PRODUCTION OF METHYL KETONES BY *STAPHYLOCOCCUS CARNOSUS* 833 STRAIN

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Background

The characteristic taste and aroma of fermented sausage are due to many different non-volatile and volatile compounds. 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 during ripening and the steady increase of different carbonyl oxidation products like aldehydes and ketones [1]. Increased levels of ketones and alcohols were recorded when *Staphylococcus carnosus* was inoculated in sausage mince as starter culture [2]. However, the staphylococcal enzymic pathway leading to methyl ketones is unknown. Fungi are known to synthesize methyl ketones. It has been postulated that the pathway incluse abortive β-oxidation sequence, the release of β-ketoacyl CoA esters that are deacylated into β-ketoacids and finally decarboxylated to methyl ketones [3].

Objectives

Concerning *S. carnosus* 833 strain, Engelvin et al. [4] have reported the existence of a β-oxidative pathway. 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 methyl ketone production in the *S. carnosus* 833 strain.

Methods

Growth of S. carnosus 833 strain

It was cultivated in chemical defined media (CDM) [5] buffered at pH 7.0 and with 0.1% of glucose for 24 h at 30°C and under stirring (150 rpm). CDM was supplemented either with β–oxidation susbstrates such as methyl esters, (lauric -C12 or oleic-C18:1) or with β–oxidation intermediates such as β-ketoester (ethylbutyryl acetate-C8) and hydroxyacid (DL-β-hydroxycaprylic acid-C6) at a final concentration of 2 mM. Biotin or pyridoxal phosphate (PLP) were omitted in the preparation of CDM to study the influence of these cofactors.
Cells were collected by centrifugation and resuspended in K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 7.0) 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.

Effect of technological conditions

Cells were grown for 24 h in complex media (CM) [4] in eight conditions that mimic technological conditions of sausage manufacturing (Table 1).

Methyl ketone production and assays

These assays were 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) in a 1 ml final volume of K_2HPO_4-KH_2PO_4 buffer (100 mM, pH 7.0). Either the bacteria or the substrate were omitted from the 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 (15000×*g*). The supernatants containing 2-pentanone were kept at -20°C until enzymic quantification by a NADPH-dependent alcohol dehydrogenase (ADH) (EC 1.1.1.2) [6]. 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 supernatant, 100 µl of NADPH (100 mM) and 21 µl of ADH solution (0.94 units ml^{-1}). The NADPH oxidation was followed continuously at 334 nm at 40°C for 10 min. A calibration curve of 2-pentanone was made from 0 to 500 µM. The results were expressed as µmoles of 2-pentanone cell g^{-1} h^{-1}.

Results and discussion

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 2). The production of 2-pentanone was reduced by 35% or 32.5% when cells were grown in presence of lauric or oleic methyl esters, respectively. However, the addition of intermediates of 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 presence of DL-β hydroxycaprylic acid. After 24 h of *S. carnosus* growth in presence of the β-hydroxyacid, the supernatant culture smelt an intense blue cheese odor related to the synthesis of 2-heptanone in the growth medium.
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 2).
In bacteria, the production of methyl ketones via β-oxidation 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* [7]. The only two β-decarboxylase enzymes described for bacteria related with the production of ketones are those of *Clostridium acetobutyicum* [8] and methylobacteria [9].

Effect of technological conditions on 2-pentanone production

The resting cells of *S. carnosus* 833 strain grown in the eight conditions that mimic technological conditions of sausage manufacturing were able to produce significant amount of methyl ketone from β-ketoacid (Table 1). The highest production, 15.49 µmol cell g^{-1} h^{-1}, was noticed when the cells were grown at pH 5.0, 15°C, high level of air, in presence of NaCl, KNO3 and glucose (batch 1). The lowest production, 2-fold less, was noticed when the cells were grown at pH6.0, 24°C, low level of air, in presence of NaCl and glucose (batch 2).

Conclusion

We have shown for the first time a β-decarboxylase activity in *S. carnosus*. We have measured this activity with a β-ketoester as substrate in *S. carnosus* grown in a complex or in a chemically defined medium. Our first results showed that the environmental conditions that *S.*

carnosus meets during sausage manufacturing would give it the ability to produce methyl ketone from β-ketoacid. The question that remains is the origin of β-ketoacid, does it come from an incomplete β-oxidation or not?

Pertinent literature

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Table 1. Effect of eight technological conditions on methyl ketone production by *S. carnosus* strain 833

Batch n°	pH	NaCl %	KNO_3 %	T °C	Air v/v	Glucose %	Pentanone µmol cell g^{-1} h^{-1}
1	5.0	0.5	0.03	15	1/2	0.5	15.49 ± 1.54
2	6.0	0.5	0	24	1/3	0.5	7.61 ± 1.16
3	6.0	3.5	0	15	1/2	0	9.20 ± 0.80
4	6.0	3.5	0.03	15	1/3	0.5	10.24 ± 1.25
5	5.0	3.5	0.03	24	1/3	0	13.69 ± 1.28
6	6.0	0.5	0.03	24	1/2	0	13.73 ± 1.10
7	5.0	3.5	0	24	1/2	0.5	12.99 ± 1.26
8	5.0	0.5	0	15	1/3	0	11.86 ± 1.04

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

Compounds	2-pentanone µmol cell g^{-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
Biotin*	7.55 ± 0.03
PLP*	6.30 ± 0.02

*Biotin and pyridoxal phosphate (PLP) were omitted in the preparation of CDM