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Production of S-Methylthioacetate by *Brevibacterium linens*

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Volatile sulfur compounds production by eight strains of *Brevibacterium linens* isolated from cheeses was demonstrated: methanethiol, dimethyldisulfide, and 2,3,4-trithiapentane. Four of these strains also produced S-methylthioacetate, an important aroma component of smear-coated cheeses. It is the first demonstrated microbiological production of a thioester.

Sulfur compounds are usually considered as key compounds in cheese flavor. Many authors (7-9, 11, 20) claimed that cheddar flavor could be related to the presence of sulfur compounds such as hydrogen sulfide, methanethiol, and dimethylsulfide. Sometimes methional is also listed (6, 14), and Manning (11) stressed the particular importance of methanethiol.

Yet another class of sulfur compounds, thioesters, has been recently shown to be an important contributor to the characteristic odor of smear-coated cheeses (1, 2). The homologs with short chains (e.g., S-methylthioacetate, thiopropanoate, thiobutyrate, and thioisovalerate) possess quite interesting organoleptic properties, which have been studied recently (to be published). The objective of that work was to look for microorganisms able to produce these compounds.

Volatile sulfur compounds can be produced by a lot of microorganisms (4). For example, it has been reported that among the strains extensively used in cheese making, *Propionibacterium shermanii* produces dimethylsulfide (5) while *Penicillium caseicolum* yields hydrogen sulfide, methanethiol, and dimethyldisulfide (18). Several strains of coryneform bacteria, isolated from dairy products (10, 16), have been shown to be capable of producing methanethiol.

We chose to study *Brevibacterium linens* as the test organism because this species, which belongs to the coryneform group, is known to be present in the cheese type in which thioesters have been found, and to produce methanethiol (16). Its growth at the surface of the cheeses is closely related to the development of a putrid odor similar to that produced by pure cultures of this organism (3). A study of the production of sulfur compounds by one strain of *B. linens* was carried out by Tokita and Hosono (17).

In the course of this work, a purely microbiological study was made of various coryneform bacteria strains (among them four *B. linens* strains we were interested in), indicating, in particular, that some of them produce methanethiol (16).

We systematically study the production of volatile sulfur compounds by various *B. linens* strains isolated from cheeses and cultured in liquid medium closely related to natural feeding media. Our objective is to know whether thioesters are produced or not.

Eight *B. linens* strains were tested. *B. linens* IP 6311 (= ATCC 9174) and IP 6312 (= ATCC 9175) were purchased from the Institut Pasteur, Paris; *B. linens* ATCC 8377 was from the American Type Culture Collection, Rockville, Md.; *B. linens* NCIB 9909 (= ATCC 9172) was from the National Collection of Industrial Bacteria, Aberdeen, Scotland; *B. linens* NIRD 1002 was from the National Institute for Research in Dairying, Reading, U.K.; the three others (Bl1, Bl2, and Bl3), generously provided by J. Castagné, Laiterie Coopérative de la Thiérache, Le Nouvion-en-Thiérache, France, were isolated from Maroilles cheeses and identifed by the Institut Pasteur, Lille, France, as different *B. linens* strains.

Strains were maintained on slants of trypticase soy agar (BioMérieux) at 4°C. The vegetative medium contained 1 g of yeast extract (BioMérieux), 1 g of pancreatic peptone (Industrie Biologique Française), 1 g of K_2HPO_4, 1 g of NaCl, and 0.5 g of Casamino Acids (Difco) in a total volume of 100 ml of distilled water. The final growth medium contained 6 g of milk ultrafiltration-dried retentate (composition: 60% proteins, 36% lactose, and 4% mineral salts) and 1 g of NaCl in a total volume of 100 ml of distilled water.

Surface growth from each slant of *B. linens* was removed by mixing with sterile water and then inoculated into 100 ml of vegetative sterile medium in a 500-ml Erlenmeyer flask. After incubation at 27°C on a rotatory shaker at 140 rpm for 24 h, 5 ml of this culture was used to

inoculate 100 ml of sterile growth medium in a 500-ml Erlenmeyer flask. Incubation was continued as before for 72 h (time corresponding to a maximum odor).

Volatile sulfur compounds in the growth medium were studied by direct headspace analysis (12) on a Girdel 3000 gas chromatograph fitted with a Tracor flame photometer detector and an all-glass and Teflon injection system; mass spectra were obtained with a low-resolution mass spectrometer (AEI MS 20).

The sulfur detection method used in this study proved much more sensitive than the colorimetric method used so far. It enabled us to establish with some certainty the methanethiol production by *B. linens* IP 6312 (= NCIB 8546), whereas previous investigations conducted with the help of colorimetric method (15, 16) were unable to come to a definite conclusion.

It appeared that for each of the strains tested, the growth medium contained large amounts of methanethiol, hydrogen sulfide, dimethyldisulfide, and 2,3,4-trithiapentane. Under the experimental conditions used in this study, *S*-methylthioacetate was produced by four of the eight strains tested. In our state of knowledge, it is the first evidenced microbiological production of a thioester. Quantitative results are indicated in Table 1.

In the same time, control experiments have been performed to check whether methanethiol and thioacetate production could be achieved in sterile medium or not: only methanethiol traces have been found in the autoclaved media, and no thioacetate has ever been identified, even when the chemical precursors, sodium acetate and methanethiol, were added.

Coryneform bacteria (10) and some other microorganisms (4) are able to produce methanethiol. Methanethiol production from methionine has been verified by means of [^{35}S]methionine (3). *S*-Methylcysteine can also yield it according to the following scheme: cysteine + methionine → *S*-methylcysteine → CH_3SH + pyruvic acid + NH_3.

In our experiments, enrichment of the culture medium with methionine led to an increase of the methanethiol concentration while addition of cysteine increased hydrogen sulfide production.

The biosynthetic pathway involved in the thioester production is not yet elucidated.

Two hypotheses can be considered about the four strains for which no measurable thioester production was observed: either they are unable to produce thioester or, if they have the ability to synthesize some thioester, the resulting product is rapidly hydrolyzed.

TABLE 1. *Absolute concentrations of S-methylthioacetate in culture media*

Strain	Origin	Culture media pH[a]	$CH_3-C(=O)-S-CH_3$ (μg/liter)
IP 6311	Romadur	8.15	0
IP 6312	Camembert	8.40	0
NCIB 9909	Harz	8.35	0
NIRD 1002		8.35	0
ATCC 8377	Brie	8.10	3
Bl1	Maroilles	7.05	19
Bl2		7.10	11
Bl3		7.15	16

[a] Determined 3 days after inoculation.

Growth media of "nonproducing thioester" strains appeared to exhibit higher pH values than those of producing strains (e.g., Table 1). Studies relative to chemical hydrolysis of thioesters in function of pH (13) led to the conclusion that it proceeds faster when pH values increase. In the same manner, acylase studies (19) pointed out that these enzymes show an acylating ability at pH below 7 and a desacylating one at pH above 7. In fact, hydrolysis tests done with the four nonproducing thioester strains show that noticeable amounts of *S*-methylthioacetate, ranging up to 15 mg/liter, added in the growth medium of the strains could be hydrolyzed in a few hours, whereas control experiments performed by adding a thioester to the sterilized culture medium (pH 6.85) resulted in a chemical hydrolysis limited to a very small extent. In a set of experiments, *B. linens* IP 6311 was grown in culture media buffered at various pH values between 5.3 and 7.3; in spite of the acidic conditions, no thioester could be evidenced with that strain.

In conclusion, it appears that the production of sulfur compounds (particularly methanethiol) is a widespread character in *B. linens* species. However the most striking feature, which occurs in some conditions and with a few strains only, is the biosynthesis of a thioester: *S*-methylthioacetate.

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