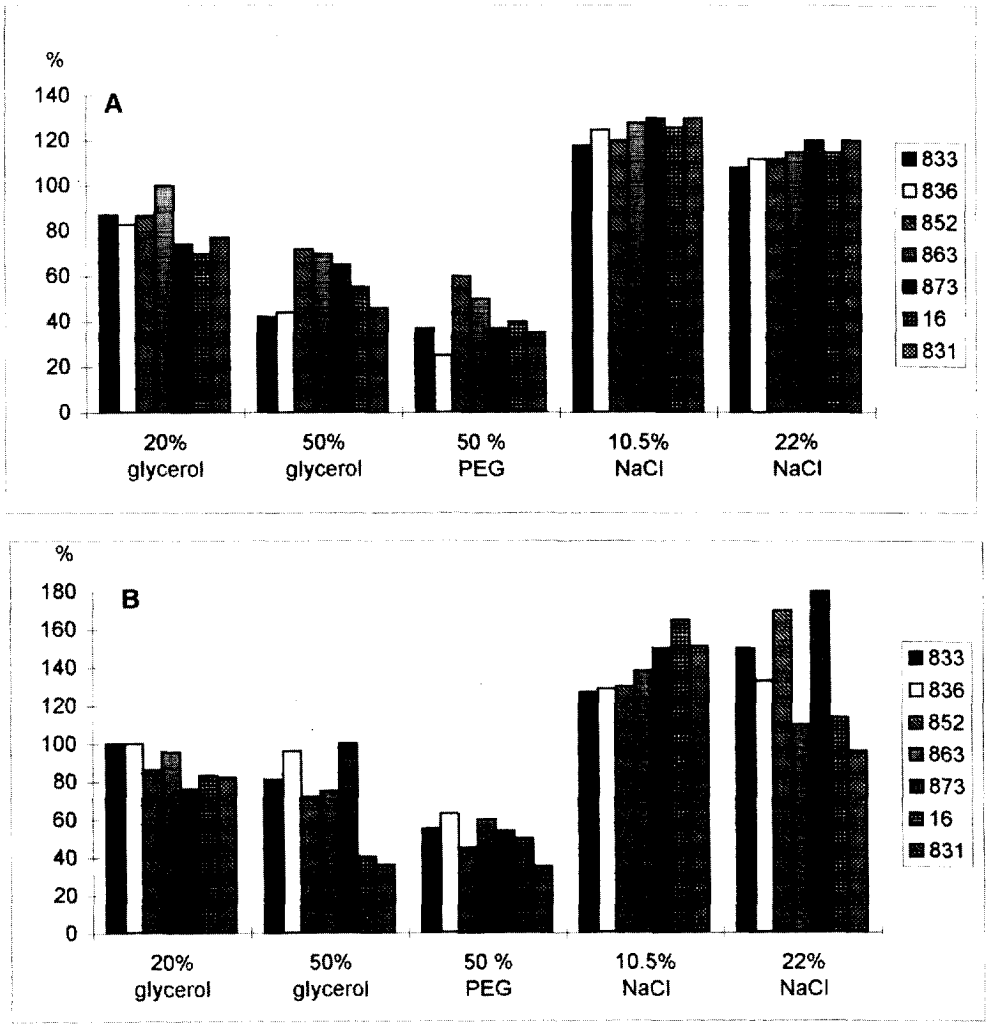

Fig. 1. Esterase activities of cell free extracts (A) and extracellular concentrates (B) of *Staphylococcus carnosus* (833, 836), *Staphylococcus saprophyticus* (852), *Staphylococcus warneri* (863) and *Staphylococcus xylosus* (873, 16, 831) in buffers with different water activities. Activities are expressed as a percentage of the activity measured in phosphate buffer. PEG = polyethylene glycol.

0.85) encountered in sausage manufacturing. However, the acidic values of pH (5.0–6.0) in sausages seem to inhibit the staphylococcal esterases.

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