ELSEVIER

PII: S0958-6946(99)00010-2

Int. Dairy Journal **8** (1998) 883–887

Printed in Great Britain
0958-6946/99/$–see front matter

Volatile Flavour Production by *Penicillium caseifulvum*

Thomas Ostenfeld Larsen*

Mycology Group, Department of Biotechnology, Building 221, Technical University of Denmark, DK-2800 Lyngby, Denmark

(Received 14 August 1998; accepted 12 December 1998)

Abstract

The production of volatile aroma compounds from the cheese associated fungus *Penicillium caseifulvum* has been compared to the aroma production by *P. camemberti* when grown on a liquid cream based medium in Petri dishes. Volatiles were collected by diffusive sampling between day 5 and 10 and between day 10 and 15 and analysed by gas chromatography coupled to mass spectrometry. During both sampling periods similar qualitative aroma profiles could be observed for the two species. In the beginning volatiles like ethanol, acetone, 2-methylpropan-1-ol, 2-pentanone, 3-methylbutan-1-ol, 2-heptanone, 2-nonanone and 2-undecanone were dominant, whereas the metabolism changed later in the growth phase. At this stage unsaturated hydrocarbons like styrene, limonene, β-caryophyllene and other terpenoids, including some possible diterpenes, were major volatiles. The results indicate the potential of *P. caseifulvum* as a new starter culture for the dairy industry or as a fermentation organism for production of natural cheese flavours by submerge fermentation.

Keywords: *Penicillium caseifulvum*; *Penicillium camemberti*; cheese fermentation; aroma; flavour; methyl ketones; terpenes

INTRODUCTION

Penicillium camemberti THOM has been used as a fungal starter for production of Brie and Camembert cheese for many years. Species names like *P. caseicola*, *P. camemberti* var. *rogeri*, *P. biforme* and *P. candidum* have been reported as synonyms for *P. camemberti* (Frisvad and Filtenborg, 1989).

More or less destructive methods, like high vacuum distillation (Jollivet and Belin, 1993), purge and trap of volatiles from heated cheese slurries (Karahadian *et al.*, 1985a) or extraction of medium filtrates by Forane 11 (Spinnler *et al.*, 1992), have almost always been used for extraction of Camembert volatiles. Alternatively to destructive methods Karahadian *et al.* (1985b) used purge and trap of headspace volatiles from pure cultures cultivated on synthetic media, while Larsen and Frisvad (1994) developed a simple method for diffusive sampling of volatiles liberated to the headspace of fungi cultivated in Petri dishes.

Important volatiles produced by *P. camemberti* on cheese are odd-chain methyl ketones from C_3 to C_{11}, 2-heptanone and 2-nonanone being the most dominant (Okumura and Kinsella, 1985). Methyl ketones are formed in a metabolic pathway that is connected to the β-oxidation pathway and are derived from free fatty acids (FFA) by loss of CO_2. FFA are themselves important in cheese aroma (Molimard and Spinnler, 1996).

* Tel.: +45 45 25 26 32; fax: +45 45 88 49 22; e-mail: TOL@IBT.DTU.DK.

Primary and secondary alcohols are also important aroma compounds. 2-Methylpropan-1-ol and 3-methylbutan-1-ol are derived by reduction of aldehydes emerging from the amino acids valine and leucine via the Strecker pathway. Ethanol, derived from lactate via the pentose phosphate pathway (Molimard and Spinnler, 1996), has only a limited aromatic role in cheeses despite being found in large quantities. However, it is the precursor of several esters. Methyl ketones are reduced to the corresponding secondary alcohols; that is why heptan-2-ol and nonan-2-ol are often dominant compounds (Molimard and Spinnler, 1996).

Another important alcohol is the mushroom alcohol 1-octene-3-ol, which together with other eight carbon aroma compounds like 3-octanone originate from the unsaturated linoleic and linolenic acids (Wurzenberger and Grosch, 1982, 1984). These compounds are biosynthesized through two enzyme catalysed reactions in the presence of atmospheric oxygen.

2-Methyl-isoborneol, which has a musty-earthy note and a very low threshold value (0.1 ppb), plays a major role in the flavour of soft and mold-ripened cheeses (Karahadian *et al.*, 1985a, b). When present in low amounts it adds a desirable flavour note to cheese. Present in excess amounts, however, it is considered an off-flavour. Styrene, another important off-flavour with a strong plastic odour, is often found as a trace element in Camembert cheeses (Adda *et al.*, 1989).

Esters, lactones, aldehydes and sulfur compounds are other types of flavour compounds which have been demonstrated to be of importance for cheese flavour.

Recently, Lund *et al.* (1998) described the new species *Penicillium caseifulvum* which is closely related to both

P. camemberti and its wildtype *P. commune*. The aim of the present study has been to further investigate this new species with special attention to its qualitative capabilities for volatile flavour production when compared to *P. camemberti*.

MATERIALS AND METHODS

Fungal isolates

The fungal isolates of *P. camemberti* (IBT 11569) and *P. caseifulvum* (IBT 15157), both originating from cheese, were taken from the Culture Collection of the Department of Biotechnology (IBT) at the Technical University of Denmark.

Medium and culture conditions

Each Petri dish was filled with 25 mL of liquid cream (fats 38.0%, proteins 2.3%, carbohydrates 3.0%). IBT 11569 and IBT 15157 were inoculated with 10^6 spores mL^{-1} and cultivated in duplicate. The spores came from 1-week old cultures on Czapek yeast autolysate agar (Frisvad and Filtenborg, 1983). The fungi were grown for 15 days at 13°C in a Forma Scientific climate chamber (model 3319), with external cooling at atmospheric conditions. Humidity was elevated in the chamber by placing an open container of water (60 cm^2 surface) on the floor of the chamber.

Collection and analysis of volatiles

Volatile metabolites were collected by diffusive sampling onto Tenax TA adsorption material placed in Perkin Elmer tubes according to the method developed by Larsen and Frisvad (1994). The method has been improved by using stainless steel lids instead of lids made of polyethylene in order to avoid adsorption of volatiles to the material. Collection of volatiles was started on day 5 at the time when mycelium started to become visible in the dishes. At day 10 the tubes were replaced with other freshly conditioned tubes, and volatiles were collected until day 15.

Volatiles collected during the two periods were thermally desorbed on a Perkin Elmer ATD 400 coupled to

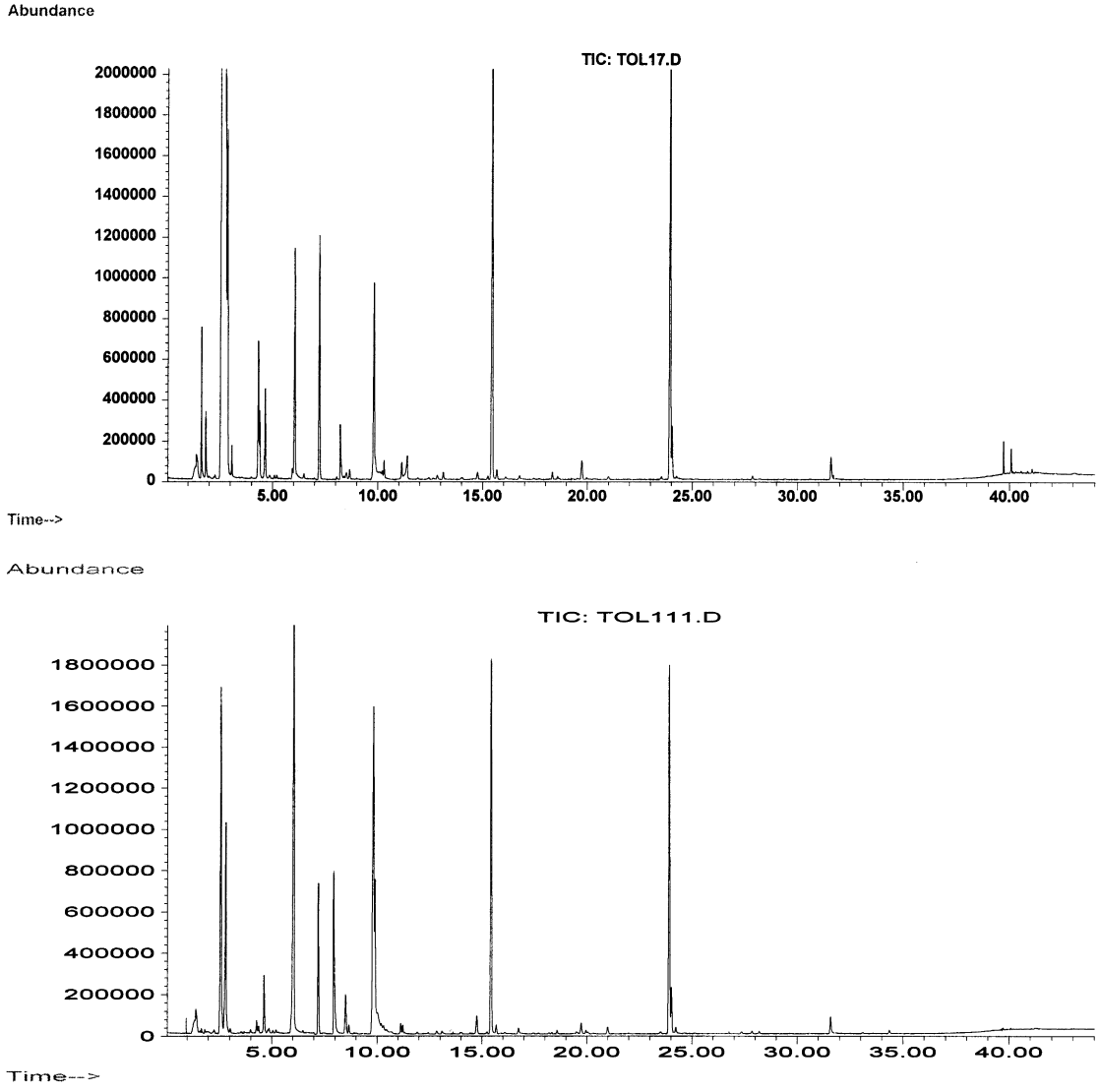

Fig. 1. Profiles of volatile compounds from *P. camemberti* (top) and *P. caseifulvum* (bottom) produced on liquid cream and sampled during day 5 and day 10 of cultivation. See Table 1 for retention times of identified volatiles.

a Hewlett Packard (HP) 5890 gas chromatograph further coupled to a HP 5972 mass selective detector. Volatiles were separated on a DB-1701 (J & W) capillary column (30 m × 0.25 mm × 1.0 μm) using He as carrier gas. Chromatographic conditions: Initial temperature 35°C for 1 min, rate 4°C min^{-1} to 175°C then rate 25°C min^{-1} to 250°C held for 10 min.

Separated compounds were characterized by their mass spectra generated by electron ionisation (EI) at 70 eV. Mass spectra were compared with spectra in the NBS/NIST database consisting of approximately 50.000 spectra.

RESULTS

Flavour compounds produced and released to the surroundings by *P. caseifulvum* and *P. camemberti* were collected by diffusive sampling between day 5 and 10 and between day 10 and 15. The first period corresponded to the time when the mold growth became visible. The second period more or less corresponded to the time when heavy sporulation could be observed. Duplicate cultures appeared identical upon visual examination during the whole period of growth. The *P. camemberti* isolate was white and the *P. caseifulvum* isolate had the grey green colour typical of the species (Lund *et al*., 1998).

The volatile profiles collected from the two species were very similar during the first period of sampling (Fig. 1). Despite the qualitative nature of the data presented in Table 1, major compounds were the alcohols ethanol, 2-methylpropan-1-ol and 3-methylbutan-1-ol together with odd-chain methyl ketones like acetone, 2-pentanone, 2-heptanone and 2-nonanone (Table 1).

During the second period of sampling the total amount of volatiles, measured as total ion count, was smaller than during the first period, as can be seen by summarizing the numbers in table 1. Compounds like ethyl acetate, 2-butanone, 2-hexanone, 2-undecanone and others, produced during the first period of sampling, could not be detected from the second period. Instead other compounds and primarily unsaturated hydrocarbons like 1-pentene, 1-nonene, styrene, 3,7-dimethyl-1,3,7-octatriene, β-caryophyllene and some possible

Table 1. Volatile Compounds Collected by Diffusive Sampling from *Penicillium camemberti* (IBT 11569) and *Penicillium caseifulvum* (IBT 15157) During Day 5–10 (I) and Day 10–15 (II) of Cultivation on Liquid Cream in Petri Dishes. Amounts of Volatiles are Given as Total Ion Counts (TIC) in Thousands at Peak Maximum in the TIC Chromatograms and are Therefore Indicative of the Actual Amounts Produced by the Fungi

Rt	Volatile	11569-I	15157-I	11569-II	15157-II
1.84	Acetaldehyde	340			
1.98	1-Pentene			52	250
2.57	Ethanol	8800	1700	500	150
2.79	Ethyl formiate	1800	200	60	24
2.81	Acetone	1700	1000	280	70
3.97	2-Methyl-furan	10	30	30	20
4.27	1-Propanol	700	80	40	
4.41	Ethyl acetate	340	50		
4.64	2-Butanone	440	300		
5.17	Sulfur dioxide			520	700
6.03	2-Methylpropan-1-ol	1150	2150	1300	525
7.21	2-Pentanone	1200	740	30	
7.96	Acetic acid	290	800	84	100
9.83	3-Methylbutan-1-ol	980	1600	440	160
9.91	2-Methylbutan-1-ol	120	730	160	240
10.31	Ethyl butanoate	125	20	22	
10.43	Delta-nonalactone				50
11.14	2-Hexanone	95	60		
11.41	Propanoic acid	125	50	33	
11.88	1-Nonene			50	38
13.09	Isobutanoic acid	45	25	28	
14.39	Styrene	5	15	20	820
15.41	2-Heptanone	2350	1600	48	18
16.76	Isopentanoic acid	30	40	20	16
18.32	Dimethyl trisulfide	40			
18.54	Limonene	25	28	20	25
19.53	3,7-Dimethyl-1,3,7-octatriene				170
19.73	2-Octanone	110	64		
19.96	Benzaldehyde	21	27	15	20
23.91	2-Nonanone	2400	1700	39	24
24.24	8-Nonene-2-one	260	240		
27.85	2-Decanone	30	25		
21.28	Unidentified sesquiterpene		18		185
31.58	2-Undecanone	125	95		
34.36	Beta-caryophyllene		30		1800
39.69	Unidentified diterpene	170	43	980	55
40.05	Unidentified diterpene	160	40	700	50

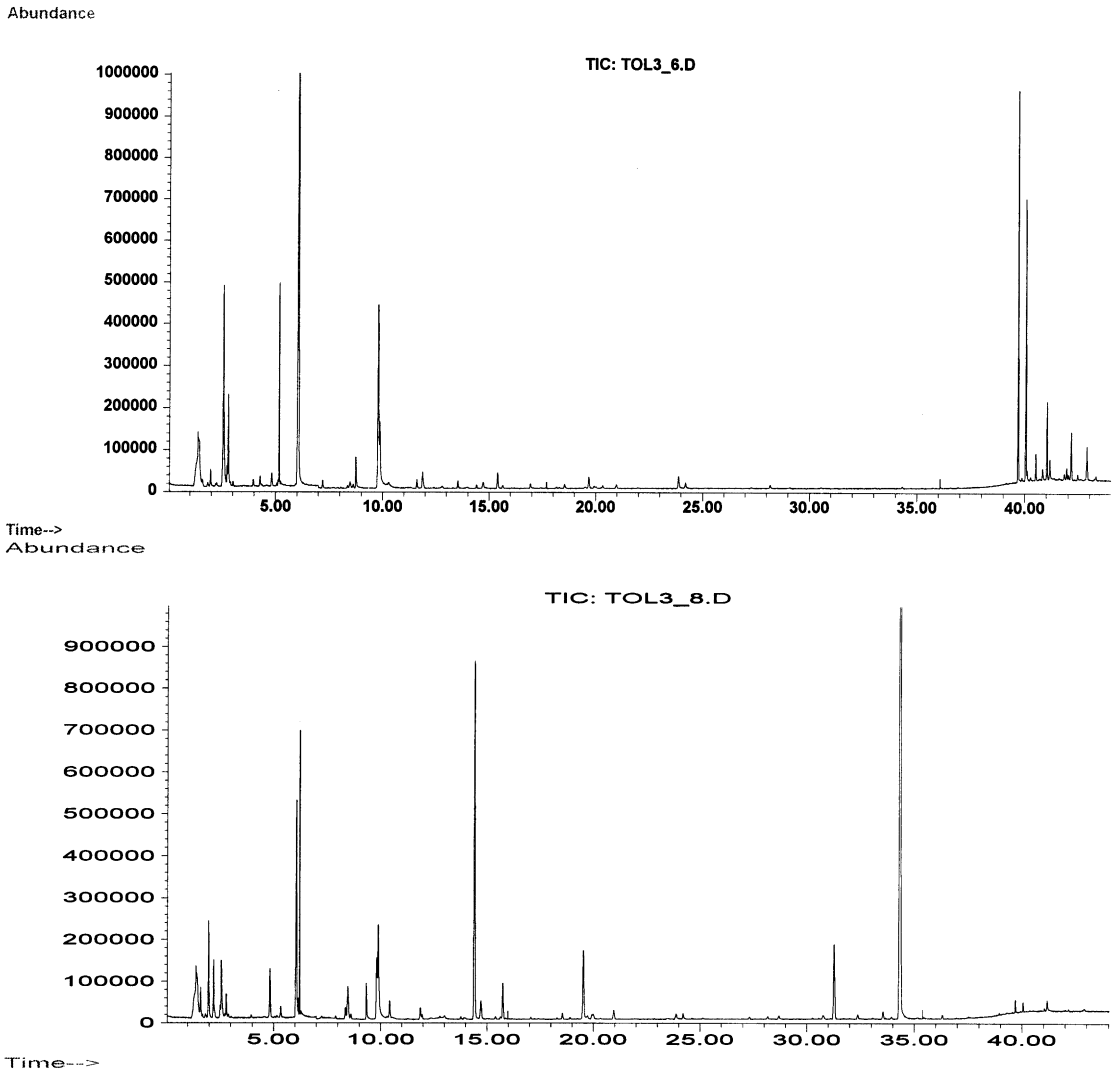

Fig. 2. Profiles of volatile compounds from *P. camemberti* (top) and *P. caseifulvum* (bottom) produced on liquid cream and sampled during day 10 and day 15 of cultivation. See Table 1 for retention times of identified volatiles.

diterpenoids ($m/z = 272$ amu corresponding to $C_{20}H_{32}$) were produced. 3,7-Dimethyl-1,3,7-octatriene, β-caryophyllene and a some other sesquiterpenes were only detected from *P. caseifulvum*, whereas *P. camemberti* produced relative large amounts of diterpenoids. Likewise *P. caseifulvum* had a the large production of styrene in comparison to the production from *P. camemberti* (Fig. 2).

DISCUSSION

Both the *P. camemberti* and the *P. caseifulvum* isolate showed strong lipolytic activity producing large amounts of especially odd-chain methyl ketones together with small primary alcohols during the first sampling period. The almost identical aroma profiles (Fig. 1), including many typical *P. camemberti* volatiles (Molimard and Spinnler, 1996), strongly support that the two species are taxonomically closely related as stated by Lund *et al.* (1998).

The non-destructive nature of the diffusive sampling method used in this study is probably the reason why typical Camembert volatiles like 1-octene-3-ol, 1,5-octadiene-3-ol, 3-octanone and 1,5-octadiene-3-one could not be detected from neither of the species in agreement with the findings of Larsen and Frisvad (1995a), who compared diffusive sampling with a destructive steam distillation and extraction method. More surprisingly no secondary alcohols like heptan-2-ol and nonan-2-ol were detected in this study. Normally they are major aroma components present in amounts up to 20% of the total Camembert aroma (Molimard and Spinnler, 1996). This may also be due to the non-destructive nature of the sampling method, or secondary alcohols, that were actually produced, may have been converted to methyl ketones by bacteria present in the non-sterilised cream as reported for *Brevibacterium linens* (Molimard and Spinnler, 1996).

2-Methyl-isoborneol previously reported to be a product from *P. camemberti* (Karahadian *et al.*, 1985b) and detected from *P. caseifulvum* (T. O. Larsen, unpublished results), when cultivated on the sucrose based medium SYES (Svendsen and Frisvad, 1994), was not detected from any of the two species. However, unsaturated hydrocarbons and other terpenes like limonene, 3,7-dimethyl-1,3,7-octatriene, β-caryphyllene and other sesquiterpenes and diterpenes were produced by both species.

Several terpenes could not be characterized by matching their mass spectra with spectra in the NIST database. Mono- and sesquiterpenes produced by *P. caseifulvum* in this investigation are also produced from pure cultures on synthetic media (T. O. Larsen, unpublished results) and identical to compounds produced by *P. commune* (Larsen and Frisvad, 1995b; Larsen, 1997) strongly indicating that the terpenes found here are true fungal metabolites and not produced by bacteria or yeasts from the cream. This is thus the first report on mono- and diterpene production by *P. camemberti* on a milk fat based medium. In a study using synthetic media Larsen and Frisvad (1995a) also observed terpene production late in the growth phase of *Penicillium vulpinum*, both when using diffusive sampling and steam distillation and extraction.

A drawback of *P. caseifulvum*, with respect to its possible industrial use, could be its strong capability for production of styrene, even though a strong styrene odour could not be smelled from the cultures investigated here. A large liberation of styrene to the surroundings during the fermentation process does not necessarily mean that a cheese fermented with *P. caseifulvum* will have a large styrene content at the time of consumption. One way of moderating a strong styrene metabolism of *P. caseifulvum* could be to produce 'Caseifulvum cheeses' at lower temperatures than the temperatures typically used for Camembert cheeses as suggested by Spinnler *et al.* (1992) for control of styrene production by *P. camemberti*. It is possible that other *P. caseifulvum* isolates may be less strong styrene producers and, therefore, better suited as cheese starters than the isolate studied here. Thus, Jollivet and Belin (1993) found large qualitative differences in volatile production between the ten strains of *P. camemberti* they studied.

The present work has demonstrated the aroma profile of *P. caseifulvum* to be very similar to the profile of *P. camemberti*, indicating *P. caseifulvum* as a possible new starter culture. Lund *et al.* (1998) also suggested that *P. caseifulvum* might be considered for cheese fermentation as the species does not produce cyclopiazonic acid like *P. camemberti* and *P. commune*. However, the species may produce other mycotoxins, a possibility that needs to be further investigated before using *P. caseifulvum* in food fermentations. So far *P. caseifulvum* has only been isolated from the very specific habitat with *P. roquefort* on blue cheeses (Lund *et al.*, 1998). However, traditional manufactured green Brie cheeses, as studied by Karahadian *et al.* (1985a), might actually be cheeses fermented with *P. caseifulvum* or *P. commune* due to the close taxonomically relationship of the three species (Lund *et al.*, 1998).

Finally, another application of *P. caseifulvum* could be in the production of natural Blue cheese flavours by submerge fermentation, as an alternative to the use of *P. roqueforti* and other fungi (Yagi *et al.*, 1990).

ACKNOWLEDGEMENTS

The Support of the Danish Dairy Research Foundation (Danish Dairy Board) and the Danish Research and Development Programme for Food Technology is acknowledged.

REFERENCES

Adda, J., Dekimpe, J., Vassal, L. and Spinnler, H. E. (1989) Production de styréne par *Penicillium camemberti* Thom. *Lait* **69,** 115–120.

Frisvad, J. C. and Filtenborg, O. (1983) Classification of terverticillate penicillia based on profiles of mycotoxins and other secondary metabolites. *Applied and Environmental Microbiology* **46,** 1301–1310.

Frisvad, J. C. and Filtenborg, O. (1989) Terverticillate penicillia: Chemotaxonomy and mycotoxin production. *Mycologia* **81,** 837–861.

Jollivet, N. and Belin, J. M. (1993) Comparison of volatile flavor compounds by ten strains of *Penicillium camemberti*. *Journal of Dairy Science* **76,** 1837–1844.

Karahadian, C., Josephson, D. B. and Lindsay, R. C. (1985a) Contribution of *Penicillium* sp. to the flavor of Brie and Camembert cheese. *Journal of Dairy Science* **68,** 1865–1877.

Karahadian, C., Josephson, D. B. and Lindsay, R. C. (1985b) Volatile compounds from *Penicillium* sp. contributing musty-earthy notes to Brie and Camembert cheese flavors. *Journal of Agricultural and Food Chemistry* **33,** 339–343.

Larsen, T. O. (1997) Identification of cheese-associated fungi using selected ion monitoring of volatile terpenes. *Letters in Applied Microbiology* **24,** 463–466.

Larsen, T. O. (1998a) Volatiles in fungal taxonomy. In *Handbook of Applied Mycology*, Vol. 6. *Chemical Fungal Taxonomy*, eds J. C. Frisvad, P. D. Bridge and D. K. Arora. Marcel Dekker, New York, pp. 263–287.

Larsen, T. O. and Frisvad, J. C. (1994) A simple method for collection of volatile metabolites from fungi based on diffusive sampling from Petri dishes. *Journal of Microbiological Methods* **19,** 297–305.

Larsen, T. O. and Frisvad, J. C. (1995a) Comparison of different methods for collection of volatile chemical markers from fungi. *Journal of Microbiological Methods* **24,** 135–144.

Larsen, T. O. and Frisvad, J. C. (1995b) Characterization of volatile metabolites from 47 *Penicillium* taxa. *Mycological Research* **10,** 1153–1166.

Lund, F., Filtenborg, O. and Frisvad, J. C. (1998) *Penicillium caseifulvum*, a new species found on fermented blue cheese. *Journal of Food Mycology* **1,** 95–101.

Molimard, P. and Spinnler, H. E. (1996) Compounds involved in the flavor of surface mold-ripened cheeses: origin and properties (Review). *Journal of Dairy Science* **79,** 169–184.

Okumura, J. and Kinsella, J. E. (1985) Methyl ketone formation by *Penicillium camemberti* in model systems. *Journal of Dairy Science* **68,** 11–15.

Spinnler, H. E., Grosjean, O. and Bouvier, I. (1992) Effect of culture parameters on the production of styrene (vinyl benzene) and 1-octene-3-ol by *P. caseicolum*. *Journal of Dairy Research* **59,** 533–541.

Svendsen, A. and Frisvad, J. C. (1994) A chemotaxonomic study of the terverticillate penicillia based on high performance liquid chromatography of secondary metabolites. *Mycological Research* **98,** 1317–1328.

Wurzenberger, M. and Grosch, W. (1982) The enzymatic oxidative breakdown of linoleic acid in mushrooms (*Psalliota bispora*). *Zeitschrift für Lebensmittel-Untersuchung und Forschung* **175,** 186–190.

Wurzenberger, M. and Grosch, W. (1984) Origin of the oxygen in the products of the enzymatic cleavage reaction of linoleic acid to 1-octene-3-ol and 10-oxo-*trans*-8-decenoic acid in mushrooms (*Psalliota bispora*). *Biochemica et Biophysica Acta* **794,** 18–24.

Yagi, T., Kawaguchi, M., Hatano, T., Fukui, F. and Fukui, S. (1990) Screening for methylalkylketone-accumulating fungi from type culture strains. *Journal of Fermentation and Bioengineering* **70,** 94–99.