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Degradation of Preservatives with the Formation of Off-Odor Volatile Compounds—The Case of Strawberry-Flavored Bottled Water

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Abstract: Foods preserved with sorbic acid or its salts can undergo spoilage with the formation of chemicals characterized by odors of plastic, hydrocarbons, or kerosene. 1,3-pentadiene, which is formed through the decarboxylation of sorbic acid or its salts, is one such compound. Numerous species of molds and yeasts have been reported as capable of degrading sorbic acid. This work is aimed to identify the off-odor compounds in samples of strawberry-flavored water preserved with potassium sorbate and sodium benzoate. In addition, the mold isolated from this drink was evaluated for the ability to form undesirable compounds, and the results revealed the presence of 1,4-pentadiene and benzaldehyde in the tested samples. The mold isolated from the samples was identified as *Penicillium corylophilum*. During its 5-day incubation at 25 °C in a liquid medium, potassium sorbate added at a final concentration of 200 and 400 mg/L was completely assimilated by the growing mycelium and converted into 1,4-pentadiene. The concentration of the latter was determined as 46.5 and 92.6 mg/L, respectively. The decrease in the concentration of sodium benzoate exceeded 53% in the broth spiked at 200 mg/L and 23% at 400 mg/L, resulting in the formation of benzaldehyde.

Keywords: potassium sorbate; 1,4-pentadiene; sodium benzoate; benzaldehyde; styrene; strawberry-flavored water

1. Introduction

Preservatives are substances that prolong the shelf-life of foodstuffs by protecting them against deterioration by microorganisms. Since soft drinks exhibit high water activity and some of them are rich in vitamins and minerals, they are an attractive environment for microorganisms. The main preservatives that are permitted to be used and commonly used in soft drinks are benzoic acid (E210), sorbic acid (E200), and their derived salts (E211–213 and E201–203, respectively) [1].

Benzoic acid is very effective against the growth of molds, yeasts, and bacteria. It is particularly well suited for soft drinks such as carbonated, still, and juice beverages because it works best at a pH value of 2–4. The benzoic acid salts are more stable than the acid form and more soluble in water, which make them a favorable choice in the soft drinks industry [2].

Sorbic acid and its salts are widely used as preservatives in the food industry to protect high-acid (low-pH) foods primarily against the growth of yeasts and molds. Their salts are more soluble than the

acid form, but addition at high levels can affect the taste of the product. Benzoates and sorbates are often used in combination, especially for highly acidic soft drinks [3].

In 1954, the fungal degradation of food preservatives was demonstrated for the first time through the disappearance of sorbic acid from cheese wrappers contaminated with mold [4,5]. Since then, many cases of spoilage have been reported in foods with added sorbate such as cheeses [6], margarine [7], marzipan [8], bakery products [9], and soft drinks [10–12].

Spoilage manifests as an off-odor, which has been referred to as the smell of plastic, hydrocarbons, or kerosene, resulting from the decarboxylation of sorbate into the volatile hydrocarbon 1,3-pentadiene. Several species of microorganisms were described as responsible for this decarboxylation process, including molds such as *Penicillium* spp. (*P. brevicompactum*, *P. caseicolum*, *P. chrysogenum*, *P. crustosum*, *P. cyaneofulvum*, *P. cyclopium*, *P. frequentans*, *P. puberulum*, *P. roqueforti*, *P. notatum*, *P. simplicissimum*), *Aspergillus niger*, *Paecilomyces variotii*, or *Trichoderm*a spp. (*T. koningii*, *T. viride*), *Cephaloascus fragrans*, and osmotolerant yeasts such as *Debaryomyces hansenii*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces rouxii* [6,7,10–17].

Styrene, an aromatic hydrocarbon, is another fungal metabolite that causes an intense and unpleasant odor in food. Previous studies have described the production of styrene using some dairy starter cultures, such as *Penicillium camemberti* [18] and *Penicillium caseifulvum* [19]. A case of styrene taint in spiced buns was reported as caused by the yeast *Hyphopichia burtonii* in the presence of cinnamon flavoring [9], with a simple decarboxylation mechanism suggested by the author. However, *P. caseicolum* produces styrene by de novo synthesis from a simple basal medium, which is independent of the carbon source, and in the absence of flavor precursors [20]. Nevertheless, it should be mentioned that in the study of Pinches and Apps [10], styrene was produced only in the presence of cinnamic acid and was not synthesized de novo.

The aim of the present work was to identify the off-odor compounds in samples of strawberry-flavored water preserved with potassium sorbate and sodium benzoate. In addition, the study evaluated the mold isolated from this drink for the ability to form such undesirable compounds.

2. Materials and Methods

2.1. Samples

Twenty bottles of strawberry-flavored bottled water preserved with potassium sorbate and sodium benzoate were delivered from the producer. According to the information after bottling and distribution, characteristic delicate, fluffy, cottony masses were suspended in the water, and a kerosene-like smell appeared in the two-month period. Strawberry-flavored water from another batch with no signs of spoilage was also tested.

2.2. Analyses of Volatile Compounds

Gas chromatography coupled to mass spectrometry (GC/MS) was used for volatile identification and quantification. Volatiles were sampled using the solid phase extraction method. The samples were extracted using a CombiPal auto-sampler (CTC Analytics AG, Zwingen, Switzerland) and introduced into a GC instrument. A 10-mL sample of strawberry-flavored water was added into a 20-mL glass auto-sampler vial and spiked with internal standard (trimethylpiridine). The vials were closed with screw caps sealed with Teflon and conditioned for 10 min at 45 °C. The headspace was extracted for 30 min using solid phase microextraction (SPME) fibers coated with 50/30 mm-thick divinylbenzene/carboxen/polydimethylsiloxane films. Before use, the fibers were conditioned for 60 min at 270 °C in the GC injector port. Then, they were desorbed for 5 min at the injector port operated at 260 °C in the splitless mode. Chromatographic analysis was carried out using an Agilent 5975 C/6890 GC/MS instrument (Santa Clara, CA, USA). Separation was performed on a ZB-WAX fused-silica capillary column (30 m × 0.25 mm id × 0.5 mm film thickness). Helium flowing at a

constant rate of 1.2 mL min^{-1} was used as the carrier gas. The oven was programmed from 6 min hold at 40 °C, ramped at 4 °C/min to 150 °C and then at 20 °C/min to 250 °C, and finally held for 5 min. Samples were analyzed in triplicates. 2,4,6-trimethylpyridine at a final concentration of 1 mg/L was used as the internal standard. Pure authentic standards (Sigma-Aldrich, St. Louis, MO, USA) were used for calibrating the quantified compounds.

2.3. Determination of Preservative Concentration

The concentrations of benzoic and sorbic acid were analyzed by applying some modifications to the high-performance liquid chromatography (HPLC) method presented in the ISO 22855:2008 standard [21]. The analysis was carried out using a Waters 2695 Separation Module and Waters 2996 Photodiode Array Detector (both from Waters, Milford, MA, USA). Before the analysis, the samples were diluted with methanol and filtered through 0.45 µm syringe filters. Then, 10 µL of samples was separated on a Sunfire C8 column (5 µm, 4.6 mm × 250 mm; Waters) thermostated to 35 °C. The samples were eluted at a flow rate of 0.8 mL/min using a gradient of ammonium acetate (analytical grade; Sigma-Aldrich, St. Louis, MO, USA) and glacial acetic acid buffer (POCh, Gliwice, Poland, pH 3.0) used as solvent A and methanol (HPLC grade; POCh, Gliwice, Poland) used as solvent B, as follows (time, corresponding composition): 0 min, 52% A; 1.5 min, 52% A; 2.25 min, 61% A; 15 min, 43% A; 22.5 min, 43% A; and 23.25 min, 52% A. The compounds were quantified by UV absorption at 235 nm.

2.4. Microbial Analysis and Identification of Molds

Three randomly selected bottles of spoiled strawberry-flavored water were examined by microscopic observation, and mold mycelia were found. Samples of water were spread on plate count agar (PCA) (Merck, Darmstadt, Germany), orange serum agar (OSA) (Merck), dichloran rose bengal chloramphenicol (DRBC) (Merck) and de Man, Rogosa and Sharpe agar (MRS) (GRASO Biotech, Starogard Gdański, Poland) for the screening of various microorganisms. After incubation (72 h at 25 °C for DRBC and 72 h, 30 °C for other media), the presence of a homogeneous culture of mold was observed. Before molecular analysis, mold was spread on malt extract agar (Merck) and incubated at 25 °C for 5 days.

The molds were identified by sequencing the internal transcribed spacer ITS region of the rDNA operon and D1/D2 region of the 26S rDNA gene. Universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG) and ITS4 (5′-TCCTCCGCTTATTGATATGC) were used for the analysis of ITS region, while NL1 (5′-GCATATCAATAAGCGGAGGAAAAG) and NL4 (5′-GGTCCGTGTTTCAAGACGG) primers were used for sequencing the 26S gene. Amplification was performed in a total volume of 40 µL, which contained 20 ng of the template DNA, 40 pM of each of the primers, and 25 µL of DreamTaq™ Green PCR Master Mix (Thermo Scientific, Wallham, MA, USA). The amplification reaction for ITS involved the following: initial denaturation at 95 °C for 2 min; six cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 35 s, and elongation at 72 °C for 1 min; 28 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 35 s, and elongation at 72 °C for 1 min; and final elongation at 72 °C for 2 min. The amplification reaction for the 26S rDNA gene included the following: initial denaturation at 95 °C for 1 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 1 min; and final elongation at 72 °C for 2 min. The amplified products were sequenced using ITS1/ITS4 or NL1/NL4 primers. The sequences were assembled by Serial Cloner 2.6 software (SerialBasic).

2.5. Assessment of the Mold's Ability to Form Off-Odor Compounds

The mold's *Penicillium corylophilum* conidia suspension (about 10^7 CFU/mL) was prepared in physiological salt from 5-day culture grown on malt extract agar (Merck, Darmstadt, Germany) at 25 °C. Potato dextrose broth (PDB; Merck, Darmstadt, Germany) with pH adjusted to 3.5 by adding sterile 10% (*m*/*v*) tartaric acid (Merck, Darmstadt, Germany) was used in this study, according to Pinches and Apps [10]. Sterile potassium sorbate (Sigma-Aldrich, St. Louis, MO, USA) and sodium benzoate

(Sigma-Aldrich, St. Louis, MO, USA) were added to achieve a concentration of 200 or 400 mg/L. Ten milliliters of PDB with or without (control media) preservatives was inoculated with a 0.1 mL conidia suspension and incubated at 25 °C for 2 and 5 days. After incubation, the headspace was analyzed to determine the presence of volatile compounds, while the medium was analyzed for the presence of sorbic and benzoic acid. The study was performed in triplicate.

2.6. Calculation of Odor Activity Values

The odor activity values (OAVs) of volatile compounds were calculated according to Guclu et al. 2016 [22] by dividing concentrations of aroma compounds with their sensory thresholds from the literature [23,24].

2.7. Statistical Analysis

The significance of differences ($p < 0.05$) between the mean values of compound concentration was tested by the analysis of variance and Tukey's multiple-range test, using StatSoft® Statistica 13.1.

3. Results and Discussion

Although fungal degradation of food preservatives has been known since the 1950s, cases of spoilage associated with the formation of unpleasant odors appearing from time to time surprise manufacturers, which force them to identify the cause of food spoilage. The present study analyzed the samples of spoiled strawberry-flavored bottled water with characteristic, visually observed delicate, fluffy, cottony masses sent by the manufacturer for investigation. Assuming that the cause of spoilage was related to the dosage of preservatives added, the concentrations of potassium sorbate and sodium benzoate were examined. In the same batch of tested water, a kerosene-like smell was also found in some samples.

The homogenous culture of mold was isolated from the samples of spoiled strawberry-flavored bottled water. No growth of other microorganisms was obtained. By comparing the sequences of ITS and 26S rDNA to the GenBank sequences database, the mold was identified as *Penicillium corylophilum*. The sequence of ITS fragment of rDNa operon isolated from analyzed strain was 100% identical to *P. corylophilum* strain reported in GenBank under accession number MW165219.1, and 99.82% to several other *P. corylophilum* strains, e.g., MK450687.1, MH861216.1, KY469027.1. The sequence of D1/D2 fragment of 26S rDNA gene isolated from analyzed strain was 100% identical to several *P. corylophilum* strains, e.g., MH878082.1, MW165060.1, MH869416.1. The sequences of the analyzed strain were deposited in the GenBank database under the following numbers: MW165060 for the D1/D2 region of the 26S rDNA gene and MW165219 for the ITS region of the rDNA operon.

To date, *P. corylophilum* fungi have been found mainly on damp building materials (paper-faced gypsum wallboard, fibrous insulation, and manufactured wood) and damp and moldy buildings [25]. It has been reported to cause spoilage of high-fat foods and jams. *P. corylophilum* has been isolated from cereals, salami, nuts, water and fruit juices [26].

In the next stage of research, the content of preservatives in the tested samples of strawberry-flavored water was determined. The concentration of preservatives was found to be significantly decreased in the samples of spoiled water compared to water with no signs of spoilage (Table 1). The decrease in sodium benzoate concentration reached 42%, while the concentration of potassium sorbate was under the limit of determination.

Compounds such as 1,4-pentadiene, benzaldehyde, and styrene were detected in the tested samples of spoiled strawberry-flavored water preserved with potassium sorbate and sodium benzoate (Table 2). It should be noted that only 1,4-pentadiene and styrene show an intense and unpleasant odor, while benzaldehyde has an almond-like odor. Accordingly, it was concluded that the mold *P. corylophilum* is responsible for the formation of off-odor volatile compounds and preservative degradation. Thus, the next part of the work investigated the ability of this strain to produce volatile organic compounds such as 1,4-pentadiene, benzaldehyde, and styrene.

Table 1. Concentration of preservatives in strawberry-flavored water.

Sample Description	Sodium Benzoate (mg/L)	Potassium Sorbate (mg/L)
Spoiled strawberry-flavored water	77.3 ± 1.2 [a]	≤2.7 [a]
Strawberry-flavored water with no signs of deterioration	133.3 ± 0.7 [b]	126.0 ± 0.2 [b]

Data are presented as mean ± standard deviation (n = 3). Means with different letters within columns ([a,b]) differ significantly ($p < 0.05$).

Table 2. Off-odor volatile compounds found in strawberry-flavored water.

Sample Description	1,4-Pentadiene	Benzaldehyde	Styrene
Spoiled strawberry-flavored water	+	+	+
Strawberry-flavored water with no signs of deterioration	-	-	-

+: present, -: absent.

After 5 days of incubation, all samples showed mycelial growth. Potassium sorbate added at a final concentration of 200 and 400 mg/L in PDB was completely assimilated by the growing mycelium (Table 3) and converted into 1,4-pentadiene (Table 4), and concentration of the latter was determined as 46.5 and 92.6 mg/L, respectively. The decrease in the concentration of sodium benzoate exceeded 53% in the broth spiked at 200 mg/L and 23% at 400 mg/L (Table 3), resulting in the formation of benzaldehyde at concentrations of 1.27 and 6.58 mg/L, respectively (Table 4). The strain produced both 1,4-pentadiene and benzaldehyde in PDB within 5 days of incubation in the presence of sorbic acid and benzoic acid, respectively. It was observed that these compounds were produced only in the presence of preservatives and were not synthesized de novo under the test conditions.

Table 3. Changes in the concentration of preservatives in potato dextrose broth (PDB) inoculated with *Penicillium corylophilum* after 5 days of incubation.

Sample Description	Initial		After 5 Days of Incubation	
	Potassium Sorbate (mg/L)	Sodium Benzoate (mg/L)	Potassium Sorbate (mg/L)	Sodium Benzoate (mg/L)
PDB control	≤2.7 [a]	≤2.4 [a]	≤2.7 [a]	≤2.4 [a]
PDB + 200 mg/L potassium sorbate	196.3 ± 0.9 [b]	≤2.4 [a]	≤2.7 [a]	≤2.4 [a]
PDB + 400 mg/L potassium sorbate	401.4 ± 2.4 [c]	≤2.4 [a]	≤2.7 [a]	≤2.4 [a]
PDB + 200 mg/L sodium benzoate	≤2.7 [a]	202.3 ± 1.6 [b]	≤2.7 [a]	95.5 ± 0.7 [b]
PDB + 400 mg/L sodium benzoate	≤2.7 [a]	401.1 ± 3.2 [c]	≤2.7 [a]	309.4 ± 0.2 [c]

Data are presented as mean ± standard deviation (n = 3). Means with different letters within columns ([a–c]) differ significantly ($p < 0.05$).

Although styrene was detected in spoiled strawberry-flavored water (Table 2), it probably did not arise from preservative degradation, as the highest concentration of the compound was found in the PDB control medium that had no preservatives (Table 4). However, the observed differences in the styrene concentration were not found statistically significant.

To determine the intensity of volatile compounds odors, the OAVs were calculated and are given in Table 5.

Table 4. Concentration of off-odor volatile compounds in PDB inoculated with *Penicillium corylophilum* after 5 days of incubation.

Sample Description	After 5 Days of Incubation		
	1,4-Pentadiene (mg/L)	**Benzaldehyde (mg/L)**	**Styrene (mg/L)**
PDB control	0.06 ± 0.02 [a]	0.35 ± 0.16 [a]	1.18 ± 0.72 [a]
PDB + 200 mg/ potassium sorbate	46.51 ± 14.78 [b]	0.16 ± 0.10 [a]	0.21 ± 0.13 [a]
PDB + 400 mg/L potassium sorbate	92.64 ± 21.20 [c]	0.28 ± 0.01 [a]	0.39 ± 0.11 [a]
PDB + 200 mg/L sodium benzoate	0.05 ± 0.02 [a]	1.27 ± 0.12 [b]	0.53 ± 0.26 [a]
PDB + 400 mg/L sodium benzoate	0.02 ± 0.01 [a]	6.58 ± 1.30 [c]	0.76 ± 0.35 [a]

Data are presented as mean ± standard deviation (n = 3). Means with different letters within columns ([a–c]) differ significantly ($p < 0.05$).

Table 5. Odor thresholds and odor activity values (OAVs) of 1,4-pentadiene and benzaldehyde.

Compounds	Odor Threshold Value	Odor Activity Value	Odor Descriptions
1,4-pentadiene in PDB + 200 mg/L potassium sorbate	100 µg/L in soft drinks [23]	465.1	odor of plastic, hydrocarbons, or kerosene
1,4-pentadiene in PDB + 400 mg/L potassium sorbate		926.4	
Benzaldehyde in PDB + 200 mg/L sodium benzoate	500 µg/L in water [24]	2.5	almond-like odor
Benzaldehyde in PDB + 400 mg/L sodium benzoate		13.2	

To the best of our knowledge, this study is the first to report the production of 1,4-pentadiene by molds in beverages. Thus far, the presence of molds producing this compound only in environments other than food and beverages has been reported [27]. In the study by Micheluz et al. (2016) [27], a total of 55 different volatile organic compounds of airborne molds and those grown on books isolated from a contaminated library, including 1,4-pentadiene, were detected. 1,4-pentadiene was produced by *P. chrysogenum*, *P. brevicompactum*, *Eurotium chevalierii*, *Eurotium halophilum*, *Aspergillus creber*, and *Aspergillus penicillioides*. The production of 1,3-pentadiene by fungi has been reported much more frequently. For instance, in 2011, the presence of this compound in 21 supermarket products such as black olives, green olives in brine, honey, bread, cookies, soy sauce, salad, and cheese was identified [28]. Recently, its production by 91 strains of the yeast *D. hansenii* was indicated [29]. Researchers found that nearly 96% strains of this yeast were able to produce this compound. This is especially important because *D. hansenii* belongs to the inventory of microorganisms with technological benefits due to its use in food fermentation and as a biocontrol agent [29]. Ten fungal strains isolated from jam, yogurt, or indoor air were grown in strawberry jam and strawberry jam agar, which produced five different volatile compounds, including 1,3-pentadiene and styrene [30].

The growth of some strains of certain mold species is not always controlled by commonly used antimycotics such as sorbate. The use of antimycotics other than sorbate, for example, natamycin [31], ethylenediaminetetraacetic acid [32], and propionate [33], or a reduced concentration of sorbate in combination with other antimycotics may be an alternative solution to prevent or retard the growth of 1,3-pentadiene-producing molds [12].

The potential of fungi to produce off-odor volatile organic compounds should be taken to account when conducting hazard analysis in a hazard analysis critical control point (HACCP) study. High-care bottling areas should be employed to prevent contamination by ubiquitous molds. In particular, strawberry flavor as a potential source of fungi should be investigated.

4. Conclusions

The study showed that *P. corylophilum* strain produced both 1,4-pentadiene and benzaldehyde in PDB within 5 days of incubation in the presence of potassium sorbate and sodium benzoate, respectively. However, these volatile organic compounds were produced only in the presence of preservatives and were not synthesized de novo under the test conditions. This study reports for the first time the conversion of potassium sorbate to 1,4-pentadiene and sodium benzoate to benzaldehyde by *P. corylophilum* strains. The presence of styrene in the tested samples might be taken from polystyrene-based materials and plastic labware commonly used in microbiological laboratories.

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