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Decarboxylase activity involved in methyl ketone production by *Staphylococcus carnosus* 833, a strain used in sausage fermentation

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Abstract

Staphylococcus carnosus strain 833, inoculated into sausage, increased the levels of methyl ketones which contributed to the cured aroma. These ketones were predicted to arise from incomplete β-oxidation followed by a decarboxylation. To check this hypothesis, we measured the β-decarboxylase activity in resting cells of *S. carnosus* grown in complex or in synthetic medium, using as substrate a β-ketoacid, which can be an intermediate of the β-oxidation pathway. This activity was present throughout the growth period. The enzyme appeared to be constitutive because no induction was observed. High aeration, a pH of 5 and the presence of nitrate promoted the production of methyl ketones.

Keywords: Fermented sausage; β-Oxidation; Methyl ketone; β-Decarboxylase; *Staphylococcus carnosus*

1. Introduction

The characteristic taste and aroma of fermented sausage are due to many different non-volatile and volatile compounds. Some of them originate from added spices, the others are products of metabolic or chemical reactions derived from carbohydrates, proteins and lipids during ripening. Microbial activities in the sausage mince, as well as the enzymic activities from the lean meat and fat, are responsible for many of those components. It is unknown which processes play the major part in the desirable aroma development. One of the processes studied is the breakdown of triglycerides into free fatty acids, di- and monoglycerides during ripening and the steady increase of different carbonyl oxidation products like aldehydes and ketones [1–6]. Increased levels of ketones and alcohols were recorded when *Staphylococcus carnosus* and *Staphylococcus xylosus* were inoculated into sausage mince as a starter culture [5,7]. However, the staphylococcal enzymic pathway leading to methyl ketones is unknown.

One of the most important compounds responsible for the typical flavor of Blue and Roquefort cheeses is 2-heptanone and its production has been associated with β-oxidation of fatty acids by filamentous fungi [8,9]. The mechanism used by fungi to synthesize methyl ketones from fatty acids has been postulated as an abortive β-oxidation sequence [10]. This pathway includes the release of β-ketoacyl-CoA esters that are deacylated into β-ketoacids and finally decarboxylated to methyl ketones.

Concerning *S. carnosus*, Engelvin et al. [11] have reported the existence of a β-oxidative pathway, so it is possible that methyl ketones can arise, as in fungi, from free fatty acids or from intermediates of β-oxidation.

The main goal of this study was to establish the metabolic origin of ketones by identifying the final step of the β-decarboxylation leading to CO_2 and methyl ketone production in the *S. carnosus* 833 strain. Induction assays and a fractional factorial design were used to study the influence of technological conditions on methyl ketone production.

2. Materials and methods

2.1. Chemicals

Ethylbutyryl acetate, DL-β-hydroxycaprylic acid, lauric

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acid methyl ester, oleic acid methyl ester, alcohol dehydrogenase from *Thermoanaerobium brockii* (5–15 units (mg protein)$^{-1}$), pyridoxal phosphate (PLP), biotin, β-NADPH and porcine liver esterase (250 units (mg protein)$^{-1}$) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The 2-pentanone (99% redistilled) and buffer salts were analytical reagent grade products from Aldrich Co., Inc. (Milwaukee, WI, USA).

2.2. Culture conditions of S. carnosus 833 strain

S. carnosus 833, isolated from French fermented sausages [12], was grown in a complex medium (CM) [13] or in a chemically defined medium (CDM) containing amino acids, vitamins, glucose and salts [14]. The growth of the strain was measured spectrophotometrically at $\lambda = 600$ nm. Bacteria were inoculated at an initial OD_{600} of 0.2 and cultured aerobically at 30°C in a 1-l Erlenmeyer flask containing 500 ml of medium on a rotary shaker at 170 rpm. For enzymic experiments bacteria were collected after 24 h of growth.

To study the effect of growth phase on methyl ketone production, the strain was collected by centrifugation from CM medium after 4, 6, 16 and 24 h.

2.3. Effect of glucose on 2-pentanone production

Different glucose concentrations (0, 0.1 and 1%) were added to the CDM buffered where stated K_2HPO_4-KH_2PO_4 (100 mM, pH 7.0).

2.4. Effect of different compounds on 2-pentanone production

The CDM was supplemented with β-ketoester (ethylbutyryl acetate, C8), hydroxyacid (DL-β-hydroxycaprylic acid, C6) or methyl esters, (lauric, C12 or oleic, C18:1) at a final concentration of 2 mM. CDM was also supplemented with 2-pentanone at 2 mM to study the effect of the presence of ketone on the β-decarboxylase activity. Biotin or PLP were omitted in the preparation of CDM to study the influence of these cofactors.

2.5. Effect of technological conditions and data analysis

Cells were grown for 24 h in CM. The effect of technological conditions used during sausage manufacture were studied with an experimental protocol using a fractional factorial design (2^{6-3}). In experimental designs, treatments are defined as the combinations of two or more levels of more than one factor or variable, but, when there is a large number of factors, even at two levels each, the total number of treatments becomes so large that it is very difficult to organize an experiment involving these treatments. In these conditions, fractional factorial designs are used: a large fraction of the treatments are not tested. The tested treatments are used to compute correctly only the main effects, the interaction effects between factors are 'sacrificed'. Each assay was a combination of the variables taken at a low level (−1) and at a high level (+1) as shown in Table 1. CM was buffered with 67 mM Na_2HPO_4-KH_2PO_4 buffer, pH 6.0 or pH 5.0 (initial pH of raw material or final pH of sausage, respectively). A multiple linear regression analysis was performed to determine the influence of each variable on methyl ketone production. Analysis to predict the conditions for optimal and minimal methyl ketone production were set up using the simplex method [15].

2.6. Preparation of cell suspensions

Cells were harvested at $7000 \times g$ for 10 min at 4°C and washed twice with K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 7.0). The resulting pellet was resuspended in the same buffer at a final concentration of 0.5 g of wet weight cells ml^{-1} (resting cells) and conserved at −20°C until assayed for methyl ketone production.

2.7. Procedure for methyl ketone production

The assay was based on the measurement of 2-pentanone formation following the incubation of the resting cells with ethylbutyryl acetate. The incubation mixture contained 400 μl of resting cells (0.2 g of wet weight cells), ethylbutyryl acetate (4 mM) as substrate, esterase (2.7 units) and K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 7.0) in a 1-ml final volume. Either the bacteria or the substrate were omitted from the two controls. The reaction mixture was incubated with shaking in a 2-ml Eppendorf tube at 30°C for 1 h. The reaction was stopped by removal of the cells by centrifugation ($15\,000 \times g$). The supernatants containing 2-pentanone were kept at −20°C until enzymic quantification. Three independent assays were carried out for each experiment.

Table 1
Study of the effect of six factors at two levels on methyl ketone production using a Plackett–Burman design

Batch No.	Factors					
	pH	NaCl	KNO_3	T	Air	G
1	−1[a]	−1	+1	−1	+1	+1
2	+1[a]	−1	−1	+1	−1	+1
3	+1	+1	−1	−1	+1	−1
4	+1	+1	+1	−1	−1	+1
5	−1	+1	+1	+1	−1	−1
6	+1	−1	+1	+1	+1	−1
7	−1	+1	−1	+1	+1	+1
8	−1	−1	−1	−1	−1	−1

[a]Analyzed levels: pH: 5.0 (−1), 6.0 (+1); NaCl: 0.5% (−1), 3.5% (+1); KNO_3: 0% (−1), 0.03% (+1); T: temperature: 15°C (−1), 24°C (+1); air: aeration: cells cultivated in 750 ml of CM in a 1-l Erlenmeyer flask (1:3 v/v) (−1), or in 500 ml of CM in a 1-l Erlenmeyer flask (1:2 v/v) (+1); G: glucose: 0% (−1), 0.5% (+1).

2.8. Enzymic method to quantify methyl ketones

The procedure employs a thermostable NADPH-dependent alcohol dehydrogenase (ADH) (EC 1.1.1.2) [16]. This enzyme reduces the methyl ketone to its secondary alcohol and the rate of NADPH oxidation is directly related to the ketone content [17]. The enzymic reduction of 2-pentanone was monitored continuously with a Shimadzu UV-Visible 160A spectrophotometer. The reaction mixture, in Tris–HCl buffer (100 mM, pH 7.8) made up to a final volume of 1 ml, contained the following reagents: 200 μl of the sample, 100 μl of β-NADPH (100 mM) and 21 μl of ADH solution (0.94 units ml^{-1}). The β-NADPH oxidation was followed continuously at 334 nm at 40°C for 10 min. The β-NADPH and enzyme solutions were kept on ice. A calibration curve of 2-pentanone was made from 0 to 500 μM. Four replicates were undertaken for each analysis. The results were expressed as μmol of 2-pentanone (g of wet weight cells)$^{-1}$ h^{-1}.

3. Results

3.1. Production of 2-pentanone in CM and CDM

The resting cells, previously grown in CM, converted in 1 h 10.75 μmoles (g cells)$^{-1}$ of substrate (ethylbutyryl acetate) to 2-pentanone. Only small amounts of 2-pentanone were produced by the resting cells previously grown in CDM (1.0 μmol (g cell)$^{-1}$ h^{-1}).

3.2. Effect of glucose on 2-pentanone production

Methyl ketone production was low when cells were cultivated in the original CDM which contained 1% glucose [14] (Table 2). The production of 2-pentanone was 1.8-fold higher with cells cultivated in buffered CDM (pH 7.0) with 0.1% glucose, but cells cultivated in the absence of glucose did not produce 2-pentanone despite their capacity to grow. As the 2-pentanone yield was optimal when cells were grown in buffered CDM (pH 7.0) containing 0.1% glucose, these conditions were used for the further assays.

Table 2
Effect of glucose concentration on 2-pentanone production by resting cells of *S. carnosus*

Glucose (%)	2-Pentanone (μmol (g cell)$^{-1}$ h^{-1})	
	Non-buffered CDM	Buffered CDM (pH 7.0)
1	1.00 ± 0.03[a]	4.60 ± 0.03
0.1	5.25 ± 0.04	8.15 ± 0.04
0	0	0

[a]Production of 2-pentanone by resting cells grown in the CDM described by Hussain [14].

3.3. Effect of physiological states on 2-pentanone production

The cells grown in CM showed a similar β-decarboxylase activity in early exponential (4 h), end exponential (6 h) and stationary growth phases (16 h, 24 h). The production of 2-pentanone varied from 9.0 to 10.75 μmol (g cell)$^{-1}$ h^{-1} in all states of growth. Similar results were observed for cells cultivated in CDM (data not shown).

3.4. Effect of different compounds on 2-pentanone production

The cells grown in CDM in the presence of β-oxidation substrates showed a lower methyl ketone production (Table 3). The production of 2-pentanone decreased by 35% or 32.5% when cells were grown in the presence of lauric or oleic methyl esters, respectively. However, the addition of intermediates of the β-oxidation pathway to the CDM stimulated the production of ketone: a 1.5-fold increase in 2-pentanone content for cells grown in the presence of ethylbutyryl acetate (β-ketoester) and a 2-fold increase for the cells grown in the presence of DL-β hydroxycaprylic acid (β-hydroxyacid). After 24 h of *S. carnosus* growth in the presence of β-hydroxyacid, the supernatant culture smelt strongly like blue cheese due to the synthesis of 2-heptanone in the growth medium.

The presence of 2-pentanone in the growth medium slightly inhibited the ethylbutyryl acetate transformation relative to the control (Table 3).

The role of the potential cofactors of the β-decarboxylase enzyme was also investigated in CDM. The cells grown in the absence of biotin or PLP had a lower β-decarboxylase activity. The absence of PLP had a greater effect, producing 22.7% less activity than in the control sample (Table 3).

Table 3
Effect of different compounds on 2-pentanone production by resting cells of *S. carnosus*

Compounds[a]	2-Pentanone (μmol (g cell)$^{-1}$ h^{-1})
Control	8.15 ± 0.03
Oleic acid (C18:1)	5.50 ± 0.02
Lauric acid (C12)	5.30 ± 0.02
Ethylbutyryl acetate (C6)	12.25 ± 0.03
DL-β-Hydroxycaprylic acid (C8)	17.25 ± 0.03
2-Pentanone	7.30 ± 0.02
Biotin[b]	7.55 ± 0.03
PLP[b]	6.30 ± 0.02

[a]The compounds were added to the CDM, buffered at pH 7.0 and with 0.1% of glucose, at the beginning of the culture.
[b]Biotin and PLP were omitted in the preparation of CDM.

3.5. Effect of technological conditions on 2-pentanone production

The results of the statistical analysis are shown in Table 4. The pH, the presence of KNO_3 and a high aeration of

Table 4
Influence of technological factors on the production of 2-pentanone by resting cells of *S. carnosus*

Constant	Coefficient[a]	Technological condition combinations[b]													
		Effect of:													
	25.27***	pH		NaCl (%)		KNO_3 (%)		Air (v/v)		G (%)		T (°C)		Min[c]	Max[c]
pH	−4.02***	**5**	**6**	5.50	5.50	5.50	5.50	5.50	5.50	5.50	5.50	5.50	5.50	6	5
NaCl	−0.33*	2.00	2.00	**0.50**	**3.50**	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	3.50	0.50
KNO_3	92.79***	0.015	0.015	0.015	0.015	**0**	**0.030**	0.015	0.015	0.015	0.015	0.015	0.015	0	0.030
Air	16.99***	0.42	0.42	0.42	0.42	0.42	0.42	**0.33**	**0.50**	0.42	0.42	0.42	0.42	0.33	0.50
G	2.17*	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	**0**	**0.50**	0.25	0.25	0	0.50
T	0.04 (NS)	19.50	19.50	19.50	19.50	19.50	19.50	19.50	19.50	19.50	19.5	**14**	**24**	14	24
2-Pentanone μmol (g cell)$^{-1}$ h^{-1}		14.45	10.45	12.95	11.95	11.05	13.85	13.85	11.05	11.90	13.00	12.25	12.65	6.40	18.50

[a]Coefficient in the variation and probability: ***$P < 0.1\%$; **$0.1\% < P < 1\%$; *$1\% < P < 5\%$; NS, not significant.
[b]Calculated variation of the methyl ketone production when each factor changed from lower to upper level keeping the others at the average level. Average level for pH: 5.5; for NaCl: 2%; for KNO_3: 0.015%; for air: 0.42 (v/v); for glucose (G): 0.25%; for temperature (T): 19.5°C.
[c]Predicted curing conditions for maximal (max) or minimal (min) production of methyl ketone. Bacteria were grown in the complex medium.

the growth medium highly influenced 2-pentanone production by resting cells. When the pH of the growth medium was increased from 5 to 6, the production of 2-pentanone decreased. Cells grown in higher aeration conditions or in the presence of KNO_3 produced 12.8 or 10.7% more 2-pentanone, respectively. Sodium chloride and glucose had a less significant effect than the former factors. The production of ketone was slightly inhibited in cells grown in the presence of 3.5% NaCl whereas it was slightly improved in the presence of 0.5% of glucose in the CM. Temperature did not affect significantly 2-pentanone production.

The model was used to find combinations of factors inhibiting or stimulating 2-pentanone production (Table 4). Validation of the model was undertaken using conditions corresponding to optimal (24°C, pH 5, aeration of 1:2 v/v, 0.5% NaCl, 0.03% KNO_3 and 0.5% glucose) and minimal (14°C, pH 6, aeration of 1:3 v/v, 3.5% NaCl, no nitrate and no glucose) production of 2-pentanone. No significant differences were observed between the experimental and the predicted values. A 16 ± 2 μmol (g cell)$^{-1}$ h^{-1} of 2-pentanone production was measured in the optimal production conditions versus a predicted value of 18.5 ± 3 μmol (g cell)$^{-1}$ h^{-1}. A value of 8.2 ± 2 μmol (g cell)$^{-1}$ h^{-1} was measured in minimal conditions and was in agreement with the predicted value: 6.4 ± 3 μmol (g cell)$^{-1}$ h^{-1}.

4. Discussion

The inoculation of *S. carnosus* into sausage mixture increased the level of some volatile compounds, including ketones [5,7], which contributed to the cured aroma. We hypothesized that *S. carnosus* can produce methyl ketones via fatty acid β-oxidation.

In previous work, Engelvin et al. [18] demonstrated the existence of the β-oxidation pathway in the *S. carnosus* 833 strain, as well as the liberation of intermediates, mainly β-hydroxyacids, throughout the cycle. In this work we showed for the first time a β-decarboxylase activity in *S. carnosus* grown in a complex or in a chemically defined medium with a β-ketoester as substrate.

In filamentous fungi, it is often mentioned that methyl ketones are formed from an incomplete β-oxidation of medium chain fatty acids implicating a β-decarboxylase activity [9,19]. Yagi et al. [20] have suggested that hydroxyacids are the substrates for the β-decarboxylation while others have postulated that the β-ketoacids are preferred precursors for methyl ketone production [9,21]. Hwang and Kinsella [9] have detected a β-ketoacyl decarboxylase in spores and mycelium of *Penicillium roquefortii* related to the enhanced production of methyl ketones using a β-ketoacid as substrate.

In bacteria, the production of methyl ketones via β-oxidation and decarboxylase activity has not been demonstrated. The β-oxidation pathway has been reported for many species, such as *Pseudomonas fragi*, *Caulobacter crescentus*, *Corynebacterium*, *Escherichia coli* and *Lactobacillus leichmanii* [22–25]. The only β-decarboxylase enzymes described for bacteria related with the production of ketones are those of *Clostridium acetobutylicum* [26] and methylobacteria [27]. In the solventogenic *C. acetobutylicum* and in *Methylomonas album*, an acetoacetate decarboxylase catalyzes the production of acetone by direct decarboxylation of acetoacetate [26–28].

In the *S. carnosus* 833 strain, a β-ketoacyl decarboxylase was detected using as substrate the β-ketoester (ethylbutyryl acetate). It was present in cells throughout the growth period and that possibly has an essential function in cell metabolism. On the contrary, the β-acetoacetate decarboxylase from *C. acetobutylicum* is produced at the end of the exponential growth phase prior to typical butyric acid fermentation [28].

For *S. carnosus*, glucose is required for the β-decarboxylase activity, but only at moderate concentrations (0.1%) as methyl ketone production was limited in the presence of 1% glucose. In *C. acetobutylicum*, glucose induced an acidic metabolism rather than the solventogenic metabolism involved in acetone production [28]. A decrease in 2-pentanone production was noticed when cells were cultivated in the presence of β-oxidation substrates such as lauric and oleic acids. This can be explained by a stimulation of the β-oxidation pathway by the methyl esters, as shown by Engelvin et al. [11], rather than by an incomplete β-oxidation cycle required for methyl ketone synthesis [19]. The highest yields of 2-pentanone were obtained when cells were cultivated in the presence of β-hydroxyacid (C8). This result can be linked to the results of Engelvin et al. [11] on the preferential release of β-hydroxyacids during the β-oxidation cycle. It also suggests, in accordance with the observation of Yagi et al. [20], that β-hydroxyacid could be the most appropriate substrate for the β-decarboxylase. The absence of the decarboxylase cofactors (biotin and pyridoxal) in growth media resulted in only a slight decrease of the production of 2-pentanone by *S. carnosus* resting cells. This suggests that these cofactors are not essential for β-decarboxylase activity, at least when resting cells are used as enzyme source.

There was a strong influence of the growth conditions such as pH, aeration and nitrate on the production of 2-pentanone by *S. carnosus*. Our results showed a noticeable increase (38.3%) in 2-pentanone production by cells cultivated at pH 5.0 compared to pH 6.0. The effect of nitrate and aeration could be related to the respiration pathways in the cell, which could stimulate β-decarboxylase production. The effect of the pH of the growth medium on the decarboxylase activities in other bacteria has not been reported but a pH near 5.0 favors decarboxylase activities such as acetoacetate decarboxylase from methylobacteria [27] and from *C. acetobutylicum* [28], tyrosine decarboxylase from *Lactobacillus brevis* [29] or glutamate decarboxylase from *Lactococcus lactis* [30].

In summary, we have identified a β-decarboxylase activity in the *S. carnosus* 833 strain. The enzyme or enzyme complex implicated is apparently constitutive. This work demonstrates that methyl ketone produced by *S. carnosus* derived from intermediates of the β-oxidation pathway. Further research in our laboratory is designed in order to characterize the enzyme implicated in methyl ketone production by the *S. carnosus* 833 strain.

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