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Short communication

Factors influencing leucine catabolism by a strain of *Staphylococcus carnosus*

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Abstract

The metabolism of leucine by resting cells of *Staphylococcus carnosus* 833 was studied according to three physico-chemical factors: preculture condition (defined medium; complex medium), nitrate concentration (0% and 0.03%) and stirring condition (static or shaking). A factorial design was set up to test the effects of these factors, each at two levels. The results showed that resting cells of *S. carnosus* 833 produced 3-methyl butanal, 3-methyl butanol and 3-methyl butanoic acid from leucine. Whatever the incubation conditions, there was greater quantity of 3-methyl butanoic acid than 3-methyl butanal and 3-methyl butanol. The preculture and incubation conditions influenced the level of production of the 3 metabolites. The highest overall production of the 3 metabolites was observed when cells were incubated without nitrate in the reaction mixture. 3-methylbutanoic acid production was enhanced when *S. carnosus* 833 was precultivated in complex medium. 3-methylbutanal was only detected when cells were precultivated in defined medium. Stirring condition had no effect on leucine catabolism of *S. carnosus* 833.

Keywords: Leucine catabolism; Odour compounds; *Staphylococcus carnosus*; CPG; Purge and trap; SPME

1. Introduction

In sausage production, starter cultures are widely used in order to shorten the ripening period, ensure colour development, enhance the flavour and improve product safety (Lücke and Hechelmann, 1987). Lactic acid bacteria (*Lactobacillus - Pediococcus*) produce lactic acid from carbohydrate by fermentation which contributes to the acidification of the product (Hammes, 1990). *Micrococcaceae (Staphylococcus - Micrococcus)* ensure colour development by nitrate reductase activity. They can also contribute to the development of dry sausage aroma. *Staphylococcus* strains have been shown to influence the composition of volatile compounds in the products (Berdagué et al., 1993; Montel et al., 1996). Indeed, sausages inoculated with *Staphylococcus warneri* were characterized by a high content

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of alkanes, aldehydes (heptanal, nonanal) whereas high desorption of 2,3-butanediol and 3-hydroxy butanol was associated with the presence of *Staphylococcus saprophyticus* (Berdagué et al., 1993). However, sausages with *Staphylococcus carnosus* and *Staphylococcus xylosus* were distinguished by their high desorption of pentanone, heptanone and branched-chain aldehydes (3-methyl butanal) and their corresponding acids and esters. The desorption of these volatile compounds was correlated with strong dry sausage aroma (Berdagué et al., 1993; Stahnke, 1995; Montel et al., 1996). Among these volatiles, 3-methyl butanal and 3-methylbutanoic acid have a strong impact on sausage aroma (Stahnke, 1995). As these molecules arise from leucine catabolism, the purpose of our study was to evaluate leucine catabolism by a strain of *S. carnosus* 833.

2. Material and methods

2.1. Strain and media

A strain of *S. carnosus* 833 was precultivated on APT (All Purpose Tween) broth (Difco). After overnight incubation at 30°C under shaking (100 rpm), the culture was centrifuged 15 min at 5000 × *g* and cells were washed twice with saline solution (NaCl:8.5%). Cells were inoculated at a density between 10^7 and 10^8 cells in 150 ml of a defined medium described by Hussain et al. (1991) containing 18 amino acids, six vitamins, two bases, four mineral salts without glucose or a complex medium (Proteose peptone: 10 g/l, Yeast extract: 5 g/l, NaCl: 5 g/l) with glucose (1%). After 10 h incubation time at 30°C under shaking (100 rpm), the media were centrifuged for 15 min at 5000 *g*, cells were harvested, washed and then suspended in phosphate buffer (KH_2PO_4–K_2HPO_4, 0.1 M, pH = 7) to obtain a final optical density (660 nm) of 0.7 in the reaction mixture (20 ml). The reaction mixture was composed of leucine (10 mM), pyridoxal phosphate (0.1 mM), vitamins (D biotin: 0.1 μg/ml; nicotinic acid: 2 μg/ml; panthotenic acid: 2 μg/ml; riboflavin: 2 μg/ml; thiamine HCl: 2 μg/ml), mineral salts ($CaCl_2$: 5 μg/ml; $MnSO_4$, $2H_2O$: 4.4 μg/ml; $FeSO_4$: 6 μg/ml). It was prepared with phosphate buffer KH_2PO_4–K_2HPO_4 (0.1 M), pH = 5.8. Its composition varied with respect to the nitrate concentration (0% and 0.03%) and shaking (300 rpm). Each incubation condition were performed twice. After 24 h incubation at 23°C, the medium was separated into two fractions of 10 ml which were frozen at −20°C until gas chromatography analysis. Controls without cells or without leucine were prepared in the same conditions.

2.2. Factorial design

The three factors –preculture condition, nitrate concentration and stirring condition –were integrated into a factorial design. Each factor was studied at two levels, preculture condition: defined medium or complex medium, incubation conditions: nitrate concentration (0% or 0.03%), stirring condition (0 rpm or 300 rpm). The experimental design consisted of a set of eight experimental runs (2^3), repeated twice. The experimental data was analysed by variance analysis with a STAT-ITCF software (Gouet and Philippeau, 1992).

2.3. Analytical methods

Overall analysis of volatile compounds from reaction incubated with cells precultivated in defined medium without stirring and without nitrate was carried out by headspace gas chromatography–mass spectrometry. A Varian gas chromatograph equipped with a Finnigan MAT ITS 40 ion trap, according to the method described by Hinrichsen and Andersen (1994). Calibration was performed with dodecane.

Aromatic compounds of the reaction mixtures were also extracted by solid phase micro extraction (SPME) as it was described by Vergnais et al. (1998). Two milliliters of the reaction mixture were placed in a sealed tube with sodium chloride (37% w/v, saturated solution) and hydrochloric acid 0.2%. The sample was heated at 70°C, under shaking (600 rpm) and aromatic compounds were adsorbed on a polyacrylamide coated phase (85 μm, Supelco Co.). After 30 min, the volatile compounds were thermally desorbed and injected into a GC capillary column (Supelcowax 10; 60 m × 0.32 mm; 0.1 μm - Supelco Co.). Standard solutions of 3-methyl butanal, 3-methyl butanol and 3-methyl butanoic acid were extracted and analysed in the same conditions.

3. Results and discussion

3.1. Identification of volatile compounds

Volatile compounds in a reaction mixture containing leucine and inoculated with *S. carnosus* cells were compared with reaction mixture with leucine alone or cells alone. The results are presented in Table 1. Traces of aldehydes, alcohols, acetone and diacetyl were detected in media containing leucine and inoculated with *S. carnosus* 833 but also in the control media (Table 1). These metabolites were present in the substrate leucine without cells or in the cells of *S. carnosus* 833 alone. However, the desorption of 3-methyl butanal, 3-methyl butanol and 3-methylbutanoic acid was 3 fold higher in reaction mixtures with cells and leucine than in controls with the substrate alone or with the cells alone. So, *S. carnosus* 833 was able to catabolise leucine in odour compounds, 3-methyl butanal, 3-methyl butanol and 3-methylbutanoic acid. Then, the bacterial origin of 3-methyl butanal, 3-methyl butanol and 3-methylbutanoic acid in fermented sausages is confirmed as it was suggested by the results of Berdagué et al. (1993), Stahnke (1994) and Montel et al. (1996).

The headspace-GC method of Hinrichsen and Andersen (1994) did not allow a high desorption of 3-methylbutanoic acid which was only quantified with dodecane. On the contrary, by the SPME–GC method (Vergnais et al., 1998), the surface response obtained for 3-methylbutanoic acid was high (data not shown) corresponding, after specific quantification, to the highest quantity desorbed.

3.2. Effect of the factors on the metabolite production

Whatever the preculture and the incubation conditions (nitrate, stirring), 3-methylbutanoic acid was quantitatively more important than 3-methyl butanal and 3-methyl butanol (Table 2). It represented 99% of the three metabolites detected. On the contrary, 3-methyl butanol was quantitatively the compound least detected (4 to 25 ng/ml). The literature data indicates that leucine in presence of α-ketoglutaric acid can be transaminated or oxidatively desaminated into α-ketoisocaproic acid which can be non oxidatively decarboxylated into 3-methyl butanal (Tucker and Morgan, 1967; Yvon et al., 1997; Bigelis et al., 1983; Couder and Vandecasteele, 1975). 3-methyl butanol and 3-methyl butanoic acid could be produced from reduction or oxidation of 3-methyl butanal by alcohol or aldehyde dehydrogenase re-

Table 1
GC–MS analysis of defined medium inoculated or not with *S. carnosus* 833 and incubated with leucine without nitrate under static conditions. Metabolites were extracted by purge and trap

Media	GC peak area		
	Inoculated with Leucine	Inoculated without Leucine	Uninoculated with Leucine
Acetone	1436	2267	1315
Isopropanol	2011	1263	1408
2-Methyl propanal	174	0	867
Diacetyl	0	53	79
3-Methyl butanal	31595	0	8341
Pentanal	0	68	0
3-Methyl butanol	62	0	6
Heptanal	0	0	200
3-Methylbutanoic acid	161	0	58
Benzaldehyde	132	124	217
Octanal	137	62	130
Octanol	189	106	74
2-Hydroxy benzaldehyde	99	105	145
Nonanal	3494	359	286
Phenol	74	82	149
Decanal	689	506	197

Table 2
Statistical analysis of 3-methyl butanal, 3-methyl butanol and 3-methylbutanoic acid production (in ng/ml) by *S. carnosus* 833[a]

Factors	3-Methyl butanal			3-Methyl butanol			3-Methylbutanoic acid			Total of the 3 metabolites		
		1[b]	2[c]		1	2		1	2		1	2
Preculture condition		**	45.5		NS	0.2		**	9.2		*	4.3
defined medium	208 a			11			1393 b			1612 b		
complex medium	13 b			10			2743 a			2766 a		
Nitrate content		*	13		x	27		****	71.8		****	74
0%	58 b			16			3948 a			4022 a		
0.03%	163 a			4			188 b			355 b		
Stirring condition		NS	4.3		NS	2.7		NS	0.5		NS	1.5
Static	80			12			1907			2000		
Shaking	141			8			2229			2378		
INTERACTIONS												
Preculture * Nitrate		*	15.4		NS	2.7		**	11.5		**	9.8
Defined * 0%	99 b			15			2520 b			2631		
Defined * 0.03%	318 a			7			266 c			591 c		
Complex * 0%	17 b			18			5376 a			5410 a		
Complex * 0.03%	9 b			2			110 c			121 c		
Preculture *Stirring		NS	3.1		NS	0.9		NS	0.3		NS	1.2
Defined * Static	152			12			1117			1281		
Defined * Shaking	264			10			1670			1944		
Complex * Static	8			13			2698			2719		
Complex * Shaking	17			7			2787			2811		
Nitrate * Stirring		NS	0.15		NS	0.2		NS	0.5		NS	1.3
0% * Static	33			18			3630			3681		
0% * Shaking	82			15			4267			4364		
0.03% * Static	127			7			185			319		
0.03% * Shaking	199			2			191			392		
Preculture * Nitrate * Stirring		NS	0.009		NS	23.6		NS	0.5		NS	1.2
Defined * 0% * Static	50			10			1930			1990		
Defined * 0% * Shaking	148			20			3112			3280		
Defined * 0.03% * Static	255			14			303			572		
Defined * 0.03% *Shaking	380			0			228			608		
Complex * 0% * Static	17			25			5330			5377		
Complex * 0% * Shaking	17			10			5422			5449		
Complex * 0.03% * Static	0			0			66			66		
Complex * 0.03% *Shaking	17			4			153			174		
Residual factor			18.4			42.6			5.6			5.8

[a] Metabolites were analysed by SPME–GC method. Means with different letters a, b, c, d were significantly different by Newmann-Keuls statistical test ($\alpha = 5\%$).

[b] 1: Significant levels: NS: Not significant: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

[c] 2: % of variance explained by the factors: (SSD of factor/SSD of total variations) $\times$ 100. SSD = Sum of the Square of the difference.

spectively. These enzymes were described for *Saccharomyces cerevisiae* (Bigelis et al., 1983), *Candida utilis* (Derrick and Large, 1993) and *Streptococcus lactis* var. *maltigenes* (Morgan et al., 1965). However, 3-methyl butanoic acid could be produced without previous production of 3-methyl butanal by oxidative decarboxylation of α-ketoisocaproic acid, involving decarboxylase, transacylase and lipoamide oxydoreductase (Rüdiger et al., 1972). Such enzymatic complex was found for *Bacillus subtilis* (Namba et al., 1969) and *Enterococcus faecalis* (Rüdiger et al., 1972). These different enzymes could exist for *S. carnosus*.

Factors effect (significant level, % variance) on 3-methyl butanal, 3-methyl butanol and 3-methylbutanoic acid production by *S. carnosus* 833 inferred from the experimental design are shown in Table 2. Nitrate was the prevailing factor ($p <$ 0.001) influencing the production of these metabolites; it affected mainly the 3-methylbutanoic acid

production and explained 72% of its variance (Table 2). The production of 3-methylbutanoic acid was 20-fold higher in reaction mixture without nitrate than with nitrate. Nitrate or its reduction in nitrite could inhibit the multienzymatic complex and the aldehyde dehydrogenase (Fig. 1b). On the contrary 3-methylbutanal production was increased with nitrate in the reaction mixture.

The preculture condition influenced ($p < 0.05$) the metabolite production (Table 2). Nevertheless the significant interaction ($p < 0.001$) between preculture condition and nitrate concentration showed that the preculture conditions had no effect when there was nitrate in the reaction mixture (Table 2). Whatever the conditions in the reaction mixture *S. carnosus* 833 produced 2-fold higher 3-methylbutanoic acid when it was precultivated in complex medium than in defined medium (Table 2). The complex medium could favoured aminotransferase or L-amino acid oxidase involved in the formation of α-ketoisocaproic acid. Detection of 3-methylbutanal was the highest when cells were precultivated in defined medium and incubated with nitrate. Consequently, in defined medium, decarboxylase and aldehyde dehydrogenase could be synthetised besides the multienzymatic complexe but this latter could be more important in complex medium (Fig. 1a, b).

Stirring conditions had no significant effect on the

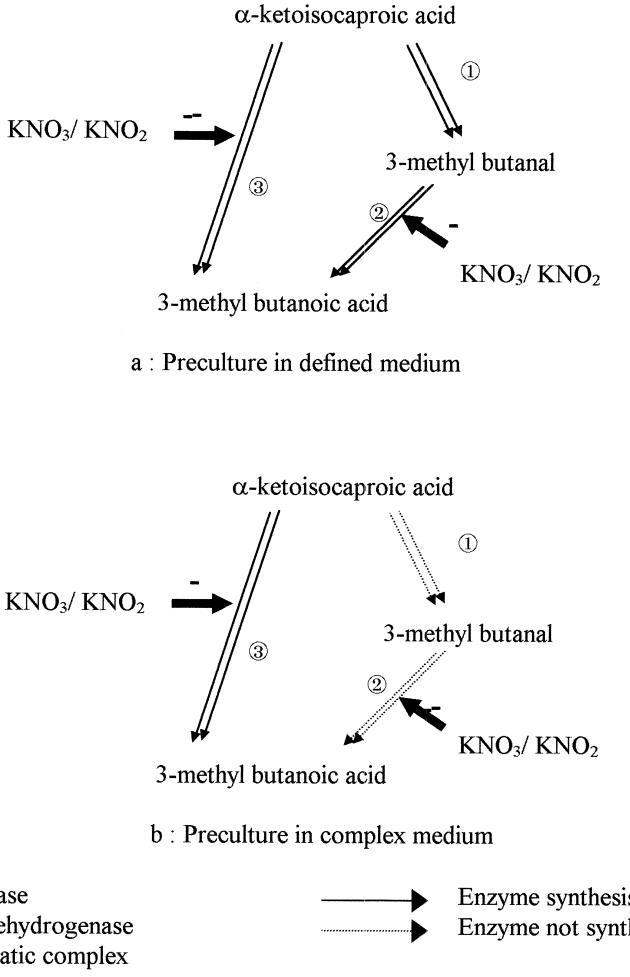

Fig. 1. Hypothesis expressed on the regulation of the enzymatic pathway of *S. carnosus* 833 involved in the conversion of α-ketoisocaproic acid into 3-methyl butanoic acid. (a): Preculture in defined medium; (b) Preculture in complex medium.

production of the three metabolites by *S. carnosus* 833.

In the technological conditions of fermented sausage, the overall production of these volatile compounds would be enhanced by the glucose added initially in the raw mixture. But the addition of nitrate would limit their optimal production. Stahnke (1995) noticed that nitrate decreased the production of 3-methyl butanol in fermented sausage inoculated with *S. xylosus*. In our experimental conditions, the amount of the 3 metabolites released by *S. carnosus* 833 could have an impact on the aroma, according to the threshold detection (in water) of 3-methyl butanal: 0.06 μg/ml (Sheldon et al., 1971), 3-methyl butanol: 4.75 μg/ml and 3-methyl butanoic acid: 0.07 μg/ml (Molimard and Spinnler, 1995).

Further studies of regulation of the enzyme activities should be undertaken for a better understanding of odour compounds production by starter cultures in sausages.

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