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Biosynthesis of eight-carbon volatiles from tomato and pepper pomaces by fungi: *Trichoderma atroviride* and *Aspergillus sojae*

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The aim of this study was to investigate the possibility of using tomato and red pepper pomaces for the production eight-carbon volatiles by *Trichoderma atroviride* and *Aspergillus sojae*. The fermentation of tomato and pepper pomace-based media by both moulds was conducted in shake flasks and bioreactors. Microbial growth behaviours and fermentation abilities of *T. atroviride* and *A. sojae* under both fermentation conditions were followed by microbial counting. The production of flavours from tomato and pepper pomaces by fungal metabolism was determined by gas chromatography–olfactometry, gas chromatography–mass spectrometry and sensory analysis. The results showed that *T. atroviride* grew faster than *A. sojae*, and the survival of *T. atroviride* in the tomato pomace was longer than that of *A. sojae*. However, *T. atroviride* grew slower than *A. sojae* in the pepper pomace. Eight-carbon flavour compounds, including (*Z*)-1,5-octadien-3-ol, 1-octen-3-ol, (*E*)-2-octenal and (*E*)-2-octenol, were produced by *T. atroviride* and *A. sojae* from the tomato and pepper pomaces. The highest production levels (265.55 ± 2.79 and 187.47 ± 0.92 μg kg^{-1}) were observed for 1-octen-3-ol in the tomato fermentation by *T. atroviride* and *A. sojae*, respectively. The relationships between volatile compounds and their flavour characteristics in tomato and pepper pomaces were analysed using principal component analysis.

[**Key words:** Flavour; Fungal metabolism; Agro-waste; Microbial fermentation; Gas chromatography-olfactometry, Sensory analysis]

Only a few of bioresources have been studied for the biotechnological production in food industry so far (1,2). Use of agro-wastes from the fruit and vegetable industries becomes very popular for the production of high-value products. Production of natural food colours, enzymes, some gums and flavours from agro-wastes by biotechnological processes has been a focus for many studies (3–6).

Tomato and red pepper pomaces, which contain seeds and peel residues, are the most abundant agro-wastes in the fruit and vegetable industries. Heuze et al. (7) have indicated that tomato waste constituted about 11 million tons, including a little more than 4 million tons of tomato pomace, in 2007. Unfortunately, we could not obtain accurate information on the volume of pepper pomace in the world. While tomato pomace has 15–24% of protein, 5–20% of fat, 28–51% of total sugar, and 3–6% of mineral substances on a dry basis, very limited compositional data are available for red pepper pomace. Pepper seeds contain 24%, 35%, 25% and 4% of protein, fibre, oil and mineral substances, respectively. The seeds can be excellent sources for biotechnological processes because of their rich nutritional content for microbial growth (7–9).

Trichoderma and *Aspergillus* are filamentous fungi belonging to Deuteromycetes, which occur in a large variety of ecosystems. They are remarkable organisms due to their rapid growth, capability of utilising diverse substrates and resistance to noxious chemicals. *Trichoderma* and *Aspergillus* contain some biotechnological workhorse species due to their high enzyme activities and easy *in vitro* culturing. Some species of *Trichoderma* produce 6-pentyl-α-pyrone associated with a characteristic sweet or coconut odour, whereas some other *Trichoderma* species are used as cellulolytic and hemicellulolytic enzyme producers for the food, textile, pulp and paper industries. *Aspergillus* species such as *Aspergillus sojae* and *A. oryzae* have been used for years for the production of traditional fermented foods such as soy sauce, Sake and Koji in Asian countries (10–14).

Biotechnological approaches such as microbial fermentation have attracted increasing attention of researchers due to a possibility of valorisation of wastes and low-cost production steps using microorganisms (15,16). In the biotechnology of natural flavours, many studies were conducted on the production of flavour compounds using enzymatic approaches (17–19) and yeast metabolism in synthetic media or certain agro-wastes (20–22). Much of what is known about the synthesis of natural flavour compounds by biotechnological processes comes from the metabolism of yeasts and bacteria (4,15,23). However, there is still lack of information on the production of natural flavours from agro-wastes by fungal metabolism, except vanillin production (24–28). From this perspective, mushroom-like flavours such as 1-octen-3-ol, 1-octen-3-one and octanol can be produced from tomato and pepper pomaces by primary and secondary metabolism of filamentous fungi such as *Trichoderma* and *Aspergillus* (5,29).

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The aim of this study was to investigate the possibility of using tomato and red pepper pomaces to produce certain aroma compounds and then comparing the flavour production behaviour of *Trichoderma atroviride* and *A. sojae* in the agro-wastes.

MATERIALS AND METHODS

Preparation of tomato and red pepper pomaces Tomato and red pepper pomaces were prepared according to Guneser et al. (30). The chemical compositions of the tomato and pepper pomaces are shown in Table 1. About 500 g of each pomace was ground using a knife mill (Restch GM 200, Haan, Germany), and 1 L of a 100 g L^{-1} suspension was prepared with distilled water for shake flask fermentation, carried out in 250-mL Erlenmeyer flasks. Each pomace solution was homogenised at 24,000 rpm using ULTRA-TURRAX (IKA-Werke GmbH, Germany). The solutions were sterilised at 121 °C for 15 min in an autoclave (Hirayama, Saitama, Japan).

Cultivation and preparation of microbial cultures and suspensions The strains of *T. atroviride* (NRRL 31396) and *A. sojae* (NRRL 1988) were obtained from the ARS (NRRL) Culture Collection (Peoria, IL, USA). *T. atroviride* NRRL 31396 was cultured on malt extract agar (MEA), while *A. sojae* NRRL 1988 was cultured on Dichloran Rose Bengal Chloramphenicol Agar (DRBC). Both moulds were incubated at 30 °C for seven days. The spores of *T. atroviride* and *A. sojae* were collected by washing the fungal growth on the surface of agar with Tween 80 (1 g kg^{-1}). The spore suspensions were filtered through two layers of sterilised cheese cloth to remove residual agar, then centrifuged at 1000 ×*g* for 5 min, and washed with a saline solution (8.5 g L^{-1} of NaCl) to remove the Tween solution. Spore concentrations in the microbial suspensions were determined by counting with a Thoma slide using a light microscope (Olympus CX 31, Olympus, Philippines) (24,31). The concentrations of the spore suspensions obtained were 10^7–10^8 spores mL^{-1}.

Fermentation experiments Fermentation experiments were conducted in 250-mL Erlenmeyer shake flasks with a 100-mL working volume. About 10^7–10^8 spores mL^{-1} were inoculated to the tomato and pepper pomace solutions, and the flasks were incubated at 30°C for 120 h in a shaking incubator at 120 rpm. The same procedure was applied to prepare a control group without the microorganisms. The shake-flask experiment was conducted in duplicate.

Batch fermentation was performed at 30°C in a 5-L stirred tank bioreactor (STR) (Biostat A-plus, Sartorius, Melsungen, Germany) with a 4-L working volume. The initial pH values of the tomato and pepper pomace solutions were 4.43 and 3.93, respectively. The aeration rate was 0.325 vvm, and the agitation speed was 120 rpm. Batch fermentation conditions were determined based on the microbial growth and aroma production results from the shake-flask fermentation. The STR was equipped with two six-blade impellers, a pH probe (EasyFerm K8/325, Hamilton) and a PT 100 temperature sensor.

Microbial growth Microbial counts of *T. atroviride* and *A. sojae* were monitored under both fermentation conditions to determine their growth behaviours and fermentation abilities on the agro-wastes. MEA was used for *T. atroviride*, and DRBC was used for *A. sojae*. Plates were incubated at 30°C for 5–7 days. Specific growth rates were calculated using growth curves to determine the characteristics of microbial growth in batch fermentation (32).

Analysis of flavour compounds Flavour compounds in the fermented tomato and red pepper pomace solutions were determined by gas chromatography–olfactometry (GCO) and gas chromatography–mass spectrometry (GC–MS).

Extraction of flavour compounds Flavour compounds for the GCO and GC–MS analyses were isolated from the fermented tomato and red pepper solutions by solid-phase microextraction (SPME) (33). Three grams of the solutions were weighed in a 40-mL amber-coloured screw-top vial with a hole-cap polytetrafluoroethylene/silicon septum (Supelco, Bellefonte, PA, USA), and 1 g of NaCl was added to the vial. The vial was kept at 40°C in a water bath for 20 min to equilibrate volatiles in the headspace. Then, an SPME fiber (2 cm, 50/30 μm DVB/Carboxen/PDMS, Supelco) was inserted into the vial. The SPME fibre was exposed at a depth of 2 cm in the headspace of the vial. Then, the SPME needle was immediately injected into a GCO or GC–MS column.

Gas chromatography–olfactometry analysis GCO analysis was conducted using HP 6890 GC (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionisation detector (FID), sniffing port and splitless injection system. A nonpolar column (HP-5, 30 m length, 0.32 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) was used for sniffing. Helium was used as a carrier gas. The inlet pressure was 48.74 kPa, and the flow rate was 1.2 mL min^{-1}. The GC oven temperature was programmed from 40°C to 230°C at a rate of 10°C min^{-1}, with the initial and final hold times of 5 and 20 min, respectively. The FID and sniffing port were maintained at temperatures of 250°C and 200°C, respectively. The GCO procedure was duplicated by two sniffers. A post-peak intensity method was used for the determination of aroma intensity using a 10-point scale anchored to the left with 'not' and to the right with 'very' (34). The sniffers had 300 h of experience with the GCO technique, scale use and odour description. Aroma-active compounds were identified by comparing the retention indices and odour quality of unknowns with those of references analysed under the same experimental conditions by the sniffers during the GCO procedure. Retention indices were calculated using an *n*-alkane series (35).

TABLE 1. Chemical compositions of tomato and pepper pomaces.

Chemical properties	Tomato pomace	Red pepper pomace
pH	4.59	4.57
Dry matter (g kg^{-1})	139.90	205.80
Total nitrogen (g kg^{-1})	3.50	4.80
Ash (g kg^{-1})	6.50	6.90

Gas chromatography–mass spectrometry analysis Volatile compounds were tentatively identified by GC–MS. A nonpolar HP5 MS column (30 m × 0.25 mm i.d. × 0.25-μm film thickness; J&W Scientific) was used for separation of flavour compounds. The GC–MS system consisted of an HP 6890 GC and 7895C mass-selective detector (MSD; Agilent Technologies, Wilmington, DE, USA). The GC oven temperature was programmed from 40 to 230°C at a rate of 10°C min^{-1}, with the initial and final hold times of 5 and 20 min, respectively. Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. The MSD conditions were as follows: capillary direct interface temperature, 280°C; ionisation energy, 70 eV; mass range, 35–350 amu; scan rate, 4.45 scans s^{-1}. The identification of flavour compounds was based on comparison of the mass spectra of unknown compounds with those in the databases of the National Institute of Standards and Technology and Wiley Registry of Mass Spectral Data. Flavour compounds were quantified based on their relative abundances. 2-methyl pentanoic acid (0.093 μg mL^{-1}) and 2-methyl-3-heptanone (0.486 μg mL^{-1}) were used as internal standards for acidic and neutral–basic compounds, respectively (36). All chemicals and reagents were of chromatographic grade and obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA) and Merck KGaA (Darmstadt, Germany).

Sensory analysis A roundtable discussion was conducted to determine descriptive sensory properties and changes in aroma profiles of fermented versus control samples (31). Seven trained panellists conducted sensory evaluation. The panellists were staff and graduate students at the Department of Food Engineering of Çanakkale Onsekiz Mart University; four were females and three were males; the age ranged from 24 to 45 years old. The panel received about 300 h of training on definition of descriptive terms for various kinds of products. The panellists quantified the attributes using a 15-point product-specific scale anchored to the left with 'not' and to the right with 'very' (37).

Statistical analysis One-way analysis of variance (ANOVA) was conducted to determine the differences in intensities of flavour compounds among the fermented and unfermented solutions, obtained by GC–MS analysis for the shake-flask and bioreactor fermentations. Moreover, two-way ANOVA was conducted to evaluate the growth of the microorganisms in each pomace during the shake-flask fermentation. Welch's test, a parametric alternative to ANOVA, was also used for the data that did not meet the prerequisites (homogeneity of variance and equality of variance) for ANOVA. Tukey's honestly significant differences test was used for separating means (38). SPSS for Windows (version 15.0) was used for the ANOVA analyses. Principal component analysis (PCA) was performed using the XLSTAT statistical program (trial version, 2015, Addinsoft, Inc., New York, NY, USA) for interpreting the quantity of produced flavour compounds and their sensory impacts or perceptions.

RESULTS AND DISCUSSION

Growth of *T. atroviride* and *A. sojae* in tomato and pepper pomaces It was determined that the microbial growth in the tomato and pepper pomaces depend on the mould type and fermentation time ($P = 0.01$). The maximum count of *A. sojae* was observed at 72 and 48 h of fermentation in the tomato and pepper pomaces, respectively. A significant increase in the count of *T. atroviride* was determined at 96 and 72 h of fermentation (Table 2). When the increases in the counts of *A. sojae* and *T. atroviride* at the end of the shake-flask fermentations of both pomaces were compared, *T. atroviride* showed slightly higher growth in the tomato pomace; meanwhile, the growth (2.28 log colony-forming units mL^{-1}) of *A. sojae* was higher than that of *T. atroviride* in the pepper pomace. These results were confirmed by the growth behaviours for the batch fermentations. Using nutrients from agro-wastes for microbial growth promotion is quite challenging. Many agro-wastes from the fruit and vegetable industries have cellulose, hemicellulose and lignin-type polysaccharides. These polysaccharides cannot be directly utilised by many microorganisms, while free glucose, lactose and sucrose in synthetic media are more effectively used by microorganisms

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