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Short communication

Production in food of 1,3-pentadiene and styrene by *Trichoderma* species

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Abstract

The ability of two strains of *Trichoderma*, isolated from food, to produce the Volatile Organic Compounds 1,3-pentadiene and styrene was investigated. One of the strains had been implicated in a case of food spoilage involving the production of both compounds. In vitro in potato dextrose broth, the strains produced both 1,3-pentadiene and styrene within 5 days in the presence of sorbic acid and cinnamic acid. The taints were produced only in the presence of sorbic acid and cinnamic acid and were not synthesised *de novo* under the test conditions. Neither the conversion of cinnamic acid to styrene, nor the conversion of sorbic acid to 1,3-pentadiene by *Trichoderma* strains in foods has been previously reported. The range of organisms implicated in these types of spoilage is thus extended.

Keywords: *Trichoderma*; Sorbic acid; 1,3-pentadiene; Cinnamic acid; Styrene

1. Introduction

Microbial spoilage is the major cause of quality loss and taints in foods and beverages. Whilst consumer descriptions of taint (and off-odours) are notoriously unreliable and subjective, some taints are sufficiently noxious as to illicit immediate and vehement consumer rejection. These microbial taints are usually described as 'chemical' with an often-assumed corollary that the food has been chemically contaminated rather than subject to microbial deterioration.

Two such noxious 'chemical' taints produced in food by microbial activity are 1,3-pentadiene and styrene. Both these hydrocarbons can be produced in food by the action of fungi. The structure of sorbic acid and 1,3-pentadiene is given in Fig. 1. It is presumed that sorbic acid is converted to 1,3-pentadiene by a decarboxylation mechanism (Saxby, 1996).

Sorbic acid is widely used as a preservative in the food industry to protect high acid (low pH) foods primarily against the growth of yeasts and moulds. The compound occurs naturally in berry fruits. The production of the taint by certain strains of moulds is a known hazard in the dairy industry in foods preserved with sorbic acid or its salts (Marth et al., 1966). The 1,3-pentadiene taint has also been reported in sorbate-preserved bakery products (Saxby, 1996), marzipan (Loureiro and Querol, 1999) and in sorbate-preserved margarine (Sensidoni et al., 1994). Typically, the taint is described as kerosene like. The organisms that have been implicated in taint production include the moulds *Penicillium chrysogenum*, *P. simplicissimum*, *P. crustosum*, *Penicillium roqueforti*, *Penicillium caseicolum* and *Aspergillus niger*, and the yeasts *Zygosaccharomyces rouxii* and *Debaromyces hansenii*. In this laboratory, foodstuffs tainted by 1,3-pentadiene have included bakery products, high moisture soft margarine, artificial cream in cakes, processed cheese and, most commonly, a variety of soft drinks (unpublished). The putative causative agents of the spoilage in the margarine and soft drinks have been strains of the mould *Trichoderma*. The agent of spoilage in the processed cheese was *P. roqueforti*, and in the artificial cream an osmophilic yeast. All the foodstuffs had been preserved with potassium sorbate. The compound 1,3-pentadiene, CAS Number 504-60-9, is not a known carcinogen.

The presence of styrene (and ethylbenzene) in foods is commonly assumed to be due to migration from packaging material (Miltz et al., 1980; Linssen et al., 1993; Tang et al., 2000). Styrene taint can, however, be associated with mould and yeast growth in food. Adda et al. (1989) reported the production of styrene by some strains of *Penicillium camemberti*, and

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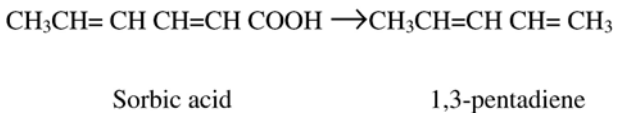

Fig. 1. Degradation of sorbic acid in foods (Saxby, 1996).

Larsen (1998) reported the in vitro production of styrene by the mould *Penicillium caseifulvum*, when it was evaluated as a dairy starter culture. Saxby (1996) cites a case of styrene taint in spiced buns caused by the yeast *Hypopichia burtonii* in the presence of cinnamon flavouring. The structures of cinnamaldehyde, one of the most commonly used cinnamon flavourants, and styrene are given in Fig. 2. Again, a simple decarboxylation mechanism is suggested by Saxby (1996).

The presence of cinnamon or cinnamon flavours is not a prerequisite for styrene production. *P. caseicolum* is fully synthetic, producing the compound by *de novo* synthesis from a simple basal medium, independent of the carbon source and in the absence of flavour precursors (Spinnler et al., 1992).

At high oral doses, styrene (CAS Number 100-42-5) causes nerve, liver and gastric damage and is mutagenic (WHO, 2003). Nonetheless, the offensive odour of styrene and the low concentrations at which it is detected and recognised by taste (Linssen et al., 1993; Miltz et al., 1980; WHO, 2003) make it unlikely that foods and beverages contaminated with this compound would be ingested in any quantity.

There are no reports of the production of either styrene or 1,3-pentadiene in food by *Trichoderma*, and conversion of cinnamon flavourings, cinnamic acid and cinnamaldehyde, to styrene has been reported principally for *Penicillium* moulds and some yeasts (Adda et al., 1989; Larsen, 1998; Saxby, 1996). The current study investigates the capacity of two food isolates of *Trichoderma* to produce 1,3-pentadiene and styrene in vitro.

2. Materials and methods

2.1. Isolates

Two fungal isolates were investigated; one, identified as *Trichoderma viride*, was obtained from a contaminated soft drink in which 1,3-pentadiene and styrene were detected by headspace analysis. The second strain was isolated from a dried instant soup powder and identified as *Trichoderma koningii*. Isolates of the organisms were identified at the Centre for Applied Mycological Studies, University of Pretoria, South Africa from the morphological characteristics of the fruiting structures, as well as colony color and texture. Morphological observations of the anamorphic structures were based on cultures grown in 90 mm Petri plates on potato dextrose agar (PDA). The plates were incubated at 25 °C with 12 h fluorescent light and 12 h darkness. Fruiting structures were microscopically examined approximately one week after incubation. Identification was based on the morphological descriptive characteristics as described by Chaverri and Samuels (2003).

Cultures for inoculation were prepared by culturing onto potato dextrose agar (Oxoid) and incubating at 25 °C for 72 h. Subcultures on the fungal growth were performed to confirm purity.

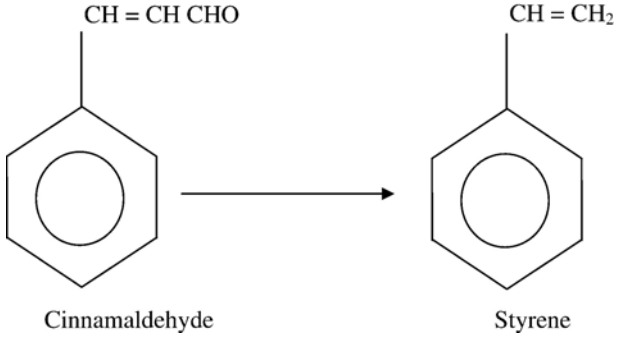

Fig. 2. Relationship of cinnamaldehyde to styrene in foods (Saxby, 1996).

2.2. Materials

Potato dextrose broth (Sigma) was prepared in 300 mL volumes in 500 mL Schott bottles and sterilised at 121 °C for 15 min. After autoclaving and cooling, the pH of the broths was adjusted to pH 3.5 by the addition of sterile 10% tartaric acid solution. Sterile potassium sorbate solution (Saarchem) was added to give sorbic acid equivalents of either 200 or 400 mg L^{-1}. Sterile cinnamic acid was added to give final concentrations of 15 or 25 mg L^{-1} respectively. Duplicate test series contained media with one or the other or both of the additives, together with additive free control media, and in each series each concentration of each additive was duplicated for each organism (Table 1).

A second series of broths was prepared, based on the formulation devised by Spinnler et al. (1992). This medium was originally devised to test the capacity of the potential dairy starter culture *P. caseicolum* to produce styrene in the absence of milk flavourants. The medium contained, per litre; glucose, 10 g; $NaNO_3$, 3 g; KH_2PO_4, 1 g; KCl, 0.5 g; $MgSO_4$, 0.5 g; $FeSO_4$, 10 mg; casamino acids (Scharlau), 5 g; yeast extract

Table 1
Production of 1,3-pentadiene and styrene in potato dextrose broth by two strains of *Trichoderma* after a 5 day incubation at 25 °C

Trichoderma strain	Sorbic acid concentration (mg L^{-1})	Cinnamic acid concentration (mg L^{-1})	1,3-pentadiene production	Styrene production
T. koningii	0	0	−ve	−ve
T. viride	0	0	−ve	−ve
T. koningii	0	15	−ve	+ve
T. viride	0	15	−ve	+ve
T. koningii	0	25	−ve	+ve
T. viride	0	25	−ve	+ve
T. koningii	200	0	+ve	−ve
T. viride	200	0	+ve	−ve
T. koningii	200	15	+ve	+ve
T. viride	200	15	+ve	+ve
T. koningii	200	25	+ve	+ve
T. viride	200	25	+ve	+ve

Key: +ve = positive for the presence of the named compound.
−ve = negative for the presence of the named compound.

Table 2
Production of styrene in the synthetic growth medium of Spinnler et al. (1992), by two strains of *Trichoderma* after a 5 day incubation at 25 °C

Trichoderma strain	Cinnamic acid concentration (mg L^{-1})	Styrene production
T. koningii	0	−ve
T. viride	0	−ve
T. koningii	15	+ve
T. viride	15	+ve
T. koningii	25	+ve
T. viride	25	+ve

Key: +ve = positive for the presence of styrene.
−ve = negative for the presence of styrene.

(Oxoid), 0.5 g. The medium was autoclaved in 300 mL volumes in 500 mL Schott bottles at 121 °C for 15 min. After autoclaving and cooling, the pH of the broths was adjusted to pH 3.5 by the addition of sterile 10% tartaric acid solution. Sterile cinnamic acid solution was added to a concentration of 15 or 25 mg L^{-1}, the levels commonly used to impart cinnamon flavours to foods.

2.3. Analyses

The sterile broth series were inoculated by touch loop technique from 72 hour *Trichoderma* cultures grown on potato dextrose agar at 25 °C. The broths were incubated at 25 °C for 5 days. After incubation, the headspace was analysed to determine the presence of 1,3-pentadiene and styrene. Samples of the container headspace (0.1 mL) were injected with a 10:1 split ratio onto 30 m × 0.25 mm × 0.25 m 5% phenylmethylsilicone and polyethylene glycol capillary columns in a Hewlett Packard 6890 GC coupled to a Hewlett Packard 5973 mass spectrometer. To identify analytes, 70 eV impact spectra were compared to the Wiley 275 mass spectral library.

Two columns with different stationary phases were used. The temperature of the silicone column was ramped from 30 °C to 110 °C at 10 °C min^{-1} and the PEG column from 40 °C to 90 °C at 10 °C min^{-1}.

3. Results

After five days of growth in potato dextrose broth containing 200 mg L^{-1} sorbic acid, both strains of *Trichoderma* produced 1,3-pentadiene, with the strong "kerosene" odour typical of spoilt foods displaying this type of microbial spoilage. 1,3-pentadiene was not detected by odour or GC–MS in the absence of sorbic acid. After five days of growth in potato dextrose broth containing 200 mg L^{-1} sorbic acid and either 15 or 25 mg L^{-1} cinnamic acid, both *Trichoderma* strains produced styrene, in addition to 1,3-pentadiene, with a strong "kerosene–plastic" odour. Neither strain of *Trichoderma* produced styrene in the absence of cinnamic acid. These results (Table 1) were identical for the two series of potato dextrose broth cultures. Sorbic acid at a concentration of 400 mg L^{-1} in potato dextrose broth was generally inhibitory to both strains, with poor growth and only occasional production of 1,3-pentadiene and a "kerosene" odour, even after prolonged incubation.

Both strains of *Trichoderma* grew well in the medium of Spinnler et al. (1992). The results are given in Table 2. Styrene was detected by GC–MS and an offensively strong odour at both 15 and 25 mg L^{-1} cinnamic acid inclusion level, but not in the absence of cinnamic acid.

4. Discussion

Sorbic acid, cinnamic acid and the hydroxycinnamates are naturally occurring antimicrobials found in a wide variety of plants (Hoskins, 1984). The ability of a microorganism to decarboxylate an antimicrobial confers a competitive advantage to an invading organism. A further advantage is conferred if the volatile break-down product is itself inhibitory to other competing organisms. Certain *Trichoderma* spp. have been reported to produce volatiles possessing an inhibitory effect against other fungi (Dennis and Webster, 1971) although the antimicrobial properties of 1,3-pentadiene and styrene are not established. In foods, the presence of either or both of the compounds imparts repellent smells and highly disagreeable off-odours inimical to consumer acceptance of the foodstuff.

We were alerted to the possible production of both 1,3-pentadiene and styrene by *Trichoderma* by the presence of these compounds in a variety of non-carbonated soft drinks that were preserved with sorbate, flavoured with cinnamon flavouring and spoiled by *Trichoderma*. The offensively strong off-odours that resulted led to consumer complaints, some of which included claims that the product had been contaminated by 'chemicals'. In vitro the two tainting compounds are indeed produced in the presence of sorbic acid and cinnamic acid, but not if the corresponding precursors are absent. *Trichoderma* moulds are known to produce a wide range of volatile organic compounds, (VOCs) (McAfee and Taylor, 1999), whose identities and concentrations depend on the identity of the *Trichoderma* isolate and the growth medium (Wheatley et al., 1997). The production of 1,3-pentadiene and styrene in food from simple food ingredients and additives by *Trichoderma* increases the number of known VOCs produced by this mould.

Sorbic and benzoic acid, and their salts have GRAS (Generally Regarded as Safe) status in the food industry where they are commonly employed as preservatives primarily to prevent the outgrowth of yeasts and moulds in processed foods. The finding that benzoic acid, in the presence of ascorbic acid (and possibly citric acid and erythorbic acid), may be converted to benzene under certain processing and storage conditions suggests that changes in formulation, processing and storage of soft drinks may result in greater use of sorbic acid and its salts. Cognisance of the potential for the production by *Trichoderma* strains of 1,3-pentadiene from sorbic acid and of styrene from flavourants in susceptible foods, particularly soft drinks, should be taken when conducting hazard analysis in a Hazard Analysis Critical Control Point (HACCP) study. The use of high care bottling areas to prevent contamination by ubiquitous *Trichoderma* strains, and the isolation of wood pulp derived packaging materials and wooden pallets, known sources of the mould, should be employed.

In conclusion, cognisance of the potential of fungi to produce highly repellent odours in foods containing sorbic acid and

cinnamon flavourants should be taken. The highly repellent nature of the taints represents both a hazard and a commercial restraint.

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