# Development of Novel Fermentation Systems for the Production of Nonalcoholic Beverages with Basidiomycetes

Cumulative dissertation

Presented by

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I declare that I have completed this dissertation without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived from published literatures, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

Date, Place

Signature

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Basidiomycetes represent the highest developed class of fungi. They are able to synthesize pharmacological relevant secondary metabolites, natural flavor compounds, and highly sought after enzymes. Because of their biochemical potential, basidiomycetes are ideal tools for the food industry.

With the recent worldwide declining consumption of beer, breweries are eagerly searching for innovative nonalcoholic fermented beverages to compensate for this negative trend. Different from microorganisms used in traditional fermented beverages, such as yeasts, lactic acid bacteria, *Acetobacter* species, and lower fungi, basidiomycetes possess the unique capability of producing a wide range of natural flavors in submerged cultures. Because of the consumers' demand for natural food and the above-mentioned biochemical potential, basidiomycetes producing natural flavor compounds are ideal candidates to develop novel fermentation systems for producing nonalcoholic fermented beverages.

In novel fermentation systems, 31 edible or medicinal basidiomycetes were cultivated in wort (without hops) for up to 48 h at 24 °C and 150 rpm. Every 2 - 4 h, samples were taken, and species exhibiting interesting flavor impressions were identified with their corresponding fermentation times by sensory evaluation. Afterwards, aroma compounds of the beverages with pleasant flavors were isolated by liquid-liquid extraction (LLE) and by headspace solid phase microextraction (HS-SPME). The key odor-active compounds were analyzed by aroma extract dilution analysis (AEDA) using a gas chromatography system equipped with a tandem mass spectrometry detector and an olfactory detection port (GC-MS/MS-O). For HS-SPME, a revised AEDA method was developed by sequentially increasing the GC inlet split ratio (aroma dilution analysis, ADA). The key odorants of the beverages were analyzed in kinetic studies, quantified, and verified by aroma reconstitution. The biosynthetic pathway of the most important odorant of a beverage fermented by shiitake was elucidated by means of isotopic labeling experiments. The results may help to control and improve the quality of the products in the future.

All of the selected basidiomycetes grew well in wort and highly interesting flavor impressions (*e.g.*, green, fruity, floral, honey-like, and toasted) were perceived. Among the 31 screened fungi, shiitake (*Lentinula edodes*) and *Trametes versicolor* were rated as the most promising species for producing novel nonalcoholic beverages after sensory evaluation by 10 panelists. The flavors of both beverages were distinctly different from the substrate wort (malty, sweetish, and aromatic).

Wort fermented by shiitake exhibited a very pleasant flavor after 48 h, which was perceived as fresh, fruity, plum-like, sweet, and slightly sour. Most of the key odor-active

compounds (*e.g.*, 2-acetylpyrrole, *ß*-damascenone, (*E*)-2-nonenal, and 2-phenylethanol) were detected with both extraction techniques. However, distinct differences between these two methods were observed. Compounds such as methyl 2-methylbutanoate and (*E*)-methyl cinnamate, which exhibit typical fruity flavors were only identified by HS-SPME as key odor-active compounds. The kinetic studies indicated that shiitake synthesized fruity and rose-like odors, and simultaneously degraded the typical odorants of the substrate wort during the fermentation. Using the standard addition method, a good linearity and high degree of precision were obtained for the quantification of twelve aroma compounds with flavor dilution (FD) factors ranging from 4 to 64. Methyl 2-methylbutanoate, produced by shiitake, showed the highest odor activity value (OAV 30) and was responsible for the typical fruity odor of the beverage. A correlation between the concentration of methyl 2-methylbutanoate and the perceived fruitiness of the beverage was observed. The biosynthesis of this important methyl ester by transformation of 2-methylbutanoic acid and L-isoleucine was confirmed by means of an isotopic labeling experiment.

Another novel beverage fermented by *T. versicolor* also possessed an appealing aroma profile (fresh, fruity, sweetish, and slightly floral). Six odor-active compounds produced by *T. versicolor*, ethyl 2-methylbutanoate (OAV 1.2), ethyl 2-methylpropanoate (OAV 31), *ß*-linalool (OAV 16.4), 2-methylbutanoic acid (OAV 2.0), 3-methylbutanoic acid (OAV 7.0), and 2-methylpropanoic acid (OAV 2.3), were responsible for the overall flavor of the beverage. In addition, the initial oxalic acid concentration of the substrate wort was decreased by 18% during the fermentation with *T. versicolor*.

Shiitake and *T. versicolor* represent ideal candidates to develop novel fermentation systems to produce nonalcoholic fermented beverages. It is well-known that the sensory and organoleptic characteristics are important for acceptance and utilization of any natural or processed food. In the novel fermentation system, unique flavors were generated in short fermentation times without formation of off-flavors. In addition, the whole fermentation procedures are easy to handle and may be performed with traditional brewery equipment. Shiitake is the second most popular edible mushroom in the global market and was the first medicinal macrofungus to enter the realm of modern biotechnology. *T. versicolor* has already been recognized as a functional food and promising dietary supplement for promoting health qualities. Therefore, a high consumer acceptance and a high market potential for the novel fermented beverages are expected.

This thesis is based on the following peer-reviewed original publications:

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2. Zhang, Y., Hartung, N. M., Fraatz, M. A., & Zorn, H. (2015). Quantification of Key Odor-Active Compounds of a Novel Nonalcoholic Beverage Produced by Fermentation of Wort by Shiitake (*Lentinula edodes*) and Aroma Genesis Studies. *Food Research International*, *70*, 23-30.

3. Zhang, Y., Fraatz, M. A., Müller, J., Schmitz, H., Birk, F., Schrenk, D., & Zorn, H. (2015). Aroma Characterization and Safety Assessment of a Beverage Fermented by *Trametes versicolor*. *Journal of Agricultural and Food Chemistry*, *63*, 6915-6921.

Peer-reviewed book chapter:

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Conference abstracts:

1. Zhang, Y., Fraatz, M. A., Quitmann, H., Czermak, P., & Zorn, H. (2014). Determination of Key Odor-Active Compounds of a Novel Beverage Fermented by Shiitake using Headspace Solid Phase Microextraction. *Lebensmittelchemie*, *68*, 66-67.

2. Zhang, Y., Fraatz, M. A., Hartung, N. M., Quitmann, H., Czermak, P., & Zorn, H. (2014). Quantification of Key Odor-Active Compounds of a Novel Non-Alcoholic Beverage Produced by Fermentation of Wort using Shiitake (*Lentinula edodes*) and Aroma Reconstitution Studies. *Lebensmittelchemie*, *68*, 154.

3. Oezdemir, S., Quitmann, H., Zhang, Y., Fraatz, M. A., Zorn, H., & Czermak, P. (2014). Einfluss Verschiedener Prozessparameter auf die Natürliche Aromaproduktion mit *Lentinula edodes* (Shiitake) für Neuartige Fermentierte Alkoholfreie Getränke. *Lebensmittelchemie*, *68*, 151.

| | |
|---|---|
| **AEDA** | Aroma extract dilution analysis |
| **ADA** | Aroma dilution analysis |
| **ADH** | Alcohol dehydrogenase |
| **CAR/PDMS** | Carboxen/polydimethylsiloxane |
| **CBS** | *Centraalbureau voor Schimmelcultures* |
| **DSMZ** | *German collection of microorganisms and cell cultures* |
| **DVB/CAR/PDMS** | Divinylbenzene/carboxen/polydimethylsiloxane |
| **FD** | Flavor dilution |
| **GC** | Gas chromatography |
| **GC-MS/MS-O** | Gas chromatography equipped with a tandem mass spectrometry detector and an olfactory detection port |
| **HS-SPME** | Headspace solid phase microextraction |
| **i.d.** | Inner diameter |
| **IS** | Internal standard |
| **LAB** | Lactic acid bacteria |
| **LLE** | Liquid-liquid extraction |
| **MS** | Mass spectrometry |
| **OAV** | Odor activity value |
| **ODP** | Olfactory detection port |
| **PDMS** | Polydimethylsiloxane |
| **PDMS/DVB** | Polydimethylsiloxane/divinylbenzene |
| **P&T** | Purge and trap |
| ***Rf*** | Response factor |
| **RI** | Retention index |
| **RSD** | Relative standard deviation |
| **SD** | Standard deviation |
| **SIM** | Selected ion monitoring |
| **SPE** | Solid phase extraction |
| **TIC** | Total ion current |
| **WBTM** | Department of molecular wood biotechnology and technical mycology |

# 1. Introduction

With the recent worldwide declining consumption of beer and the fast increasing consumers' demand for nonalcoholic beverages, breweries are eagerly searching for innovative nonalcoholic alternatives with highly competitive potential to expand their market share.

## 1.1 Fermentation Systems for Traditional Beverages

Fermentation is generally viewed as a simple, economical, and natural way for improving foods' sensory properties, nutritional value, and functional qualities as well as for preservation (Blandino, Al-Aseeri, Pandiella, Cantero, & Webb, 2003; Simango, 1997). During fermentation, polysaccharides, proteins, and lipids are often hydrolyzed to non-toxic metabolites by a diverse set of enzymes (*e.g.*, amylases, proteases, and lipases) from edible microorganisms. Additionally, the aroma, taste and texture of food may become more pleasant and more attractive to consumers (Steinkraus, 1996). At the same time, the nutritional value of food may be improved by supplementing certain essential amino acids, increased starch availability, and degradation of anti-nutrients (*e.g.*, phytic acid and oxalic acid) (Chavan & Kadam, 1989). In some fermentation processes, secondary metabolites (*e.g.*, organic acids and antibiotics) are produced, which can be effective against food spoilage and therefore extend the shelf-life.

Due to the potential of fermentation, the technology is widely applied in food processes. Throughout history and around the world, fermented beverages are produced by various manufacturing techniques, raw materials, and microorganisms (McGovern et al., 2004). Until now, four main processes (alcoholic, lactic acid, acetic acid, and alkali fermentations) are applied in the production of traditional and newly developed fermented foods. Apart from the well-known traditional fermented beverages like beer, wine, kwass, kombucha, and Chinese liquor, a series of innovations created novel fermented beverages (Table 1). The most often used microorganisms are yeasts, lactic acid bacteria (LAB), *Acetobacter* species, and lower fungi. For the majority of nonalcoholic fermented beverages, the microorganisms used are lactic acid bacteria or co-cultures mixed with yeasts or filamentous fungi (Chavan & Kadam, 1989; Hesseltine, 1979). Because of the formation of lactic acid, the flavor of the nonalcoholic beverages is typically perceived as sourish (Chavan & Kadam, 1989; Fleet, 1998).

**Table 1. Development of novel fermented beverages during the last decade**

| Raw material | Microorganism | Ethanol | Reference |
|---|---|---|---|
| Barley | LAB | - | Rathore, Salmerón, & Pandiella, 2012 |
| Wheat | LAB | - | Coda, Rizzello, Trani, & Gobbetti, 2011 |
| Oat | LAB | - | Muñoz-Insa, Gastl, Zarnkow, & Becker, 2011; Gokavi, Zhang, Huang, Zhao, & Guo, 2005 |
| Soybean | LAB | - | Champagne, Tompkins, Buckley, & Green-Johnson, 2010 |
| Banana | Yeast | * | Riana, Antonio, & Deborah, 2007 |
| Apple juice | LAB (Yeast) | -/* | Pereira, Maciel, & Rodrigues, 2011; Herrero, García, & Díaz, 2006 |
| Herbal mate leaves | LAB | - | Lima, De Dea Lindner, Soccol, Parada, & Soccol, 2012 |
| Seaweed | LAB | - | Ratanaburee, Kantachote, Charernjiratrakul, Penjamras, & Chaiyasut, 2011 |
| Potato | LAB | - | Kim, Jang, & Yoon, 2012 |
| Sweet potato | Yeast | * | Saigusa, Kawashima, & Ohba, 2007 |
| Whey | Kefir | - | Magalhães et al., 2010; Pescuma, Hébert, Mozzi, & De Valdez, 2010 |

- < 1%, * > 5%

## 1.2 Beer

Because of its pleasant sensory attributes, favorable nutritional characteristic, as well as low costs, beer is consumed worldwide as a popular beverage. However, in the last 16 years the per capita consumption in Germany, the largest European beer market, dropped from 127.3 liters in 1996 to 99.2 liters in 2012 (Statistisches Bundesamt, 2015). In contrast, the per capita consumption of nonalcoholic beverages, in particular of soft drinks, in Germany increased from 253.1 to 290.2 liters (Springer, 2011). Two main reasons for the annual decrease in beer sales are discussed:

*Increasing awareness of consumers about their health.*

Ethanol is metabolized to acetaldehyde mainly in the liver. Acetaldehyde is highly toxic and binds cellular constituents generating harmful acetaldehyde adducts (Rajendram & Preedy, 2009). It has been reported that alcohol causes approximately 4.6% of the global burden of disease and accounts for 3.6% of all deaths world-wide (Rehm et al., 2009).

Although alcoholics and heavy drinkers are at great risk of alcohol-related diseases, large problems arise from low or moderate drinkers (Bagnardi et al., 2011).

*Legal restrictions for alcohol consumption.*

Alcohol consumption is limited by safety regulations at the workplace or by increasing restrictive traffic laws regarding the blood alcohol content. In order to decrease risks of crimes, public disorder, and health damages, the consumption of alcohol in factories and shops is prohibited by labor protection laws or under certain individual circumstances (pregnancy, medication, cardiovascular and hepatic pathologies, and professional athletes) (Brányik, Silva, Baszczyňski, Lehnert, & Almeida e Silva, 2012).

In order to compensate for this negative trend, breweries are proposing a variety of different efforts for the industrial production of nonalcoholic beers with acceptable organoleptic properties. These strategies may be distinguished into two main groups: physical processes and biotechnological processes (Figure 1). However, nonalcoholic beers often suffer from having an artificial, immature, and dull (also known as flat or bland) flavor as well as an inappropriate body and foaming properties. During the dealcoholization process with physical methods, losses of volatiles occur. Although some flavor compounds that are lost during distillation can be trapped and re-added to the product after dealcoholization, it is still difficult to provide the initial balance of flavor compounds as found in normal beer (Caluwaerts, 1995; Dziondziak, 1989; Huige, Sanchez, & Leidig, 1990). Moreover, this procedure adds extra costs to the whole process. Various biotechnological approaches are based on a limited formation of ethanol during the beer fermentation. They are usually performed with traditional brewery equipment without additional investments. However, their products are often characterized by a worty off-flavor. Limited fermentation times during the production decrease the capability of yeast cells for the absorption and reduction of aldehydes such as acetaldehyde, 2,3-dimethylbutanal, hexanal, and heptanal in wort to alcohols. Relatively high amounts of residual aldehydes in nonalcoholic beers form worty-off flavors (Hardwick, 1994; Zurcher & Gruss, 1991). In order to enhance ‘worty’ aldehyde reduction, some special mutant yeast strains were applied in the production of nonalcoholic beer, such as an alcohol dehydrogenase-free (ADH-free) non-revertible mutant of *Saccharomyces cerevisiae* (Evellin, Perpète, & Collin, 1999). However, genetically modified yeasts are very difficult to introduce in industrial food applications because of both legal obstacles and particularly the consumers’ negative perception.

Driven by the disadvantages of nonalcoholic beers and the increasing demand of consumers for nonalcoholic beverages, breweries are eagerly searching for innovative nonalcoholic alternatives to expand their share in the beverage market.

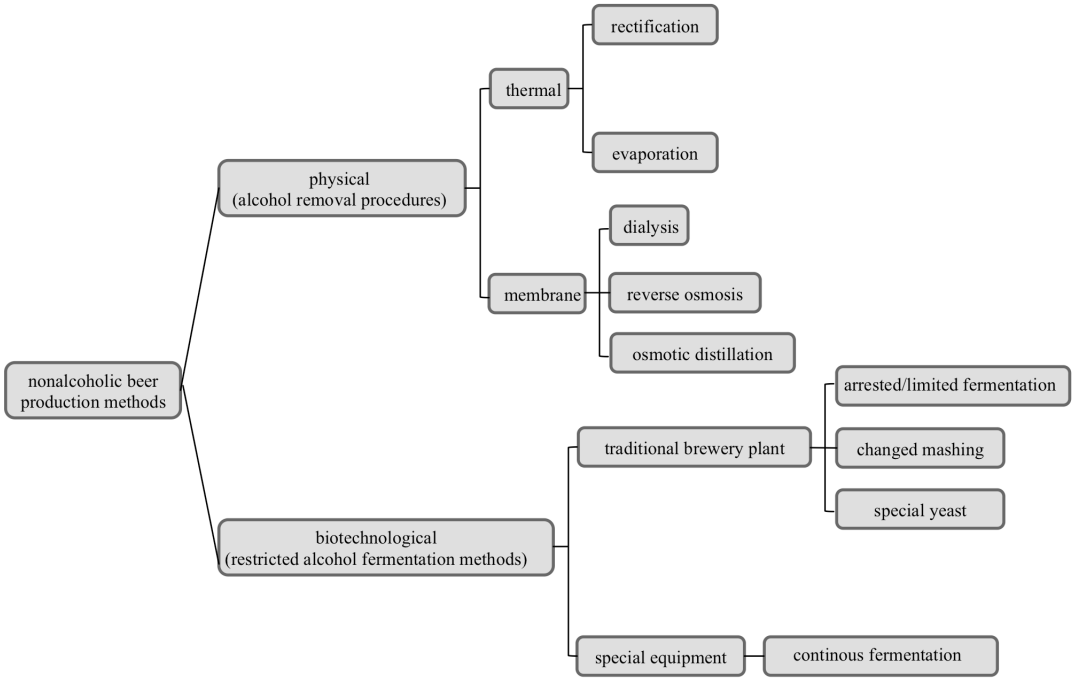


**Figure 1**. Scheme of the most common alcohol-free beer production methods (modified after Brányik et al., 2012; Sohrabvandi, Mousavi, Razavi, Mortazavian, & Rezaei, 2010)

Wort is the main raw material of beer and obtained as shown in Figure 2. It represents a complex mixture of fermentable carbohydrates, peptides, proteins, lipids, metal and non-metal ions, and polyphenols (Fantozzi et al., 1998). Furthermore, various potential flavor precursors such as phenolic acids, fatty acids, and amino acids are found in wort (Floridi, Montanari, Marconi, & Fantozzi, 2003; Kennedy, Taidi, Dolan, & Hodgson, 1997; Kobayashi, Kaneda, Kano, & Koshino, 1993). As it provides all essential nutrients and a proper environment for the growth of microorganisms, wort has already been used for the production of a wide range of low and nonalcoholic fermented beverages by yeasts, lactic acid bacteria (Brányik et al., 2012; Rathore et al., 2012; Zannini et al., 2013), and fungi from the genus *Monascus* (Lin, Chen, & Wang, 2005). To the best of our knowledge, until now there is no report about producing nonalcoholic beverages fermented by basidiomycetes.

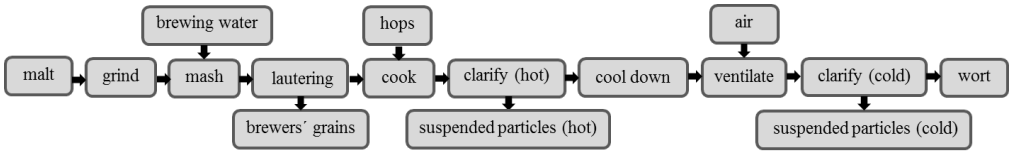


**Figure 2**. Manufacturing process of wort (modified after Eßlinger & Narziß, 2005)

## 1.3 Basidiomycetes

The phylum basidiomycota, with three subphyla (*Agaricomycotina*, *Pucciniomycotina*, and *Ustilaginomycotina*), comprises 31,515 described species (Kirk, Cannon, Minter, & Stalpers, 2008). Almost all edible fungi such as *Agaricus*, *Amanita*, *Boletus*, *Cantharellus*, *Coprinus*, *Lactarius*, *Macrolepiota*, *Pleurotus*, *Russula*, *Termitomyces,* and *Tricholoma* belong to basidiomycetes (Kirk et al., 2008). They have been collected or cultivated worldwide as an important source of food for thousands of years due to their nutritional value and delicious taste (Lorenzen & Anke, 1998). For example, the three most important edible fungi worldwide, the field mushroom (*Agaricus bisporus*), shiitake (*Lentinula edodes*, Figure 3A), and the oyster mushroom (*Pleurotus* spp.), exceed an annual production of 3.4 billion tons (Berger & Zorn, 2004). Recently, fruiting bodies of mushrooms or mycelia (*e.g.*, *A. bisporus*, *Grifola frondosa*, *Hypsizygus marmoreus*, *Pholiota nameko*, *Pleurotus eryngii*, *Shiitake*, *Tremella fuciformis*, or *Tricholoma matsutak*) have been added to various products as extracts or powder as functional ingredients to increase the quality of processed foods (*e.g.*, bread and cookies), meat products (*e.g.*, pork patties), meat analogs, soup mix, and seasonings and to impart beneficial health effects (Cha, Heo, Lee, Lo, & Moon, 2014; Kim et al., 2011; Kim, Jung, & Kwak, 2010; Lee, Kyung, & Chang, 2004; Singh, Ghosh, & Patil, 2003; Yoo, Kim, Choi, & Oh, 2007). An Enokitake (*Flammulina velutipes*) extract was applied in the production of apple juice as a natural food additive for the prevention of browning caused by polyphenol oxidase or tyrosinase (Jang, Sanada, Ushio, Tanaka, & Ohshima, 2002). Ergothioneine from Enokitake, *L. edodes*, *Pleurotus cornucopiae*, or *Pleurotus eryngii* was used as a color stabilizer in processed fish meat as well (Bao, Osako, & Ohshima, 2010). Based on their pharmacological active secondary metabolites, various fungi have been used for medical applications (Kirk et al., 2008). Shiitake was the first medicinal macrofungus that was employed in modern biotechnology (Bisen, Baghel, Sanodiya, Thakur, & Prasad, 2010). Lentinan, lectins, and eritadenine from shiitake are used medicinally against diseases, including cancer and allergies. *Agaricus subrufescens* with high concentrations of *ß*-glucans is used in cancer therapies (Zied et al., 2014). A variety of promising dietary supplements, such as protein-bound polysaccharides, have been produced by *Trametes versicolor* (Figure 3B)

and marketed in the form of capsules, tablets, or powder (Chang & Buswell, 1996; Sullivan, Smith, & Rowan, 2006).

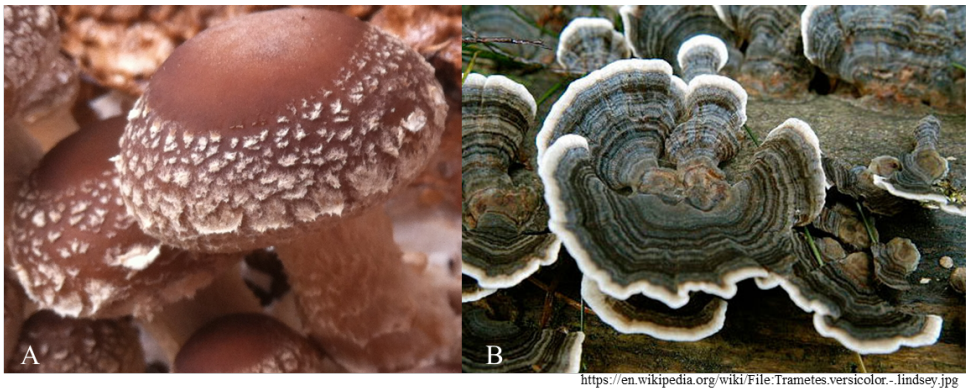


**Figure 3**. *Lentinula edodes* (A) and *Trametes versicolor* (B)

### *1.3.1 Biotechnological Production of Flavors by Basidiomycetes*

The generation of natural flavors with basidiomycetes by *de novo* synthesis or by bioconversion in submerged cultivation (Figure 4) has been investigated intensely. Janssens et al. (1992) reported that odorants generated *de novo* by basidiomycetes were identical to valuable flavors isolated from plants. Furthermore, compared to all other microorganisms (*e.g.*, yeasts, lactic acid bacteria, *Acetobacter* species, and lower fungi), the "spectrum of volatiles" (or "volatilome") of basidiomycetes is the closest to those of plants. Diverse volatiles found in basidiomycetes include highly potent aliphatic compounds (*e.g.*, 1-octen-3-ol, 3-methylbutanol, 2-methylpentanal, and methyl 3-methylpentanoate), aromatics (*e.g.*, anisaldehyde, benzaldehyde, methyl benzoate, methyl phenylacetate, and phenylacetaldehyde), terpenoids (*e.g.*, citronellol and linalool), lactones and sulfurous compounds. The formation of these flavors is closely related to a complex set of extra and intracellular enzymes of basidiomycetes (Bouws, Wattenberg, & Zorn, 2008; Fraatz et al., 2009; Zelena, Hardebusch, Hülsdau, Berger, & Zorn, 2009).


**Figure 4**. Submerged cultures of basidiomycetes (A) and pellets of *Lentinula edodes* (B)

The best-known fungal volatile is 1-octen-3-ol, which originates mainly from the enzymatic oxidative degradation of linoleic acid. Shiitake, which is widely consumed in China and Japan, has a very intense aroma due to 1,2,3,5,6-pentathiepane (lenthionine), as well as sulfur containing degradation products of S-alkyl cysteine sulfoxide (lentinic acid) (Belitz & Grosch, 1987). Beyond these well-known representatives, basidiomycetes can also produce precursors or intermediates via different reactions, such as oxidations, hydrolysis, reductions, dehydration, formation of new C-C bonds, and several degradation reactions, to form a wide spectrum of flavor compounds (Figure 5). (+)-Valencene can be transformed to the grapefruit flavor compound (+)-nootkatone by a novel dioxygenase (lipoxygenase) from *Pleurotus sapidus* (Fraatz et al., 2009). Extracelluar peroxidases (MsP 1 and MsP 2) of *Mycetinis scorodonius* (garlic mushroom) transform *ß*-carotene to the floral compound *ß*-ionone (Zelena et al., 2009). Ferulic acid as a precursor may be converted to vanillin by *Pycnoporus cinnabarinus* (Falconnier et al., 1994), and raspberry ketone is synthesized by *Nidula niveo-tomentosa* (bird's nest fungi) from L-phenylalanine as precursor (Zorn, Fischer-Zorn, & Berger, 2003). *Ischnoderma benzoium* generates benzaldehyde by transformation of L-phenylalanine (Krings, Abraham, & Berger, 1995). The use of appropriate culture conditions, the supplementation with cheap natural precursors, the immobilization of basidiomycetes, the continuous extraction of volatiles, and the design of more suitable bioreactors open the way to high-yield processes for the industrial flavor production (Lomascolo, Stentelaire, Asther, & Lesage-Meessen, 1999).

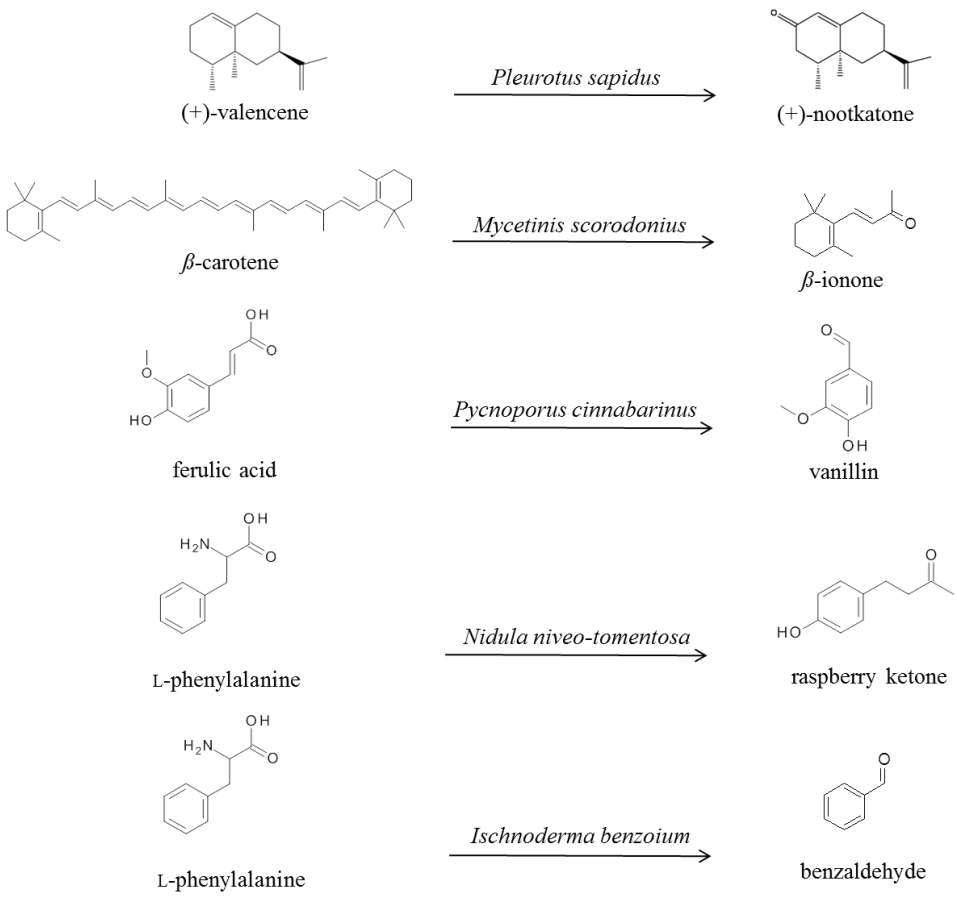


**Figure 5**. Biotransformation of precursors to aroma compounds by basidiomycetes

Nowadays, the consumer preference for natural food additives has led to an increasing demand for natural flavors. The market price for natural flavor compounds is much higher than for their synthetic counterparts (Janssens et al., 1992). For example, compared to the costs of synthetic vanillin (~15 US $ $kg^{-1}$), natural vanilla extract is much more expensive, with a current price of US ~ 4,000 $\$kg^{-1}$. According to the US and European legislation (Janssens et al., 1992; Lomascolo et al., 1999), the term 'natural flavors' includes those flavors obtained from living cells, food-grade microorganisms, and their enzymes. Therefore, basidiomycetes, which serve as a biological aroma factory, offer an alternative to natural plant sources. The use of the unique characteristics of basidiomycetes represents a potent and promising alternative to industrially produce foods with natural flavors.

### *1.3.2 Beverages Produced with the help of Basidiomycetes*

Until now, only few studies on the production of beverages using basidiomycetes have been published. Lin et al. (2010) developed a novel beverage by *Saccharomyces* using shiitake stipe extract as a nitrogen source. Hou and coworkers (2008) prepared a novel beverage by mixing polysaccharides extracted from BaChu mushroom with hawthorn juice and apple

juice. Yang and Zhang (2009) produced a soy milk beverage fermented by *Ganoderma lucidum*, a medicinal fungus, The contents of thiamin, riboflavin, and niacin were increased, whereas the flatulence factor (stachyose and raffinose) was significantly decreased. Okamura et al. (2001 and 2003) reported on beer-like, wine-like, and sake-like fermented drinks using *Pleurotus ostreatus*, *Tricholoma matsutake*, or *Agaricus subrufescens* instead of *Saccharomyces cerevisiae* as all of these fungi possess an alcohol dehydrogenase activity. However, the unique capabilities of basidiomycetes in producing a wide range of natural flavors in beverages have been ignored for submerged cultivations so far.

## 1.4 Flavor Analysis

Flavor is regarded as one of the most important product attributes for consumers (Yu & Bogue, 2013). An efficient aroma analysis provides reliable information on the relationship between the manufacturing processes/ingredients and the sensory attributes of the product (Sidel & Stone, 2006). In the food industry, flavor analysis is a key tool used to design new products and for evaluation of the food's quality.

A state of the art flavor analysis is typically performed according to Grosch (2001):

1. Isolation of the volatiles from the sample and separation by gas chromatography.
2. Detection of highly potent odorants by gas chromatography-olfactometry and aroma extract dilution analysis.
3. Enrichment and identification of potent odorants.
4. Quantification of potent odorants and calculation of their odor activity values.
5. Preparation of a synthetic blend of the potent odorants on the basis of the quantitative data and comparison of the aroma profile of the synthetic aroma model with that of the original sample.

Flavors (*e.g.*, acids, alcohols, aldehydes, esters, and ketones) from food represent compounds of different polarity and chemical character. Generally, they are present in very low concentrations and possess a comparatively high vapor pressure. Their molecular weight usually does not exceed 300 Da. The high variety of food matrices (solid, aqueous, and oily) poses many challenges for the extraction, separation, quantification, and reconstitution of the volatiles. In order to obtain a mixture of volatiles representing the authentic odor of the original food, various isolation techniques have been developed.

Liquid-liquid extraction (LLE) as a traditional extraction method has been widely used for the analysis of volatile flavors of food and beverages as it can be easily carried out manually and traps a wide spectrum of volatile components (Baltussen, 2000; Marais, 1986). Diethyl

ether is often used as the organic solvent due to its high extraction capacity and its low boiling point. However, diethyl ether has a weak capacity for the extraction of lower alkanols. In order to compensate for this disadvantage, less polar solvents such as isopentane, pentane, dichloromethane, or fluorocarbons are mixed with diethyl ether to improve the extraction. In some cases, the boiling point of the solvent is very close to the boiling points of some of the analytes in the samples. Therefore, a partial loss of low-boiling volatiles during the removal of the solvent and potential contaminations represent the main drawbacks of LLE (Schreier, 1984). The disposal of used solvents adds extra costs to the analytical procedure, creates health hazards for the laboratory personnel, and may cause environment problems (Vas & Vékey, 2004).

As an alternative to LLE, solid phase extraction techniques have been developed for flavor isolation. They include, amongst others, solid phase extraction (SPE), solid phase microextraction (SPME), stir bar sorptive extraction, and solid phase dynamic extraction. Especially microextraction techniques are considered attractive tools and have been widely used for food flavor analysis for two decades. Apart from being solvent-free, SPME is characterized by a fast sample preparation, a high sensitivity, and the possibility of automation (Jeleń, Majcher, & Dziadas, 2012). Based on the number of publications from 2006 - 2011 (Jeleń et al, 2012), SPME was the technique most often used for food flavor analysis , and a wide variety of compounds from different food matrices has been analyzed.

SPME has been developed by Pawliszyn in 1989. Headspace SPME (HS-SPME) is based on two partition equilibriums. Typically, HS-SPME was used to analyze liquid samples. The first equilibrium is established between the liquid and the gas phase. The second one between the fiber coating (adsorption / absorption) and the gas phase (desorption). The main steps of the solid phase microextraction process are incubation, extraction, adsorption / absorption, and desorption. Although SPME possesses a number of advantages, the amount of extraction medium (*e.g.*, polydimethylsiloxane coated on the fiber) is limited, which might negatively affect the extraction capacity (David & Sandra, 2007).

Each extraction procedure offers specific advantages, and on the other side has particular drawbacks under certain circumstances (Zhou, Riesen, & Gilpin, 1996). Therefore, no single extraction procedure is uniformly satisfactory. The chosen isolation method influences the actual composition of volatiles. In order to obtain all representative volatiles from a sample, at least two different isolation methods should be applied and compared. For example, solid phase microextraction and purge and trap (P&T) methods were compared to evaluate their effectiveness for the extraction of cheese aroma compounds (Mallia, Fernández-García, & Olivier Bosset, 2005). P&T was more suitable than SPME to extract 2,3-butanedione as a key

odor active compound of Gruyère Switzerland. Ebeler, Terrien, & Butzke (2000) found that aroma volatiles of brandy were trapped in a wider range with a combination of solid phase microextraction and liquid-liquid extraction.

In order to understand the contribution of a volatile compound to the odor quality, gas chromatography-olfactometry (GC-O) was used by Fuller et al. as early as in 1964. A GC specifically set-up for GC-O has 1 to 4 odor ports, and additionally typically either a flame ionization detector (FID) or a mass spectrometry (MS) detector (Figure 6) (Debonneville, Orsier, Flament, & Chaintreau, 2002). In general, the carrier gas flow is splitted after the analytical column and enters the olfactory port and a chemical detector in parallel. This provides a simultaneous identification of odor-active compounds (Delahunty, Eyres, & Dufour, 2006). GC-O has shown to be a valuable method for the selection of odor-active compounds from complex mixtures. Nowadays, it is commonly used to assign a relative importance to each of the volatile compounds. Three different techniques are distinguished: dilution of the extract to the threshold concentration (aroma extraction dilution analysis and charm analysis), determination of the detection frequency, and direct rating of the intensity (posterior intensity method and time-intensity method) (Delahunty et al., 2006). However, these approaches are not able to provide information on their behavior in a mixture, although they indicate the relevance of some compounds for the aroma of a food. As a final step in aroma analysis, reconstitution studies should be used to prove the correct selection of odor active compounds (Van Ruth, 2001).


**Figure 6**. Gas chromatography equipped with a tandem mass spectrometry detector and an olfactory detection port (GC-MS/MS-O)

The absolute configuration of volatiles is closely related to their odor. The sensorial character of optical isomers often differs from one another. For example, the aroma of (*R*)-linalool is described as floral, whereas the (*S*)-enantiomer imparts an intense green note

(Tamogami, Awano, Amaike, Takagi, & Kitahara, 2004). Additionally, determination of enantiomeric excess (ee) values of chiral odorants is a key step to characterize the authenticity and quality of products, especially in the area of flavors, fragrances, and essential oils (Mosandl, 1995). Therefore, chiral analysis has been recognized as one of the most important items in the field of flavor research. Multidimensional GC (MDGC) has proven to be an efficient tool for chiral analysis. It was first described by Schomburg et al. (1984) and commonly consists of a multi Dean's switch and two GC ovens, which are connected via a heated transfer line, a flame ionization detector in the first dimension, and an MS as second-dimension detector (Figure 7). In Dean's-switch MDGC systems, target components are multi cut from the primary (achiral) column and delivered to the (chiral) analytical column. An example is shown in Figure 8.

**Figure 7**. Multidimensional gas chromatography – mass spectrometry (MDGC-MS)

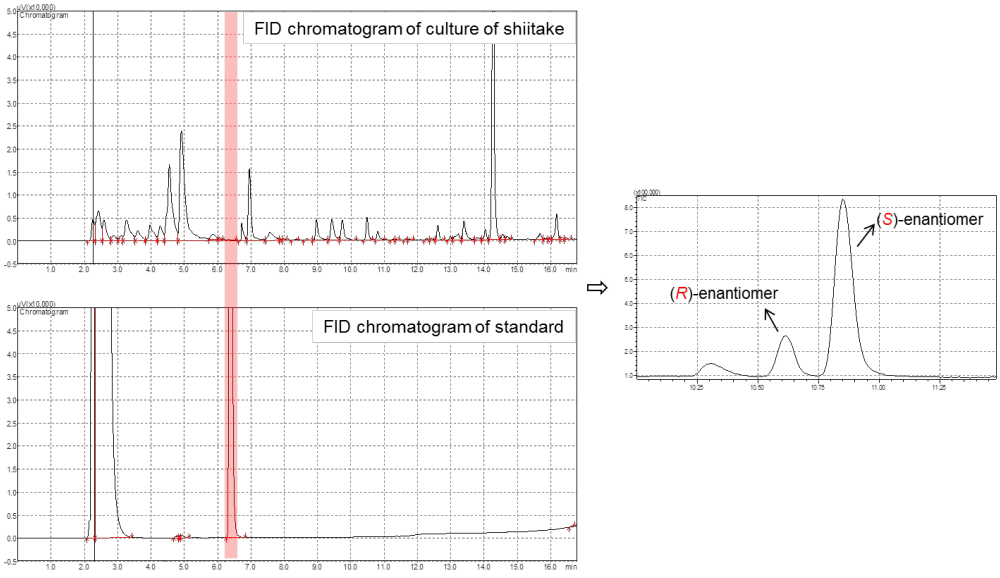


**Figure 8**. Chiral analysis of methyl 2-methylbutanoate

## 1.5 Aim of the thesis

Basidiomycetes represent the highest developed class of fungi. They are able to synthesize pharmacological relevant secondary metabolites, natural flavor compounds, and highly sought after enzymes. Because of their unique biochemical capabilities, basidiomycetes represent ideal candidates to develop novel fermentation concepts.

In order to compensate for the negative trend of beer consumption for breweries, a novel fermentation system utilizing basidiomycetes should be developed for the production of nonalcoholic fermented drinks with interesting flavor properties. Using wort (without hops) as the substrate, numerous basidiomycetes should be screened. The optimal species and fermentation conditions should be determined by sensory evaluation. Interesting fermented beverages should be subjected to a sophisticated flavor analysis. Additionally, the biosynthetic pathways of the key odorants of the beverages should be further investigated in order to control and improve the quality in the future.

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# Identification of Potent Odorants in a Novel Nonalcoholic Beverage Produced by Fermentation of Wort with Shiitake (*Lentinula edodes*)

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# Identification of Potent Odorants in a Novel Nonalcoholic Beverage Produced by Fermentation of Wort with Shiitake (*Lentinula edodes*)

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Supporting Information

**ABSTRACT:** Novel refreshments with pleasant flavors were developed by fermentation of wort with basidiomycetes. Among 31 screened fungi, shiitake (*Lentinula edodes*) produced the most pleasant flavor. It was perceived as fruity, slightly sour, and plum-like. Flavor compounds were isolated by liquid−liquid extraction (LLE) and by headspace solid phase microextraction (HS-SPME). The key odor-active compounds were analyzed by a gas chromatography system equipped with a tandem mass spectrometry detector and an olfactory detection port (GC-MS/MS-O) and aroma extract dilution analysis (AEDA). For HS-SPME, a revised method of increasing the GC inlet split ratio was used. Most of the key odor-active compounds (e.g., 2-acetylpyrrole, $\beta$-damascenone, (*E*)-2-nonenal, and 2-phenylethanol) were detected with both extraction techniques. However, distinct differences between these two methods were observed.

**KEYWORDS:** *shiitake, beverage, fermentation, olfactometry, headspace solid phase microextraction, liquid−liquid extraction, wort*

## INTRODUCTION

Basidiomycetes are able to synthesize pharmacologically relevant secondary metabolites, natural flavor compounds, and highly sought after enzymes.[1] Because of their unique biochemical potential, basidiomycetes are ideal tools for the food industry. In addition to their nutritional value, therapeutic applications of basidiomycetes, based on their antibiotic, antioxidative, and anticarcinogenic properties, have been discussed.

Because of their high nutritional value and good microbial stability, fermented beverages are becoming increasingly popular all over the world.[2] They are produced by various manufacturing techniques, raw materials, and microorganisms.[3] The most common microorganisms used in traditional fermented beverages such as beer, wine, kwass, kombucha, and Chinese liquor are yeasts, lactic acid bacteria, acetobacter species, and lower fungi.

Only a few studies on beverage production using basidiomycetes have been published. Hill produced a soluble tea product with champagne-like properties in combination with Manchurian mushroom.[4] Lin et al. developed a novel beverage by *Saccharomyces* using shiitake stipe extract as a nitrogen source.[5] Hou and co-workers prepared a novel beverage by mixing polysaccharides extracted from BaChu mushroom with hawthorn juice and apple juice.[6] Okamura et al. reported on beer-like fermented drinks using *Pleurotus ostreatus*, *Tricholoma matsutake*, or *Agaricus subrufescens* instead of *Saccharomyces cerevisiae* as all of these fungi possess an alcohol dehydrogenase activity.[7,8] However, the unique capabilities of basidiomycetes in producing a wide range of natural flavors in beverages have been ignored for submerged cultivations so far. Because of consumers' demand for natural food, basidiomycetes producing natural flavor compounds are ideal candidates for developing novel fermentation systems. With the recent worldwide declining consumption of beer,[9] breweries are eagerly searching for innovative nonalcoholic alternatives to compensate for this negative trend. In a previous study, complex and pleasant flavor mixtures were formed by fermentation of byproducts of the food industry with basidiomycetes.[10] This approach was adopted and transferred to the development of novel beverages by utilizing basidiomycetes for the fermentation of wort.

Traditional liquid−liquid extraction (LLE) and headspace solid phase microextraction (HS-SPME) are commonly used in flavor analysis. HS-SPME is a rapid and solvent-free method with low detection limits, which has been used for the analysis of a wide variety of compounds. Key odor-active compounds may be determined by aroma extract dilution analysis (AEDA).[11] In contrast to LLE, no dilutable organic flavor extract is obtained by using HS-SPME. Therefore, successively diluted original samples were analyzed by SPME in previous studies.[12−16] A different approach, namely, increasing gas chromatograph (GC) inlet split ratios, has only been reported by Kim et al.[17]

In this study, novel refreshments with pleasant flavors were developed by fermentation of wort with basidiomycetes. The cultures were extracted using two different extractions techniques (LLE and HS-SPME). The key odor-active compounds were determined and compared using a GC equipped with a tandem mass spectrometry detector and an olfactory detection port (GC-MS/MS-O) and AEDA. For

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Table 1. Odor Impression, Intensity, and Color of Wort after Fermentation with Basidiomycetes

| no. | species | odor impression | fermentation time (h) | intensity[a] | color |
|---|---|---|---|---|---|
| 1 | *Agaricus arvensis* | metallic | 6 | 4 | reddish-brown |
| 2 | *Agrocybe aegerita* | toasted, cereals, sweetish | 22 | 3 | dark yellow |
| 3 | *Clitocybe geotropa* | odorless | 24 | 1 | yellow |
| 4 | *Fistulina hepatica* | metallic | 4 | 2 | yellow |
| 5 | *Flammula alnicola* | moldy | 24 | 4 | yellow |
| 6 | *Flammulina velutipes* | sweetish | 18 | 2 | dark yellow |
| 7 | *Fomes fomentarius* | wort (weak), less sweetish | 40 | 2 | reddish-brown |
| 8 | *Ganoderma applanatum* | playdough, medicinal | 16 | 3 | yellow |
| 9 | *Hericium erinaceus* | odorless | 24 | 1 | reddish-brown |
| 10 | *Hypsizygus tesselatus* | stale beer | 23 | 4 | yellow |
| 11 | *Kuehneromyces mutabilis* | grassy | 16 | 4 | reddish-brown |
| 12 | *Lactarius deliciosus* | wort (weak) | 40 | 3 | yellow |
| 13 | *Laetiporus sulphureus* | grassy, smoky | 16 | 4 | reddish-brown |
| 14 | *Lentinula edodes* | sweetish, fruity, plum-like, sourish | 48 | 3 | reddish-brown |
| 15 | *Lentinus squarrosulus* | sweetish, banana peel | 22 | 3 | yellow |
| 16 | *Lepiota procera* | soapy | 16 | 2 | yellow |
| 17 | *Lepista nuda* | cereal, popcorn, sweetish | 8 | 3 | yellow |
| 18 | *Meripilus giganteus* | wort (weak) | 44 | 2 | reddish-brown |
| 19 | *Panellus serotinus* | fresh, honey, sweetish | 24 | 2 | reddish-brown |
| 20 | *Pholiota aurivella* | earthy, medicinal | 18 | 3 | dark yellow |
| 21 | *Pholiota nameko* | chlorine | 20 | 3 | reddish-brown |
| 22 | *Pleurotus cornucopiae* | fruity, floral | 14 | 3 | yellow |
| 23 | *Pleurotus eous* | wort (weak) | 16 | 2 | reddish-brown |
| 24 | *Pleurotus flabellatus* | wort (weak) | 21 | 2 | reddish-brown |
| 25 | *Pleurotus floridanus* | fruity, moldy (weak) | 22 | 2 | reddish-brown |
| 26 | *Pleurotus ostreatus* | chemical | 24 | 4 | reddish-brown |
| 27 | *Pleurotus pulmonarius* | odorless | 20 | 1 | reddish-brown |
| 28 | *Polyporus squamosus* | malty, fruity (weak), sweetish | 32 | 3 | reddish-brown |
| 29 | *Polyporus umbellatus* | raspberry, kiwi, sourish | 42 | 4 | pale yellow |
| 30 | *Termitomyces albuminosus* | creamy, sourish | 9 | 2 | yellow |
| 31 | *Trametes versicolor* | honey, fermented fruit, tea, fresh | 38 | 3 | reddish-brown |

[a]1, odorless; 2, weak; 3, moderately intense; 4, strong; 5, very intense; the odor of unfermented wort was perceived as malty, sweetish, and aromatic (intensity 4).

aroma dilution analysis (ADA) by HS-SPME, increasing GC inlet split ratios were used.

## MATERIALS AND METHODS

**Chemicals and Materials.** Thirty-one different basidiomycetes were obtained from the Centraalbureau voor Schimmelcultures (CBS, Utrecht, The Netherlands), the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the Department of Molecular Wood Biotechnology and Technical Mycology (WBTM, Göttingen, Germany).

Authentic flavor standards were purchased from Alfa Aesar (Karlsruhe, Germany), Fisher Scientific (Schwerte, Germany), Sigma-Aldrich (Steinheim, Germany), TCI Deutschland (Eschborn, Germany), Th. Geyer (Hamburg, Germany), and VWR (Darmstadt, Germany). The chemicals were of analytical grade. For GC helium 5.0 and nitrogen 5.0 were used.

**Wort Preparation.** Wort (Kölsch type, 13 °Plato) was provided by the University of Applied Sciences (Giessen, Germany). Pilsner (80%, w/w), Munich (13%, w/w), and wheat (7%, w/w) malts were used for the production of the wort with a KB50 pilot-scale brewing device (Alfred Gruber GmbH, Eugendorf, Austria). After cooking, the wort was filtered (0.2 μm), filled into aluminum bottles, and pasteurized at 80 °C for 30 min. The wort was stored at −20 °C prior to usage.

**Precultures and Fermentation of Wort.** Culture flasks containing standard nutrition solution (100 mL/250 mL, medium volume/flask volume) were inoculated with fungal mycelium as published previously.[18] This preculture was incubated on a rotary shaker (24 °C, 150 rpm, 25 mm shaking diameter) in the dark until sufficient growth was obtained. Mycelia of 10 mL preculture broth were precipitated by means of centrifugation (4000 rpm, 2150*g*, 10 min, 20 °C) and washed three times with sterile water. The fungal pellets were resuspended in 10 mL of heat sterilized wort. This suspension was added into an Erlenmeyer flask (250 mL) containing 100 mL of wort. The fermentation was carried out at 24 °C for up to 48 h under aerobic conditions in a rotary shaker (150 rpm). During the fermentation, samples were taken every 2−4 h and were subsequently analyzed by sensory evaluation.

**Sensory Evaluation of Cultures.** The mycelium was removed by centrifugation (4000 rpm, 2150*g*, 5 min, 4 °C), and the supernatant was transferred to odorless snap-cap vials (20 mL). After storage for 15 min at 4 °C, the odor of the supernatant was assessed by at least three experienced sensory assessors. The odor intensity was rated on a scale of 1−5 (1, low intensity; 5, intense, strong odor). Samples with a distinctive and potent odor were additionally evaluated by tasting. Promising fermentations were repeated at least three times. In the final analysis the flavor was described by 10 panelists.

**Headspace Solid Phase Microextraction.** For HS-SPME, PDMS fibers (polydimethylsiloxane, film thickness = 100 μm, fiber length = 1 cm) (Supelco, Steinheim, Germany), PDMS/DVB fibers (polydimethylsiloxane/divinylbenzene, 65 μm, 1 cm), CAR/PDMS fibers (carboxen/polydimethylsiloxane, 75 μm, 1 cm), and DVB/CAR/PDMS fibers (divinylbenzene/carboxen/polydimethylsiloxane, 50/30 μm, 1 cm) in combination with an MPS 2XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany) were used. Ten milliliters of the culture broth was transferred into a headspace vial (20 mL). The samples were agitated for 20 min (250 rpm) at 50, 55, or 60 °C, followed by headspace extraction, at the same temperature as

during the incubation, for 40, 45, or 50 min. Afterward, the analytes were directly desorbed in the split/splitless inlet at 250 °C using a SPME liner with 0.75 mm i.d. (Supelco) of the GC-MS/MS-O system for 1 min. After desorption, the fiber was heated at the respective recommended conditioning temperature in the SPME fiber conditioning station for 20 min.

**Liquid−Liquid Extraction.** The mycelium was removed from the fermentation broth by centrifugation (4000 rpm, 2150*g*, 5 min, 4 °C), and the culture supernatant was extracted three times with equal amounts of pentane/diethyl ether (1:1.12, v/v). The organic phases were combined, dried over anhydrous $Na_2SO_4$, and concentrated by means of a Vigreux column (water bath temperature = 45 °C) to a final volume of approximately 1 mL. Afterward, 100 μL of thymol (300 mg/L in pentane/diethyl ether) was added as internal standard (IS). The sample was used for AEDA in combination with GC-MS/MS-O analysis.

**Aroma (Extract) Dilution Analysis.** For LLE the flavor dilution (FD) factors were determined by AEDA. The organic extracts of the cultures were diluted with pentane/diethyl ether (1:1.12, v/v) in a series of 1:1 dilutions, and each dilution was sniffed twice until no odorants were perceived at the ODP 3 olfactory detection port (GERSTEL) of the GC-MS/MS-O system.

For HS-SPME, aroma dilution analysis was performed by increasing GC inlet split ratios.[17] Prior to sample analysis, the method was validated using six authentic flavor standards. Ten milliliters of the standard solution [43.9 mg of 2-acetylpyrrole (**37/2**), 24.6 mg of β-damascenone (**32/2**), 46.1 mg of methyl 2-methylbutanoate, 39.1 mg of 2-phenylacetaldehyde, 44.5 mg of 2-phenylethanol, and 46.5 mg of 3-phenylpropanol dissolved in 50 mL of ethanol and subsequently diluted 1:1000 with distilled water] was transferred into a HS vial (20 mL) and sealed with a Teflon screw cap. The standard solution was incubated at 60 °C for 20 min and extracted at the same temperature by means of HS-SPME with a CAR/PDMS fiber for 45 min. The volatiles were diluted stepwise by controlling the split ratio as follows: splitless (splitless time = 2 min), 2:1, 4:1, 8:1, 16:1, 32:1, 64:1, 128:1, and 256:1. The same procedure was applied for the samples.

**Gas Chromatography.** Gas chromatography was carried out using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 7000B triple-quadrupole mass spectrometry (MS/MS) detector (Agilent Technologies). The column used was a polar 30 m × 0.25 mm i.d., 0.25 μm Agilent J&W VF-WAXms unless stated otherwise. Helium was used at a constant flow rate of 1.56 mL/min as the carrier gas. At the end of the capillary column, the carrier gas was split 1:1 into the Agilent 7000B detector and into the ODP 3. The oven temperature was programmed from 40 to 240 °C at 5 °C/min, with initial and final hold times of 3 and 12 min, respectively. Other conditions were as follows: inlet temperature, 250 °C; septum purge flow rate, 3 mL/min; scan mode, total ion current (TIC) in Q1; scan range, $m/z$ 33−330; electron ionization energy, 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; MS/MS transfer line temperature, 250 °C; He quench gas, 2.25 mL/min; $N_2$ collision gas, 1.5 mL/min; ODP 3 transfer line temperature, 250 °C; ODP 3 mixing chamber temperature, 150 °C; ODP 3 makeup gas, $N_2$.

GC-MS analyses were performed using an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with a model 5975C mass spectrometry detector (Agilent Technologies), under the following conditions: carrier gas, helium; constant flow rate, 1.2 mL/min; inlet temperature, 250 °C; split ratio, 10:1; septum purge flow rate, 3 mL/min; 30 m × 0.25 mm i.d., 0.25 μm Agilent J&W DB-5MS column; temperature program, 40 °C (3 min), 5 °C/min to 300 °C (12 min); scan mode, TIC; scan range, $m/z$ 33−330; electron ionization energy, 70 eV; source temperature, 230 °C; quadrupole temperature, 150 °C; transfer line temperature, 250 °C.

**Compound Identification.** For the identification of the flavor compounds, the odor impressions, the retention indices (RI), and the mass spectra of each compound were compared to authentic standards on two columns of different polarities (VF-WAXms and DB-5MS columns), published data, and the NIST 2011 MS library.

## RESULTS AND DISCUSSION

**Sensory Evaluation.** Thirty-one different basidiomycetes were screened for the fermentation of wort. With most of the fungi significant changes of the aroma were observed. During the fermentation process the odor varied in quality and intensity. The most characteristic and intense odor impressions of the fermentations are listed in Table 1.

The odor of the cultures may be classified into three groups: pleasant, unpleasant, and odorless. The main pleasant odors included fruity (e.g., *Lentinula edodes*, *Polyporus umbellatus*, and *Trametes versicolor*), toasted (e.g., *Agrocybe aegerita* and *Pleurotus cornucopiae*), and honey-like (e.g., *Trametes versicolor* and *Panellus serotinus*) notes. Unpleasant odors, such as chlorine, medicinal, metallic, moldy, and smoky, were produced by a few basidiomycetes (e.g., *Agaricus arvensis*, *Flammula alnicola*, *Ganoderma applanatum*, *Laetiporus sulphureus*, and *Pholiota nameko*). Some basidiomycetes (e.g., *Meripilus giganteus*, *Pleurotus eous*, and *Pleurotus flabellatus*) diminished the typical odor of wort, some of them to completely odorless (e.g., *Clitocybe geotropa* and *Hericium erinaceus*). The cultures produced this variety of different odor impressions within the short fermentation time of up to 48 h. This phenomenon is remarkably different from previous investigations. Gallois et al. studied the capability of 29 different ligninolytic basidiomycetes to produce odors in 6 different media.[19] Flavors were perceived after an incubation period of 7−10 days. Kawabe and Morita reported that *Polyporus tuberaster* K 2606 produced a fruity and floral odor on day 13 in peptone−glucose−yeast medium.[20] The fast formation of flavor compounds in this study might be related to the available nutrients and flavor precursors in the wort. Wort represents a complex mixture of fermentable carbohydrates, peptides, proteins, lipids, metal and nonmetal ions, and polyphenols.[21] Therefore, it provides all essential nutrients and a proper environment for the growth of basidiomycetes. Furthermore, various potential flavor precursors such as phenolic acids, fatty acids, and amino acids were found in wort.[22−24] Hence, transformation time and transformation products strongly depend on the composition of medium used.

The generation of flavor compounds was often accompanied by a change of the color of the fermentation broth. Compared to the substrate wort, the color of most culture supernatants turned darker and reddish-brown (Table 1). The simultaneous production of aroma compounds and pigments by macrofungi was already reported by Badcock.[25] However, in the current study, the synthesis of odor and pigments was not correlated in all cases. *P. umbellatus* generated a strong and interesting fruity odor reminiscent of cherry and kiwi, but the color of the culture supernatant became lighter and more transparent.

After sensory evaluation by 10 panelists, wort fermented by shiitake was classified as the most interesting one. Shiitake produced a fresh, fruity, plum-like, sweet, and slightly sour flavor. During the fermentation the broth turned reddish-brown in color.

Shiitake represents an ideal candidate to develop a novel fermentation system. Due to its flavor, nutritional value, and presumed medicinal properties, shiitake has attracted high interest in recent years.[26] To the best of our knowledge, no studies on the volatile compounds of submerged-grown shiitake cultures in wort have been published so far. Shiitake is the second most popular edible mushroom in the global market and was the first medicinal macrofungus to enter the realm of

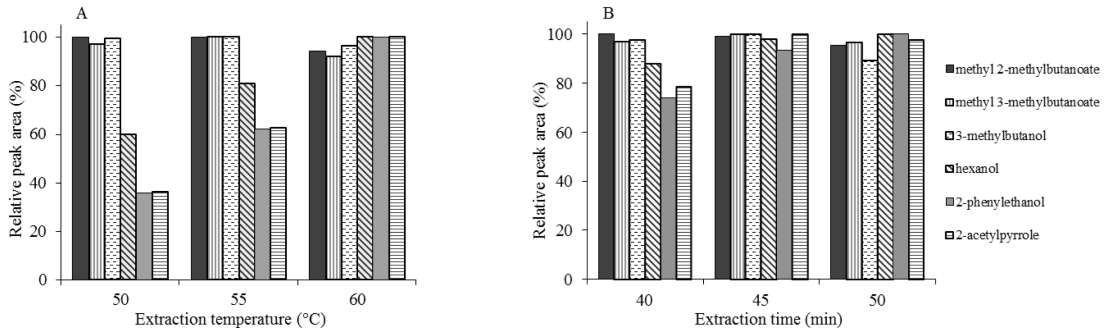


**Figure 1.** Influence of the extraction temperature (A) (CAR/PDMS fiber, 45 min extraction time) and extraction time (B) (CAR/PDMS fiber, 60 °C extraction temperature) on the extraction efficiency of selected odor compounds, expressed in percent of the maximum area.

modern biotechnology.[27] Therefore, high consumer acceptance and high market potential for the novel fermented beverage are expected.

**Optimization of HS-SPME Parameters.** SPME was invented by Pawliszyn in 1989.[28] This solvent-free extraction technique simplifies the sample preparation procedure considerably and has been widely used for food analysis. For the optimization of the HS-SPME method, six previously identified flavor compounds from the fermented wort were chosen. All of them matched the following criteria: baseline separation; distributed over the chromatographic run; and different chemical families.[15] In our study, common SPME parameters, including extraction temperature, extraction time, and fiber type, were found to greatly affect the number and intensity of perceived aroma compounds.

*Extraction Temperature.* The influence of different extraction temperatures (50, 55, and 60 °C) on the extraction efficiency was evaluated with a CAR/PDMS fiber. The peak areas of methyl 2-methylbutanoate, methyl 3-methylbutanoate, and 3-methylbutanol changed only slightly with elevated temperatures. The extraction efficiencies for 2-phenylethanol, 2-acetylpyrrole (**37**/**2**), and hexanol were enhanced significantly at increased temperatures (Figure 1A). With increased extraction temperature, the extraction efficiency for compounds with higher boiling points improved. This was in good agreement with a previous study.[29] Maximum relative peak areas were observed at 60 °C. Higher temperatures were not considered to avoid the formation of artifacts (e.g., Maillard reaction products) in the complex matrix wort. Therefore, 60 °C was selected as the optimum extraction temperature for further studies.

*Extraction Time.* For most compounds the extraction efficiency increased with extraction time, until equilibrium was reached after 45 min (Figure 1B). A prolonged extraction time led to a slight decrease of the areas of methyl 2-methylbutanoate, methyl 3-methylbutanoate, and 3-methylbutanol. Therefore, further experiments were carried out with an extraction time of 45 min.

*Fiber Type.* The extraction efficiencies of different SPME fiber types (CAR/PDMS, DVB/CAR/PDMS, PDMS/DVB, and PDMS) were evaluated by analyzing fermented wort samples by means of HS-SPME-GC-MS/MS-O. The coating material of the SPME fibers strongly influenced the extraction efficiency of odor-active compounds.

The number of odor-active compounds perceived at the ODP after extraction with the CAR/PDMS, DVB/CAR/PDMS, PDMS/DVB, and PDMS fibers were 30, 23, 19, and 8, respectively. The CAR/PDMS fiber was the most sensitive one, although two odors (octanal and an unidentified compound) could not be perceived. With the DVB/CAR/PDMS fiber a slightly reduced number of flavor compounds were detected during the first 15 min of the GC run (RI $\leq$ 1329). Seven of 32 flavor compounds [2-phenylacetaldehyde, 2-acetylthiazole, 2-phenylethanol acetate, $\beta$-damascenone (**32**/**2**), 2-phenylethanol, $\gamma$-nonalactone, and *o*-aminoacetophenone] were identified with all four fibers tested. DVB/CAR/PDMS fibers are widely used for flavor analysis, as the combination of three different materials can catch a wider range of polarities and volatilities.[30] However, for the analysis of fermented wort, a CAR/PDMS fiber was most efficient. This also indicates that the selection of the SPME fiber used for analysis depends strongly on the characteristics of the flavor compounds of the respective matrix. Due to its overall better performance, the CAR/PDMS fiber was chosen for further analysis of odor-active compounds.

**Aroma (Extract) Dilution Analysis by LLE and HS-SPME.** To evaluate the aroma profile of the fermented beverage, changes of the main odor-active compounds during the fermentation of wort were analyzed by two different extraction methods (LLE and HS-SPME) followed by an aroma (extract) dilution analysis. Different from LLE, no liquid dilutable aroma extract is obtained by SPME. For dilution analysis the original sample may be diluted. However, this might influence phase equilibrium due to a modified matrix composition. Therefore, ADA was performed after microextraction by applying different split inlet ratios. In preparation, the linear relationship between the peak area of the respective analyte and the split ratio of GC-MS/MS-O system was investigated by using a standard solution comprising six different flavor compounds. The regression plots showed a linear relationship with $R^2$ >0.989. Slopes of the respective equations ranged from −1.0 to −1.3. Kim et al. reported insufficient linearity for low and high split ratios due to an unstable carrier gas flow.[17] In our study, linear responses for all tested substances were obtained for a wide range of split ratios from splitless up to 256. Thus, increasing GC inlet split ratios was a fast and reliable tool for determining key odor-active compounds. Although the maximum split ratio of the GC was limited by the pneumatic controller, changing GC inlet split ratios combined with successive dilution of the original sample makes this method suitable even for very concentrated samples. The odor-active compounds identified by means of GC-MS/MS-O after LLE and HS-SPME of unfermented wort and wort fermented by shiitake are summarized in Table 2. A selection of

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**Table 2. Odor-Active Compounds Identified by Means of GC-MS/MS-O after LLE and HS-SPME of Unfermented Wort and Wort Fermented by Shiitake**

| | RI[a] | | | | | FD factor[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | wort | | fermented wort | |
| no. | VF-WAXms | DB-5MS | compound | odor impression | identification | LLE | HS-SPME | LLE | HS-SPME |
| 1 | 931 | <800 | 3-methylbutanal | green | MS, RI, odor | –[i] | 1 | – | 1 |
| 2 | 996 | <800 | 2,3-butanedione | buttery | MS, RI, odor | – | 32 | – | 4 |
| 3 | 1018 | <800 | methyl 2-methylbutanoate[d] | fruity | MS, RI, odor | – | – | – | 64 |
| 4 | 1026 | <800 | methyl 3-methylbutanoate | fruity | MS, RI | – | – | – | – |
| 5 | 1074 | <800 | 2,3-pentanedione | caramel | MS, RI, odor | – | 2 | – | – |
| 6 | 1091 | <800 | hexanal | green | MS, RI, odor | – | 1 | – | – |
| 7 | 1102 | <800 | (*E*)-2-methyl-2-butenal[d] | toasted, sweetish | MS, RI, odor | – | – | – | 2 |
| 8 | 1126 | 877 | 3-methylbutyl acetate | fruity, sweetish | MS, RI, odor | – | – | – | 2 |
| 9 | 1218 | <800 | 3-methylbutanol | fresh, sourish | MS, RI, odor | – | – | 1 | 1 |
| 10 | 1271 | <800 | thiazole (**10**) | rubber | MS, RI, odor | – | 2 | – | – |
| 11 | 1291 | nd[h] | unknown | caramel | | 2 | – | 1 | – |
| 12 | 1294 | nd | 1-octen-3-one[g] | mushroom | RI, odor | – | 4 | – | 4 |
| 13 | 1348 | 910 | 2,5-dimethylpyrazine | baked, nutty | MS, RI, odor | 2 | 64 | 2 | 32 |
| 14 | 1367 | 873 | hexanol | green | MS, RI | – | – | – | – |
| 15 | 1454 | 1051 | (*E*)-2-octenal | toasted | MS, RI, odor | 2 | – | 2 | – |
| 16 | 1464 | 907 | methional | boiled potato | MS, RI, odor | – | 256 | – | 64 |
| 17 | 1468 | <800 | acetic acid | sourish, vinegar | MS, RI, odor | 2 | 1 | 4 | 1 |
| 18 | 1474 | 883 | furfural | woody | MS, RI | – | – | – | – |
| 19 | 1493 | 1029 | 2-ethylhexanol | green | MS, RI, odor | – | – | – | 1 |
| 20 | 1522 | 1163 | (*E*)-2-nonenal | green | MS, RI, odor | 128 | 4 | 16 | 4 |
| 21 | 1528 | 964 | benzaldehyde | almond, cereal | MS, RI, odor | – | 4 | – | 2 |
| 22 | 1551 | 1099 | linalool (**22**) | floral | MS, RI, odor | – | 4 | – | – |
| 23 | 1563 | 1074 | 1-octanol | herbal, green | MS, RI | – | – | – | – |
| 24 | 1583 | <800 | 2-methylpropanoic acid | cheesy, sourish | MS, RI, odor | – | – | 1 | – |
| 25 | 1593 | nd | 5-methylfurfural | caramel | MS, RI, odor | – | 1 | – | – |
| 26 | 1655 | 1046 | 2-phenylacetaldehyde | floral | MS, RI, odor | 16 | 4 | 4 | – |
| 27 | 1658 | 1020 | 2-acetylthiazole | toasted, sweetish | MS, RI, odor | 16 | 128 | 1 | 8 |
| 28[c] | 1684 | 867 | 2-methylbutanoic acid | cheesy, stinky, | MS, RI, odor | 256 | 4 | 1 | 1 |
| | | 860 | 3-methylbutanoic acid[e] | sourish | | | | | |
| 29 | 1717 | 1154 | (*Z*)-3-nonen-1-ol[d] | green | MS, RI, odor | – | – | – | 16 |
| 30 | 1727 | 1184 | naphthalene | medicinal | MS, RI, odor | – | 16 | – | – |
| 31 | 1750 | nd | unknown | burnt | | 64 | 32 | 32 | 16 |
| 32[c] | 1827 | 1256 | 2-phenylethanol acetate | fruity, like pear | MS, RI, odor | 16 | 64 | 4 | 64 |
| | | 1380 | *β*-damascenone (**32**/**2**) | | | | | | |
| 33 | 1872 | 993 | hexanoic acid | floral | MS, RI, odor | 8 | 4 | – | – |
| 34 | 1881 | 1086 | 2-methoxyphenol | floral, sweetish | MS, RI, odor | 64 | 16 | 32 | 1 |
| 35 | 1892 | 1036 | benzyl alcohol | leaf, floral | MS, RI, odor | – | – | – | 1 |
| 36 | 1926 | 1113 | 2-phenylethanol | rose | MS, RI, odor | 16 | 4 | 256 | 64 |
| 37[c] | 1983 | 1109 | maltol[f] (**37**/**1**) | burnt, sweetish | MS, RI, odor | 256 | 32 | 32 | 4 |
| | | 1064 | 2-acetylpyrrole (**37**/**2**) | | | | | | |
| 38 | 2040 | 1362 | *γ*-nonalactone | caramel | MS, RI, odor | 256 | 16 | 1 | 2 |
| 39 | 2046 | 1232 | 3-phenylpropanol | floral | MS, RI | – | – | – | – |
| 40 | 2083 | 1383 | (*E*)-methyl cinnamate[d] (**40**) | fruity | MS, RI, odor | – | – | – | 16 |
| 41 | 2149 | 1817 | hexadecanal | cardboard | MS, RI, odor | – | 8 | – | 4 |
| 42 | 2179 | nd | unknown | toasted | | 4 | – | 1 | – |
| 43 | 2202 | 1314 | 2-methoxy-4-vinylphenol (**43**) | sweetish | MS, RI, odor | 2 | 2 | – | – |
| 44 | 2230 | 1920 | methyl hexadecanoate | toasty, sweetish | MS, RI, odor | – | 256 | – | 64 |
| 45 | 2241 | 1301 | *o*-aminoacetophenone | grape | MS, RI, odor | – | 16 | – | 16 |
| 46 | 2395 | nd | *γ*-dodecalactone | sweetish | MS, RI, odor | – | 16 | – | 2 |
| 47 | 2450 | 1173 | benzoic acid | balsamic | MS, RI, odor | 1 | – | – | – |
| 48 | 2529 | 1562 | dodecanoic acid | fatty | MS, RI, odor | – | 1 | – | 1 |
| 49 | 2575 | 1252 | 2-phenylacetic acid | toasted, pungent, sweetish | MS, RI, odor | 128 | – | 64 | – |
| 50 | 2828 | 1430 | cinnamic acid | honey | MS, RI | – | – | – | – |
| 51 | 2864 | 1962 | palmitic acid | oily | MS, RI | – | – | – | – |

[a]Mean of RI values from LLE and HS-SPME. [b]Flavor dilution (FD) factor of the corresponding compound. [c]Odor corresponded to coeluting compounds. [d]Compound was detected only in the fermented beverage. [e]Compound was detected only in the unfermented wort. [f]Compound was



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**Table 2. continued**

detected only in LLE. [g]MS spectrum was ambiguous; the compound was tentatively identified on the basis of RI, odor, and literature data,[33] [h]nd, no distinct MS spectrum on DB-5MS column. [i]–, odor was not perceived.

identified structures of aroma compounds with FD factors >1 are shown in Figure 2.

*Main Odor-Active Compounds Identified after LLE.* In total, 18 odor impressions were perceived in the liquid–liquid extracts of wort fermented with shiitake at the ODP during GC-MS/MS-O analysis (Table 2). The corresponding odor-active compounds were identified by comparison of the Kovats indices and mass spectra with those of the database and literature data and were confirmed by authentic standards on both polar and nonpolar GC columns. On the basis of the determined FD factors, 2-phenylethanol was the most important flavor compound in the fermented wort (FD 256). The compound with its typical rose odor was biosynthesized by shiitake during fermentation, possibly by biotransformation of L-phenylalanine. It has been shown that this metabolic pathway exists in basidiomycetes such as *Ischnoderma benzoinum*.[31] Other key odor compounds were 2-phenylacetic acid (FD 64), 2-methoxyphenol (FD 32), maltol (**37**/**1**) coeluting with 2-acetylpyrrole (**37**/**2**) (FD 32), a yet unknown compound with a burnt odor (FD 32), and (*E*)-2-nonenal (FD 16). The unknown compound was present in such low concentrations that a distinct mass spectrum could not be obtained. Further investigations are needed for a reliable identification.

All major odor-active compounds identified in the organic extract of the fermented wort after LLE (FD $\geq$ 16) were also present in the unfermented wort, but in different concentrations. The aroma compounds of wort with the highest FD factors were 2-methylbutanoic acid coeluting with 3-methylbutanoic acid (FD 256), maltol (**37**/**1**) coeluting with 2-acetylpyrrole (**37**/**2**) (FD 256), and $\gamma$-nonalactone (FD 256). $\gamma$-Nonalactone, maltol (**37**/**1**), and 2-acetylpyrrole (**37**/**2**) exhibited odor impressions typical for wort, such as malty, caramel, and toasted. After fermentation these predominant odors became less important ($1 \leq FD \leq 32$). 2-Methylbutanoic acid and $\gamma$-nonalactone could be perceived only in the undiluted fermented wort extract (FD 1).

Besides 2-phenylethanol, shiitake produced 3-phenylpropanol (39) (Figure 3), which was detected in the fermented beverage by mass spectrometry but not perceived at the ODP. Shiitake also produced 3-methylbutanol and 2-methylpropanoic acid. On the other hand, the peak areas of 3-methylbutanoic acid, hexanoic acid, cinnamic acid (50) (Figure 3), and palmitic acid (51) (Figure 3) decreased significantly during the fermentation. None of these compounds had a high impact on the overall flavor of fermented wort. Certain compounds (e.g., 3-methylbutanoic acid and cinnamic acid) were even below their limit of detection after fermentation. This implies that a wide variety of extracellular fungal enzymes, including laccases, lipoxygenases, and hydroperoxide lyases, might have catalyzed the degradation of these carboxylic acids, which can serve as an immediate carbon source for fungal growth.[18] However, these carboxylic acids might also have been precursors of some of the odor compounds, as shiitake produced aroma compounds such as 3-methylbutanol, 2-methylpropanoic acid, and 3-phenylpropanol. Krings et al. observed that 3-phenylpropanol was produced from cinnamic acid and L-phenylalanine.[31] The observed increase of the concentration of 3-methylbutanol might be associated with the simultaneous decrease of 3-methylbutanoic acid concentration.

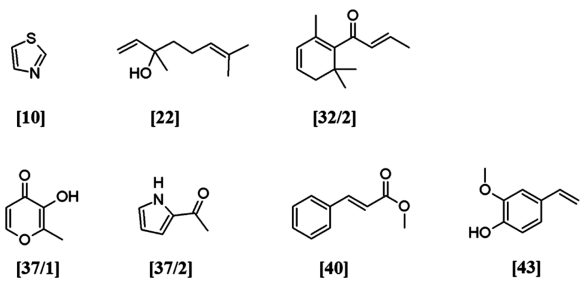


**Figure 2.** Selection of identified aroma compounds.

Although most of the odor impressions were caused by a single compound, two impressions (28 and 37) (Figure 3) originated from a mixture of compounds in both extracts. 2-Methylbutanoic acid and 3-methylbutanoic acid coeluted and represented a potent odor of wort (FD 256). They imparted a cheesy and sourish odor impression (28). During fermentation the FD factor was reduced to 1. Impression 37 (sweetish, burnt) was identified as a mixture of maltol (**37**/**1**) and 2-acetylpyrrole (**37**/**2**), which both impart a typical sweetish and burnt odor (Table 2).

The dominant odor impression of wort fermented with shiitake was fresh and fruity and thereby strikingly different from that of nonfermented wort. However, a single representative fruity flavor compound was not perceived by GC-MS/MS-O experiments after LLE. One possibility was that the fruity aroma did not result from the biosynthesis of new compounds, but from quantitative changes of several compounds. All of the most potent odor-active compounds of the culture were found in the wort extract as well, only the intensity of the aroma compounds changed remarkably. The intensity of typical aroma compounds of wort such as $\gamma$-nonalactone, maltol (**37**/**1**), 2-acetylpyrrole (**37**/**2**), and 2-acetylthiazole was very low in the fermented beverage.[32−34] The other possibility was that the compounds responsible for the fruity aroma were lost during either the extraction process or the GC-MS/MS-O analysis.[35] Hence, another extraction method was chosen for analyzing the fermentation system.

*Main Odor-Active Compounds Identified by HS-SPME.* By means of HS-SPME, 30 odor impressions were perceived for the unfermented wort and 29 from the fermented wort (Table 2). Methional (FD 256), methyl hexadecanoate (FD 256), 2-acetylthiazole (FD 128), $\beta$-damascenone (**32**/**2**) (FD 64), and 2,5-dimethylpyrazine (FD 64) were determined as the most potent odor-active compounds in wort. After fermentation by shiitake, the most important odorants (FD 64) were methional, methyl 2-methylbutanoate, methyl hexadecanoate, 2-phenylethanol acetate coeluting with $\beta$-damascenone (**32**/**2**), and 2-phenylethanol. Among them, methyl 2-methylbutanoate was detected only in the fermented beverage. The FD factor of 2-phenylethanol increased from 4 to 64 during the fermentation. It is noteworthy that especially the FD factors of the well-known typical wort flavors methional (FD 256), methyl hexadecanoate (FD 256), and 2-acetylthiazole (FD 128) decreased significantly during the fermentation (FD 64, 64,


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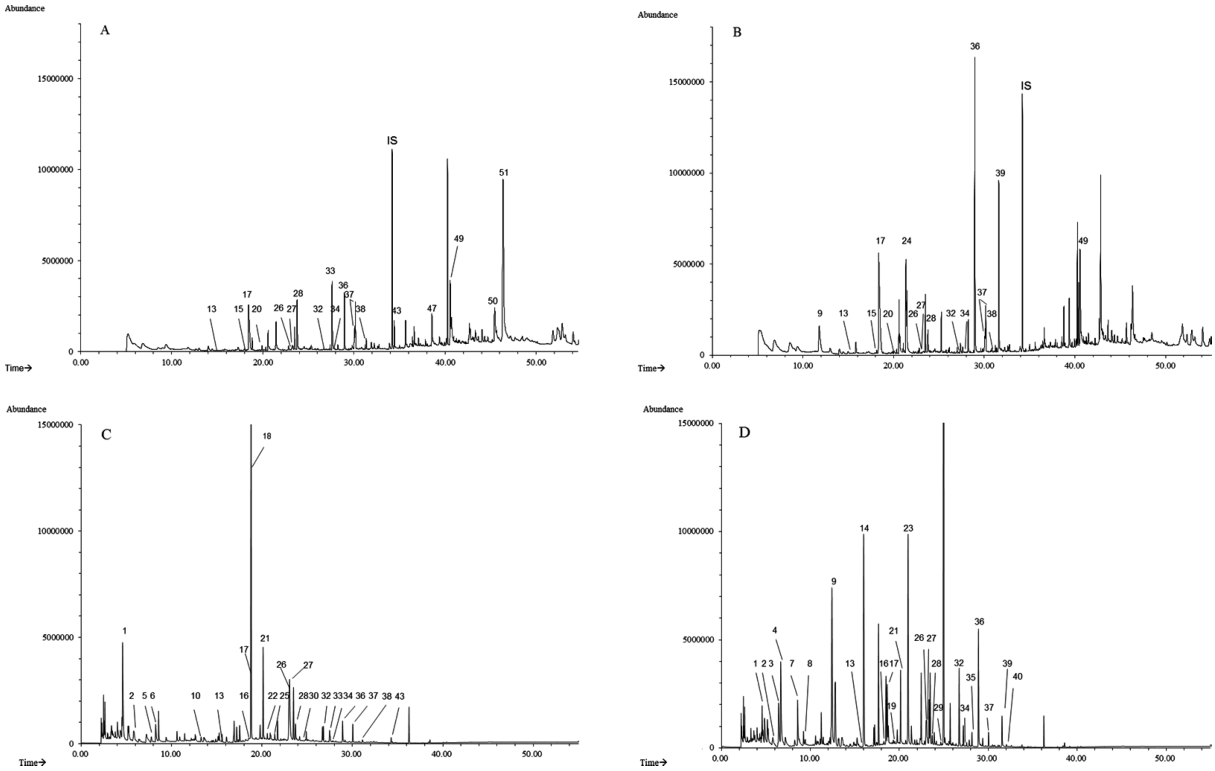


**Figure 3.** GC-MS/MS-O total ion current chromatograms of organic extracts of unfermented wort (A), of wort fermented by shiitake (B), after HS-SPME of unfermented wort (C), and of wort fermented by shiitake (D).

and 8, respectively). In addition, (*Z*)-3-nonen-1-ol (green), a yet unknown compound (burnt), (*E*)-methyl cinnamate (**40**) (fruity), and *o*-aminoacetophenone (grape) were also identified as potent odors in fermented wort (FD 16).

Despite not being perceivable at the ODP, shiitake produced methyl 3-methylbutanoate (4) (Figure 3), hexanol (14) (Figure 3), and 1-octanol (23) (Figure 3) de novo, whereas the peak area of furfural (18) (Figure 3) decreased significantly during the fermentation.

Analysis of the key odor-active compounds revealed the formation of methyl 2-methylbutanoate and (*E*)-methyl cinnamate (**40**) by shiitake. Both compounds have a typical fruity flavor and could not be detected in unfermented wort. In contrast to LLE, it was thus possible to identify the flavor compounds imparting the fruity aroma by means of HS-SPME. This is the first time that the formation of these compounds by shiitake is reported. As 2-methylbutanoic acid and cinnamic acid were detected in the LLE of wort, shiitake might transform these organic acids into their corresponding esters. Branched carboxylic acid and phenolic acids are known as flavor precursors in microbial cultures.[36]

*Comparison of Potent Odor-Active Compounds Identified by LLE and HS-SPME.* The extraction method may strongly influence the results of olfactometric analyses.[37] Depending on the extraction technique, an aroma compound might be detected or not during olfactometric analysis.[14] In our study, most of the determined key odor-active compounds of the fermented beverage were similar after LLE and HS-SPME. However, the FD factors of the individual compounds differed and some distinct differences were observed. LLE allowed the recovery of some key aroma compounds, such as 2-phenylacetic acid, a polar compound with a high boiling point (266 °C), which was hardly extracted by HS-SPME. This implied that HS-SPME was less sensitive for trapping low volatile and polar acids. This is in good agreement with a study of Yang and Peppard, who reported the adsorption of fatty acids on SPME fibers with low affinity.[38] On the other hand, HS-SPME was more appropriate for other main aroma compounds of the fermented beverage (e.g., methyl 2-methylbutanoate, methional, methyl hexadecanoate, and *o*-aminoacetophenone), which were not perceived in olfactometric analysis after LLE. The ester methyl 2-methylbutanoate is highly volatile as indicated by a RI of 1018. *o*-Aminoacetophenone and methional have low odor thresholds.[39,40] In the case of methional, panelists could recognize the potato-like smell above an olfactory threshold of 0.2 ppb in water.[40] These results suggest that HS-SPME has a better capacity for extracting highly volatile and trace compounds, which are easily lost during the extraction or concentration process of LLE, as already observed by Mebazaa et al.[41] The high sensitivity of SPME enabled trapping and identification of flavor compounds with low odor thresholds present in low concentrations with potentially high impact on the overall aroma of the sample.

In conclusion, all of the selected basidiomycetes grew well in wort, and many interesting odors were produced. Among them, shiitake was rated as the most promising species for producing a novel fermented beverage, exhibiting an interesting plum-like aroma. Odor-active compounds of the fermented beverage were identified using a combination of two different extraction methods. Odor-active compounds produced by shiitake, such as


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2-phenylethanol, methyl 2-methylbutanoate, 2-phenylethanol acetate, and (*E*)-methyl cinnamate (**40**), were responsible for the overall flavor.

## ■ ASSOCIATED CONTENT

### Supporting Information

Tables S1 and S2; Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION


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### Notes

The authors declare no competing financial interest.

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Supporting Information

Table S1. Odor-active Compounds Perceived at the ODP after HS-SPME-GC-MS/MS-O Analysis of Fermented Wort Utilizing Different Types of SPME Fibers (*cf.* Figure S1)

| No. | RI (VF-WAXms) | Compound | Odor impression | CAR/ PDMS | DVB/CAR/ PDMS | PDMS/ DVB | PDMS |
|---|---|---|---|---|---|---|---|
| 1 | 931 | 3-methylbutanal | green | + | - | - | - |
| 2 | 996 | 2,3-butanedione | buttery | + | - | - | - |
| 3 | 1,018 | methyl 2-methylbutanoate | fruity | + | + | + | - |
| 4 | 1,102 | (*E*)-2-methyl-2-butenal | toasted, sweetish | + | - | - | - |
| 5 | 1,126 | 3-methylbutyl acetate | fruity, sweetish | + | - | - | - |
| 6 | 1,237 | 3-methylbutanol | fresh, sourish | + | - | - | - |
| 7 | 1,282 | octanal[a] | soapy | - | + | + | - |
| 8 | 1,294 | 1-octen-3-one[b] | mushroom | + | + | - | - |
| 9 | 1,352 | 2,5-dimethylpyrazine | baked, nutty | + | + | + | - |
| 10 | 1,438 | not identified | sourish | - | - | + | - |
| 11 | 1,464 | methional | boiled potato | + | + | + | - |
| 12 | 1,474 | acetic acid | sourish, vinegar | + | - | - | - |
| 13 | 1,493 | 2-ethylhexanol | green | + | - | - | - |
| 14 | 1,512 | (*E*)-2-nonenal | green | + | + | + | - |
| 15 | 1,528 | benzaldehyde | almond, cereal | + | + | + | - |
| 16 | 1,652 | 2-phenylacetaldehyde | floral | + | + | + | + |
| 17 | 1,655 | 2-acetylthiazole | toasted, sweetish | + | + | + | + |
| 18 | 1,685 | 2-methylbutanoic acid | stinky, sourish, cheesy | + | - | + | - |
| 19 | 1,717 | (*Z*)-3-nonen-1-ol | green | + | + | - | - |
| 20 | 1,741 | not identified | burnt | + | + | + | + |
| 21[c] | 1,815 | 2-phenylethanol acetate *ß*-damascenone | fruity, like pear | + | + | + | + |
| 22 | 1,876 | 2-methoxyphenol | floral, | + | + | + | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | sweetish | | | | |
| 23 | 1,892 | benzyl alcohol | leaf, floral | + | + | - | - |
| 24 | 1,926 | 2-phenylethanol | rose | + | + | + | + |
| 25 | 1,990 | 2-acetylpyrrole | burnt, sweetish | + | + | + | - |
| 26 | 2,048 | *γ*-nonalactone | caramel | + | + | + | + |
| 27 | 2,083 | (*E*)-methyl cinnamate | fruity | + | + | + | - |
| 28 | 2,149 | hexadecanal | cardboard | + | + | - | + |
| 29 | 2,230 | methyl hexadecanoate | toasted | + | + | + | - |
| 30 | 2,241 | *o*-aminoacetophenone | grape | + | + | + | + |
| 31 | 2,395 | *γ*-dodecalactone | sweetish | + | + | - | - |
| 32 | 2,529 | dodecanoic acid | fatty | + | + | - | - |
| | total | | | 30 | 23 | 19 | 8 |

[a]: compound was tentatively identified by RI on VF-WAXms by DVB/CAR/PDMS, [b]: MS spectrum was ambiguous, the compound was tentatively identified on the basis of RI, odor, and literature data, [33] [c]: odor corresponds to two co-eluting compounds, +: perceived, -: not perceived

Table S2. Linear Equations and $R^2$ of the Plots (*cf*. Figure S2)

| Compound | Linear Equation[a] | Linear Range (µg/L) | $R^2$ |
|---|---|---|---|
| 2-acetylpyrrole | y = -1.1787x + 23.600 | 3 - 860 | 0.998 |
| *ß*-damascenone | y = -1.2991x + 22.450 | 0.2 - 59 | 0.997 |
| methyl 2-methylbutanoate | y = -0.9972x + 28.212 | 4 - 904 | 0.991 |
| 2-phenylacetaldehyde | y = -1.0865x + 27.339 | 3 - 774 | 0.989 |
| 2-phenylethanol | y = -1.1659x + 24.837 | 3 - 881 | 0.997 |
| 3-phenylpropanol | y = -1.2599x + 23.365 | 4 - 911 | 0.990 |

[a]: $y = \log_2$ (peak area), $x = \log_2$ (split ratio)

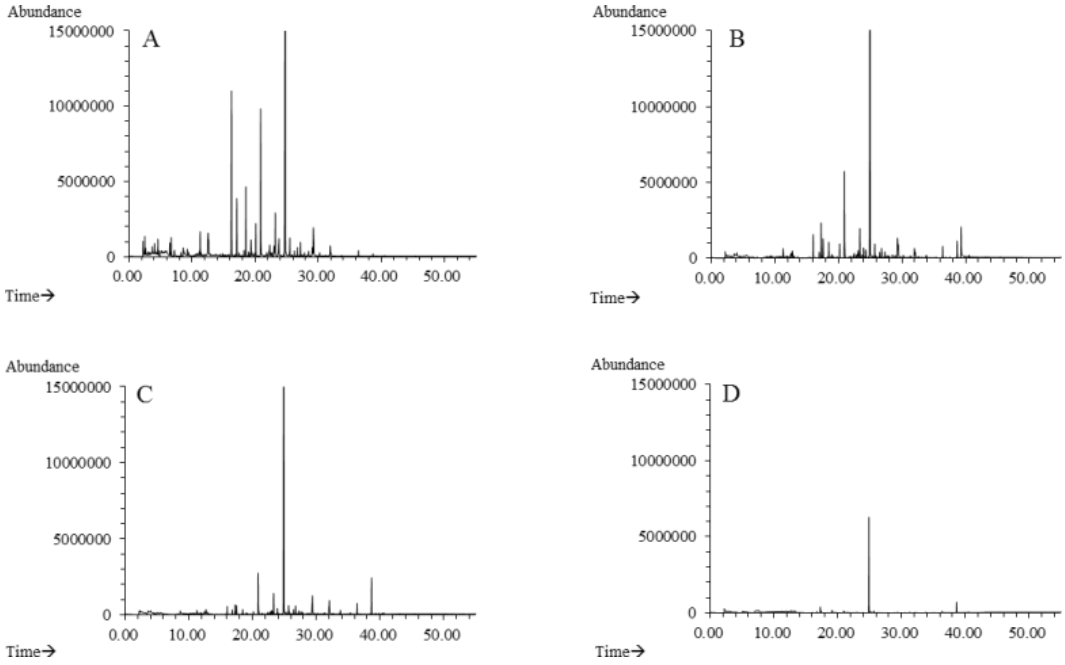


Figure S1. Total ion chromatograms of volatile compounds extracted from wort fermented with shiitake analyzed by means of HS-SPME-GC-MS/MS-O using CAR/PDMS (A), DVB/CAR/PDMS (B), PDMS/DVB (C), and PDMS (D) fiber

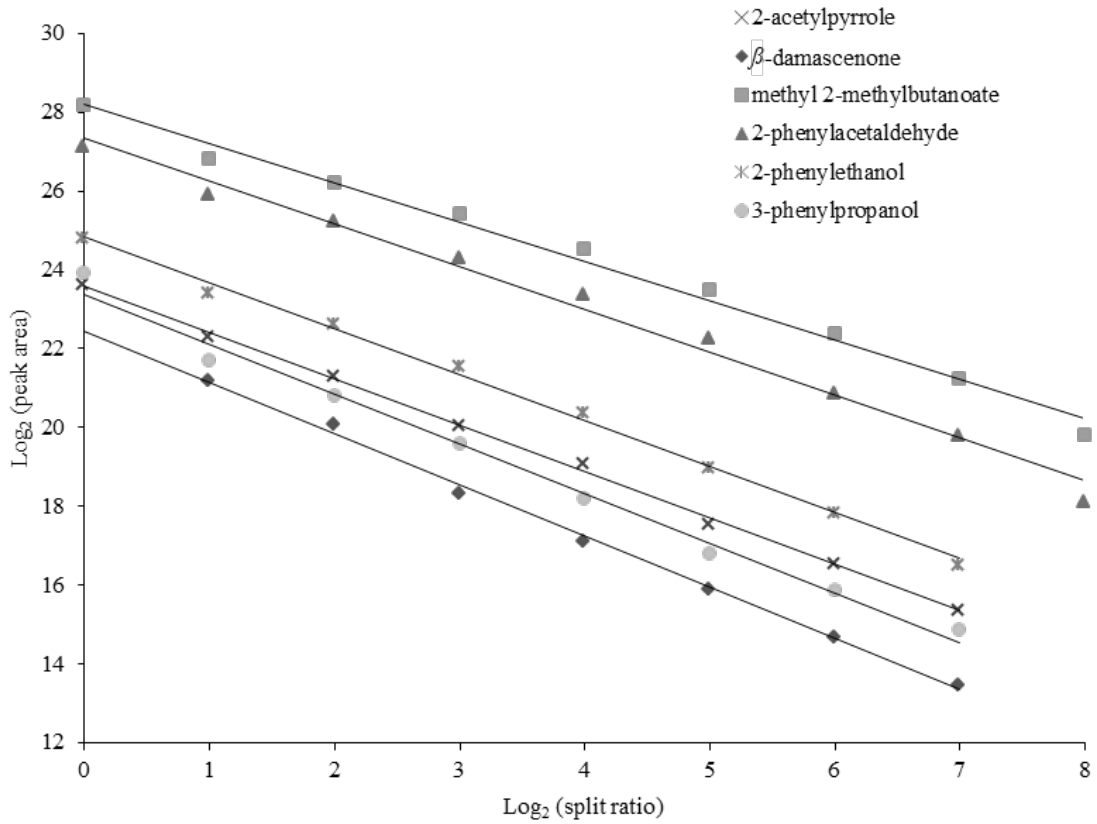


Figure S2. Regression plots of $\log_2$ (peak area) versus $\log_2$ (split ratio) for flavor compounds analyzed by HS-SPME-GC-MS/MS-O

# Quantification of Key Odor-Active Compounds of a Novel Nonalcoholic Beverage Produced by Fermentation of Wort by Shiitake (*Lentinula edodes*) and Aroma Genesis Studies

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# Quantification of key odor-active compounds of a novel nonalcoholic beverage produced by fermentation of wort by shiitake (*Lentinula edodes*) and aroma genesis studies

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2-Methylbutanoic acid (PubChem CID: 8314)
(*E*)-Methyl cinnamate (PubChem CID: 637520)
Methyl 2-methylbutanoate (PubChem CID: 13357)
2-Phenylethanol (PubChem CID: 6054)

ABSTRACT

A novel non-alcoholic beverage was produced by fermentation of wort with shiitake (*Lentinula edodes*), and the key aroma compounds were quantified. Reconstitution studies confirmed their contribution to the overall aroma of the beverage. Kinetic studies indicated that shiitake synthesized the key odor-active compounds imparting fruity and rose-like odors, while the typical odorants of the substrate wort were degraded during the fermentation. By means of the standard addition method, a good linearity ($R^2 \geq 0.983$) and degree of precision (RSD < 13%) were obtained for quantification of twelve aroma compounds. Their respective odor activity values (OAVs) were calculated to estimate their contribution to the overall aroma. Methyl 2-methylbutanoate, produced by shiitake, showed the highest OAV (30) and was responsible for the typical fruity odor of the beverage. A correlation between the concentration of methyl 2-methylbutanoate and the perceived fruitiness of the beverage was observed. The biosynthesis of this methyl ester by transformation of 2-methylbutanoic acid and L-isoleucine was confirmed by means of an isotopic labeling experiment.



## 1. Introduction

Shiitake (*Lentinula edodes*), as the second most popular edible mushroom in the world, represents a good source of carbohydrates, proteins, essential amino acids, poly-unsaturated fatty acids, and vitamins, especially vitamin D (Bisen, Baghel, Sanodiya, Thakur, & Prasad, 2010; Mattila, Suonpää, & Piironen, 2000). Furthermore, shiitake produces various secondary metabolites with potential pharmacologic relevance, such as lentinan, lectins, and eritadenine (Jasrotia, Sharma, Badhani, & Prashar, 2012). Last but not least, shiitake is highly valued because of its pleasant flavor.

Due to the worldwide declining consumption of beer (Colen & Swinnen, 2010), breweries are eagerly searching for innovative non-alcoholic beverages to broaden their product portfolio. A novel non-alcoholic beverage fermented by shiitake was developed using wort as substrate (Zhang, Fraatz, Horlamus, Quitman, & Zorn, 2014). It is nutritious and exhibits a pleasant fruity, slightly sour and plum like flavor. The key odor-active compounds of the novel fermented beverage were identified in our previous study by liquid–liquid extraction (LLE) and headspace solid phase microextration (HS-SPME) in combination with a gas chromatography system equipped with a tandem mass spectrometry detector and an olfactory detector port (GC–MS/MS–O) (Zhang et al., 2014). For further product development, i.e. up-scaling and controlling the quality of product, it is important to obtain quantitative data for the key aroma compounds and to perform aroma reconstitution studies. The latter is carried out by mixing pure aroma compounds in the concentrations determined in the food product in an appropriate matrix. Aroma reconstitution is widely accepted to finally proof the typical food aroma in regard to interactions between key odor-

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0963-9969/

active constituents (Grosch, 2001). It has been successfully applied to a variety of food, such as wine (Ferreira, Ortín, Escudero, López, & Cacho, 2002), orange and grapefruit juices (Buettner & Schieberle, 2001), roasted pistachio (Aceña, Vera, Guasch, Busto, & Mestres, 2010), and strawberry (Schieberle & Hofmann, 1997).

In the present study, twelve key odor-active compounds including 2-acetylpyrrole, 2-acetylthiazole, *o*-aminoacetophenone, β-damascenone, 2,5-dimethylpyrazine, methional, (*E*)-methyl cinnamate, methyl hexadecanoate, methyl 2-methylbutanoate, (*Z*)-3-nonenol, 2-phenylethanol, and 2-phenylethyl acetate with flavor dilution factors of 4 to 64, were selected based on the data of an aroma extract dilution analysis (Zhang et al., 2014). A kinetic study was conducted to investigate the changes of the concentrations of the key odorants during the fermentation process. Furthermore, the corresponding odor activity values were calculated, and reconstitution studies were carried out. Finally, the biosynthetic pathway of the most important odorant was elucidated by means of a labeling experiment.

## 2. Materials and methods

### 2.1. Chemicals and materials

*L. edodes* (shiitake) was obtained from the *Centraalbureau voor Schimmelcultures* (CBS, Utrecht, Netherlands).

L-isoleucine (U-$^{13}$C) (98%) was purchased from Euriso-Top GmbH (Saarbrücken, Germany). 2-Acetylpyrrole (98%), L-isoleucine (99%), methional (98%), 2-methylbutanoic acid (98%), and (*E*)-methyl cinnamate (99%) were obtained from Alfa Aesar (Karlsruhe, Germany). *o*-Aminoacetophenone (98%), and thymol (99%) were bought from TCI (Eschborn, Germany). Ethanol (99.5%), (*Z*)-3-nonenol (95%), and L-phenylalanine (99%) were obtained from Carl Roth (Karlsruhe, Germany). 2-Acetylthiazole (99%), β-damascenone (1.1–1.3 wt.% in 190 proof ethanol), 2,5-dimethylpyrazine (98%), methyl 2-methylbutanoate (98%), β-ionone (95%), and 2-phenylethyl acetate (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Methyl hexadecanoate (99%) was obtained from Cayman Chemical (Ann Arbor, USA). 2-Phenylethanol (99%) and (*E*)-cinnamic acid (98%) were purchased from Acros Organics (Nidderau, Germany). For gas chromatography (GC), helium 5.0 and nitrogen 5.0 were used.

### 2.2. Pre-culture and wort fermentation

Wort (Kölsch type, 13° Plato) was provided by the University of Applied Sciences (Giessen, Germany) (Zhang et al., 2014). The agar plug (1 × 1 cm) from the leading mycelial edge of the stock culture was transferred to flasks containing standard nutrition solution (100 mL/250 mL, medium volume/flask volume) and homogenized using an Ultra Turrax homogenizer (IKA, Staufen, Germany) as published previously (Fraatz et al., 2009). The pre-culture was incubated on a rotary shaker (24 °C, 150 rpm, 25 mm shaking diameter) for 9 days in the dark. The mycelium of 10 mL pre-culture broth was precipitated by means of centrifugation (4000 rpm, 2150 × *g*, 10 min, 20 °C) and washed three times with sterile water. The fungal pellets were resuspended in 10 mL sterilized wort, and the suspension was transferred into an Erlenmeyer flask (250 mL) containing 100 mL wort. To identify potential precursors of the biotransformation products, (*E*)-cinnamic acid (0.7 mg/100 mL), L-isoleucine (61.0 mg/100 mL), 2-methylbutanoic acid (0.4 μL/100 mL), and L-phenylalanine (62.6 mg/100 mL) were supplemented to the wort prior to the fermentation. The fermentation was carried out at 24 °C for 48 h under aerobic conditions on a rotary shaker (150 rpm). To elucidate the biogenetic pathway of methyl 2-methylbutanoate formation, labeled L-isoleucine (U-$^{13}$C) (0.45 mM) was added to wort, and the culture supernatant was analyzed by HS-SPME–GC–MS/MS–O after fermentation for 48 h by shiitake.

### 2.3. HS-SPME

For HS-SPME, CAR/PDMS (75 μm carboxen/polydimethylsiloxane, fiber length 1 cm; Supelco, Steinheim, Germany) and DVB/CAR/PDMS fibers (50/30 μm divinylbenzene/carboxen/polydimethylsiloxane, fiber length 1 cm) in combination with a GERSTEL MPS 2XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany) were used. Ten mL of the culture broth (2.2) were transferred into a headspace vial (20 mL). The sample was agitated for 20 min (250 rpm) at 60 °C, followed by headspace extraction for 45 min at the same temperature. Afterwards, the analytes were directly desorbed in the split/splitless inlet (250 °C; SPME liner, 0.75 mm i.d.; Supelco) of the GC–MS/MS–O system for 1 min (2.4).

After desorption, the fiber was heated at 300 °C for the CAR/PDMS fiber and at 270 °C for the DVB/CAR/PDMS fiber, respectively, in the fiber conditioning station for 20 min.

### 2.4. GC

GC was carried out using an Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an Agilent 7000B triple quadrupole mass spectrometry (MS/MS) detector (Agilent Technologies). A polar Agilent J&W VF-WAXms column (30 m × 0.25 mm × 0.25 μm) was used for analysis. He at a constant flow rate of 1.56 mL/min was used as the carrier gas. At the end of the capillary column, the carrier gas was split 1:1 into the triple quadrupole mass spectrometer and into an olfactory detector port (ODP 3, GERSTEL, Mülheim an der Ruhr, Germany). Further conditions were as follows: temperature program, 40 °C (3 min)/5 °C/min to 240 °C (5 min); injector temperature, 250 °C; septum purge flow rate, 3 mL/min; MS modes, selected ion monitoring (SIM) and scan mode in Q1; scan range, *m/z* 33–

**Table 1**
Compounds, internal standards, MS modes, *m/z* fragments, and fiber type used for quantitative analysis.

| Compound | MS mode | Quantifier ion (*m/z*) | Qualifier ion (*m/z*) | Fiber type | Internal standard |
|---|---|---|---|---|---|
| Methyl 2-methylbutanoate | Scan | 33–300 | – | CAR/PDMS | Thymol |
| (*E*)-methyl cinnamate | Scan | 33–300 | – | CAR/PDMS | Thymol |
| (*Z*)-3-nonenol | Scan | 33–300 | – | CAR/PDMS | Thymol |
| 2-Phenylethanol | Scan | 33–300 | – | CAR/PDMS | Thymol |
| 2-Acetylpyrrole | SIM | 109 | 94 | CAR/PDMS | Thymol[a] |
| 2-Acetylthiazole | SIM | 99 | 43 | CAR/PDMS | Thymol[a] |
| Methional | SIM | 104 | 76 | CAR/PDMS | Thymol[a] |
| *o*-Aminoacetophenone | SIM | 120 | 92 | DVB/CAR/PDMS | β-ionone[b] |
| β-Damascenone | SIM | 69 | 190 | DVB/CAR/PDMS | β-ionone[b] |
| 2,5-Dimethylpyrazine | SIM | 108 | 42 | DVB/CAR/PDMS | β-ionone[b] |
| Methyl hexadecanoate | SIM | 143 | 129 | DVB/CAR/PDMS | β-ionone[b] |
| 2-Phenylethyl acetate | SIM | 104 | 43 | DVB/CAR/PDMS | β-ionone[b] |

[a] : Quantifier ion and qualifier ion of thymol were 135 and 120, respectively.
[b] : Quantifier ion and qualifier ion of β-ionone were 177 and 43, respectively.

330; electron ionization energy, 70 eV; source temperature, 230 °C; quadrupoles temperatures, 150 °C; MS/MS transfer line temperature, 250 °C; He quench gas, 2.25 mL/min; $N_2$ collision gas, 1.5 mL/min; ODP 3 transfer line temperature, 250 °C; ODP 3 mixing chamber, 150 °C; ODP 3 make up gas, $N_2$.

### 2.5. Quantitative analysis by standard addition

Standard mixture solutions were prepared by diluting the aroma compounds in their respective concentrations in a defined amount of ethanol and finally preparing an aqueous solution (supplementary material Table 1). Ten mL samples were spiked with 0, 10, 25, 50, 100, 150, and 200 μL of the standard solution, respectively. Additionally, internal standard solutions of thymol (50 μL, 8.88 mg/L) or β-ionone (100 μL, 0.09 mg/L) were added to the samples (Table 1). The calibration curve for each target compound was determined by plotting the area ratio of the chosen ion currents and the respective internal standard against the corresponding concentrations. For each calibration curve, the regression correlation coefficients ($R^2$) and standard deviations (SD) were calculated based on duplicate analyses.

### 2.6. Kinetic analysis of the concentrations of the key odor-active compounds during the fermentation process

The relative concentrations of the key odor-active compounds were determined by means of HS-SPME–GC–MS/MS–O (2.3, 2.4) after 0, 24,

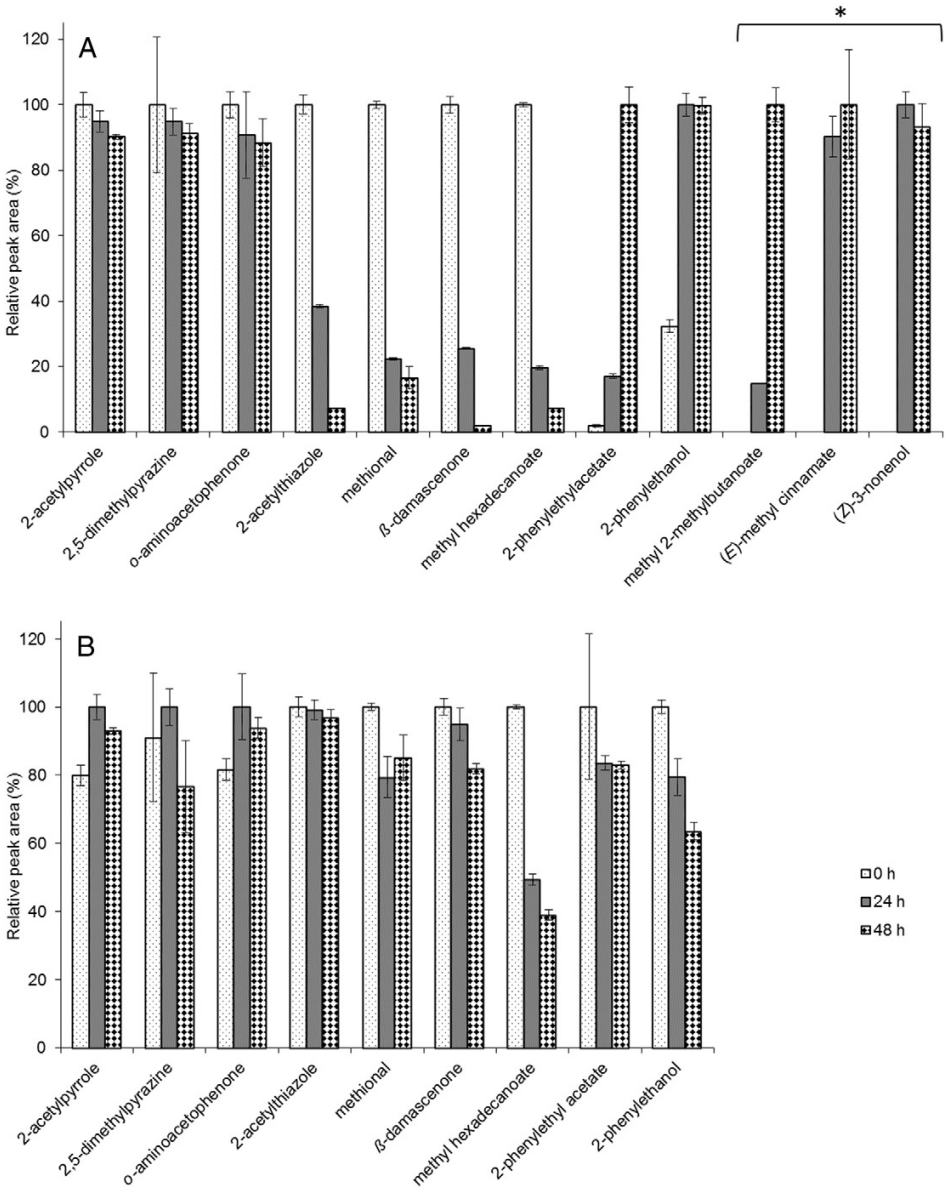


**Fig. 1.** Relative concentrations of the key odor-active compounds in wort fermented by shiitake (A) and in non-fermented wort (B) (*: compounds only detected in fermented wort).

and 48 h fermentation, and changes were expressed by the relative peak areas (peak area of each target compound/peak area of internal standard). Each sample was analyzed in duplicate.

*2.7. Calculation of odor activity values (OAVs)*

The OAV of each target compound was calculated as the ratio of the concentration in the sample to its respective odor threshold in water.

*2.8. Aroma reconstitution*

An aqueous aroma model was prepared based on the quantitative analytical data of the twelve selected target compounds. Six odor qualities (fruity, caramel, sourish, malty, sweetish, and fresh) were selected to describe the odor of the fermented beverage as well as the corresponding aroma model.

The fermented beverage and the model mixture (10 mL each) were evaluated in medicine cups (capacity, 30 mL; bottom diameter, 26 mm; top diameter, 38 mm) by a sensory panel consisting of 24 experienced persons. They were asked to rate the given odor qualities of the fermented beverage and of the aroma model using a five point intensity scale ranging from 1 to 5 (1: very weak, 2: weak, 3: medium intense, 4: strong, 5: very intense). The values given by the panelists were averaged and analyzed by *t*-test.

## 3. Results and discussion

*3.1. Changes of the concentrations of the key odor-active compounds during the fermentation process*

The key odor active compounds of the wort subjected to fermentation and of the fermented wort were identified previously (Zhang et al., 2014). In the present study, the kinetic changes of these compounds were studied by HS-SPME over the fermentation process at 0, 24, and 48 h. Three of the main flavor compounds were not detectable in unfermented wort and were thus synthesized de novo by shiitake.

2-Acetylpyrrole, 2-acetylthiazole, 2,5-dimethylpyrazine, β-damascenone, methional, and *o*-aminoacetophenone were reported as the typical odorants in wort (Beal & Mottram, 1994; De Schutter et al., 2008a,2008b; Fickert & Schieberle, 1998; Kishimoto, Wanikawa, Kono, & Shibata, 2006; Vandecan, Saison, Schouppe, Delvaux, & Delvaux, 2010). In wort and beer, these compounds are derived from Maillard type reactions (Coghe, Martens, D'Hollander, Dirinck, & Delvaux, 2004; Osamu, Takeo, Yutaka, & Motoo, 2006), oxidation of carotenoids (Bezman et al., 2005; De Schutter et al., 2008b), Strecker degradation (Gijs, Perpète, Timmermans, & Collin, 2000; Soares da Costa et al., 2004), and degradation of tryptophan (Gijs, Chevance, Jerkovic, & Collin, 2002). Their concentrations decreased with increasing fermentation time (Fig. 1A). Especially the concentration of 2-acetylthiazole, β-damascenone, methional, and methyl hexadecanoate dropped significantly.

Compared to non-fermented wort, which was incubated under the same conditions, striking differences were observed (Fig. 1B). The complex set of enzymes secreted by shiitake into the culture medium well explains the degradation of some flavor compounds (Mata & Savoie, 1998; Tsujiyama, Muraoka, & Takada, 2013). Enzymatic processes (e.g. aldo-keto reductase and alcohol dehydrogenase) for the reduction of Strecker aldehydes (e.g. methional) to alcohols have been observed in yeasts (Peppard & Halsey, 1981; Perpète & Collin, 1999). β-Damascenone may be reduced and/or adsorbed rapidly by yeast as well (Chevance, Guyot-Declerck, Dupont, & Collin, 2002). Methional, as an important odorant of wort, is derived from the amino-carbonyl reaction and the following Strecker degradation of L-methionine during the processes of wort boiling and wort clarification (Gijs et al., 2000; Soares da Costa et al., 2004). Methional has been described to impart the wort-like off-flavor of alcohol-free beer (Perpète & Collin, 1999). 2-Acetylthiazole as a key odorant of malted barley possesses a toasted and popcorn-like odor (Beal & Mottram, 1994; Pripis-Nicolau, De Revel, Bertrand, & Maujean, 2000). The decreasing concentrations of methional and 2-acetylthiazole correlate well with the decreased intensity of the wort like smell during the fermentation of wort by *L. edodes*.

The concentrations of 2-phenylethanol, 2-phenylethyl acetate, (*E*)-methyl cinnamate, methyl 2-methylbutanoate, and (*Z*)-3-nonenol increased significantly during the short fermentation time of 48 h (Fig. 1A). Among them, the latter three were only detected in fermented wort (24 h and 48 h) and not in unfermented wort (0 h). (*E*)-Methyl cinnamate was biosynthesized in considerable amounts by shiitake. The de novo synthesis of (*E*)-methyl cinnamate was also observed with *Lenziles frabea*, *Poria suboennispora*, and *Vararia effuscata* (Gallois, Gross, Langlois, Spinnler, & Brunerie, 1990). Transmethylating enzymes for the alkylation of the carboxyl group with *S*-adenosylmethionine (SAM) which catalyzed the formation of methyl cinnamate from the

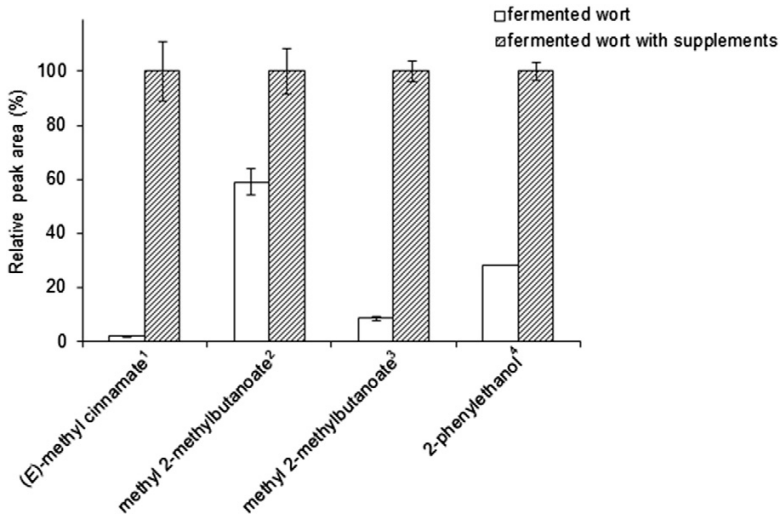


**Fig. 2.** Relative concentrations of volatiles produced by shiitake in wort supplemented with different precursors ([1]: supplemented with (*E*)-cinnamic acid, [2]: supplemented with L-isoleucine, [3]: supplemented with 2-methylbutanoic acid, [4]: supplemented with L-phenylalanine).

**Table 2**
Parameters of calibration curves of the quantified aroma compounds.

| Compound | Slope | Intercept | $s_m$ | $s_b$ | $R^2$ | Calibration range (μg/L) |
|---|---|---|---|---|---|---|
| Methyl 2-methylbutanoate | 70,098 | 525,657 | 2614 | 44,329 | 0.983 | 2.9–57.3 |
| (*E*)-methyl cinnamate | 140,910 | 301,343 | 5135 | 18,292 | 0.993 | 0.64–12.87 |
| (*Z*)-3-nonenol | 469,811 | 907,207 | 17,592 | 7759 | 0.997 | 0.63–12.64 |
| 2-Phenylethanol | 15,429 | 7,864,375 | 364 | 306,037 | 0.998 | 159–3178 |
| 2-Acetylpyrrole | 2709 | 498,486 | 37 | 6604 | 0.995 | 24–488 |
| 2-Acetylthiazole | 7794 | 33,288 | 505 | 377 | 0.994 | 0.4–7.3 |
| Methional | 1003 | 14,007 | 266 | 1999 | 0.998 | 1–22 |
| *o*-Aminoacetophenone | 2589 | 16,128 | 34 | 1794 | 0.989 | 0.7–14.8 |
| *β*-Damascenone | 436,337 | 28,228 | 22,724 | 350 | 0.986 | 0.01–0.12 |
| 2,5-Dimethylpyrazine | 11,235 | 74,078 | 29 | 1002 | 0.993 | 1.46–29.21 |
| Methyl hexadecanoate | 67,801 | 35,483 | 9883 | 4212 | 0.992 | 0.11–2.14 |
| 2-Phenylethyl acetate | 292,908 | 1,367,864 | 1351 | 122,038 | 0.993 | 1.0–19.5 |

$s_m$: SD of slope. $s_b$: SD of intercept.

free acid were detected in *Lentinus lepideus* and *Trametes suaveolens* (Lomascolo et al., 2001; Wat & Towers, 1975). Cinnamic acid has been described as a constituent of wort by Floridi (Floridi, Montanari, Marconi, & Fantozzi, 2003) and Zhang (Zhang et al., 2014). Additionally, (*E*)-methyl cinnamate was synthesized by shiitake after supplementing the culture medium with (*E*)-cinnamic acid (Fig. 2). This indicates that a similar enzymatic transmethylating activity may exist in shiitake. With methyl 2-methylbutanoate, shiitake produced a further methyl ester during the fermentation. This methyl ester has been found in fruiting bodies of *Boletus pseudocalopus* and wild *Polyporus sulfureus* as well (Kim, Choi, & Lee, 2012; Peppard & Halsey, 1981). Methyl 2-methylbutanoate was synthesized by shiitake from L-isoleucine and 2-methylbutanoic acid as substrates (Fig. 2). The responsible metabolic pathway has not yet been fully elucidated. To the best of our knowledge, (*E*)-methyl cinnamate and methyl 2-methylbutanoate have been detected in culture supernatants of shiitake for the first time. Additionally, shiitake synthesized 2-phenylethanol and 2-phenylethyl acetate during the fermentation of wort (Fig. 1A). The biotransformation of L-phenylalanine to 2-phenylethanol was described for *Ischnoderma benzoinum* by Krings et al. (Krings, Hinz, & Berger, 1996), and Cho et al. detected 2-phenylethanol in fruiting bodies of shiitake (Cho, Seo, & Kim, 2003). L-phenylalanine is a typical constituent of wort (Kennedy, Taidi, Dolan, & Hodgson, 1997), and its biotransformation to 2-phenylethanol by shiitake was confirmed by supplementing wort with L-phenylalanine (Fig. 2). 2-Phenylethyl acetate was identified by Kawabe et al. in submerged cultures of *Polyporus tuberaster* (Kawabe & Morita, 1993). Again, the metabolism has still not been fully elucidated.

During the fermentation, the concentrations of the key odorants of wort decreased while the fruity and rose-like aroma produced by shiitake increased. Shiitake synthesized sensory relevant concentrations of esters and of an aromatic alcohol by biotransformation of organic acids and amino acids present in wort. This indicated that the composition of the wort and the vitality of the fungus are the key factors affecting the characteristic odor of the novel fermented beverage.

### 3.2. Quantitative analysis of key odor-active compounds by standard addition

Stable isotope dilution analysis (SIDA) represents a precise quantification method widely used in flavor analysis (Grosch, 2001; Maraval et al., 2010). Comparative studies on the quantification of odor-active compounds of roasted coffee were performed by Bicchi et al. using SIDA and the standard addition method coupled with HS-SPME and GC–MS, respectively (Bicchi et al., 2011). The study confirmed that both methods are appropriate for precise quantification of flavor compounds in complex matrices. The standard addition method showed a good performance in avoiding matrix effects when used for quantification of aroma compounds in wort (De Schutter et al., 2008b). Considering the reliability of the standard addition method and the high costs of labeled reference compounds, standard addition was used for the quantification of the target compounds in the present study.

Depending on the characteristics of the flavor compounds, CAR/PDMS fibers or DVB/CAR/PDMS fibers were used for quantification by HS-SPME. Some trace compounds or co-eluting compounds were determined by selected ion monitoring (SIM) in order to improve the

**Table 3**
Quantitative data, odor thresholds, and odor activity values (OAVs).

| Compound | Odor impression[a] | FD factor[b] | Concentration in culture (μg/L) | Odor threshold (μg/L) | OAV |
|---|---|---|---|---|---|
| Methyl 2-methylbutanoate | Fruity | 64 | 7.5 ± 0.35 | 0.25[c] | 30.0 |
| (*E*)-methyl cinnamate | Fruity | 16 | 2.14 ± 0.050 | 11[d] | 0.2 |
| (*Z*)-3-nonenol | Green | 16 | 1.93 ± 0.060 | – | – |
| 2-Phenylethanol | Rose | 64 | 510 ± 32 | 390[e] | 1.3 |
| 2-Acetylpyrrole | Sweetish, burnt | 4 | 184 ± 5.0 | 170,000[f] | <0.1 |
| 2-Acetylthiazole | Toasted, sweetish | 8 | 4.3 ± 0.23 | 10[g] | 0.4 |
| Methional | Boiled potato | 64 | 14 ± 1.8 | 1.4[e] | 10.0 |
| *o*-Aminoacetophenone | Grape | 16 | 6.2 ± 0.78 | 0.64[e] | 9.7 |
| *β*-Damascenone | Fruity | 64 | 0.06 ± 0.004 | 0.056[e] | 1.1 |
| 2,5-Dimethylpyrazine | Baked, nutty | 32 | 6.59 ± 0.070 | 1700[f] | <0.1 |
| Methyl hexadecanoate | Toasted, sweetish | 64 | 0.53 ± 0.014 | >2000[f] | <0.1 |
| 2-Phenylethyl acetate | Fruity | 64 | 4.7 ± 0.44 | 250[h] | <0.1 |

[a] : Odor impressions were cited from Zhang et al. (2014).
[b] : Odor impressions and Flavor dilution (FD) factors were cited from Zhang et al. (2014).
[c] : Rychlik, Schieberle, and Grosch (1998).
[d] : Buettner and Welle (2004).
[e] : Czerny et al. (2008).
[f] : Buttery, Turnbaugh, and Ling (1988).
[g] : Susan (1983).
[h] : Guth (1997).

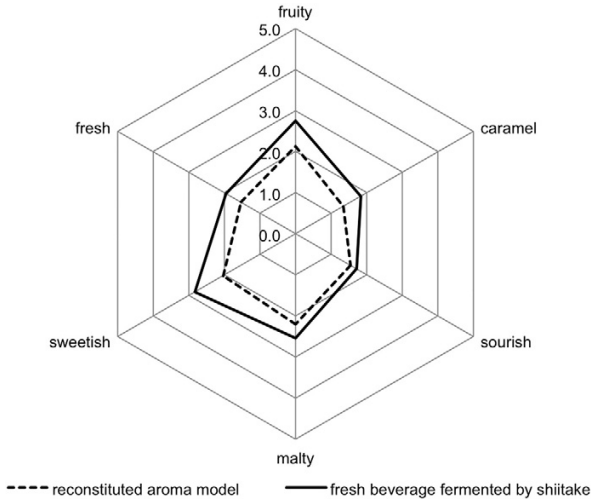


**Fig. 3.** Comparative flavor profile analysis of the reconstituted aroma model in water and the fresh beverage fermented by shiitake.

accuracy of the quantification (Table 1). The linearity was tested within a specific range for each compound (Table 2), and the obtained correlation coefficients ($R^2$) were considered acceptable ($R^2 \geq 0.983$) for the standard addition method. The key odor-active compounds quantified in the novel fermented beverage included esters, alcohols, ketones, a sulfur containing aldehyde, a pyrazine, a pyrrole, and a thiazole (Table 3). The HS-SPME method showed a good performance in the quantification of a wide range of different analytes, and a good reproducibility with a relative standard deviation (RSD %) of less than 10% for most of the compounds.

### 3.3. Odor activity values

Despite the well-known limitations of the OAV concept, OAVs are used to estimate the contribution of each compound to the overall aroma (Liu, Li, Li, & Tang, 2012). Methyl 2-methylbutanoate (OAV 30.0), methional (OAV 10.0), *o*-aminoacetophenone (OAV 9.7), 2-phenylethanol (OAV 1.3), and *β*-damascenone (OAV 1.1) were present in concentrations higher than their corresponding odor thresholds. Among them, methyl 2-methylbutanoate showed the highest OAV, and it thus contributed significantly to the characteristic fruity aroma of the novel beverage. A comparison of FD factors and OAVs showed that some odor-active compounds with high FD factors had OAVs far below 1, such as 2,5-dimethylpyrazine and methyl hexadecanoate. This phenomenon has been described by Escudero and Ferreira (Escudero et al., 2004; Ferreira et al., 2002) as well.

### 3.4. Aroma reconstitution

To compare the aroma model and the aroma of the fermented beverage, a panel of 24 assessors evaluated the odor of each sample using selected descriptors: fruity, caramel, sourish, malty, sweetish, and fresh.

The results of the aroma reconstitution study confirmed that the characteristic aroma of the novel fermented beverage may well be simulated by combining twelve key odor-active compounds in their respective concentrations using water as matrix (Fig. 3). With the exception of the sweetish odor all intensities of the descriptors of the reconstituted aroma model had no significant difference ($P > 0.05$) compared to the fresh novel beverage, although all of them were slightly weaker. Therefore, the most potent odorants of the novel beverage have been identified and quantified accurately by HS-SPME. The significantly different perception of the sweetish odor might be caused by the loss of some odorants due to the limitations of HS-SPME (e.g. discrimination of less volatile or thermally labile compounds) (Kataoka, Lord, & Pawliszyn, 2000) or by yet unidentified odorants (Zhang et al., 2014).

### 3.5. Elucidation of the biogenetic pathway of methyl 2-methylbutanoate generation and its role as a quality parameter

Methyl 2-methylbutanoate was the most important aroma compound of the fermented beverage (3.3), and L-isoleucine and 2-methylbutanoic acids were identified as biogenetic precursors. Supplementation of wort with either of the two precursors prior to the fermentation led to significantly higher concentrations of methyl 2-methylbutanoate after fermentation (Fig. 2). Subsequently, a correlation between the concentration of the fermented beverage and its typical fruity odor was observed after spiking experiments and sensory evaluation (supplementary material Table 2, supplementary material Table 3, supplementary material Fig. 1 and supplementary material Fig. 2). In samples supplemented with L-isoleucine or 2-methylbutanoic acid prior to the fermentation, the fruity and sweetish odor impressions became more distinct with increasing concentrations of methyl 2-methylbutanoate. With prolonged fermentation times (>4 days), the odor of the beverage turned uninteresting and non-fruity. At the same time, the concentration of methyl 2-methylbutanoate decreased significantly. These results indicated that methyl 2-methylbutanoate represents a suitable quality parameter for controlling and improving the quality of the novel non-alcoholic beverage in the future.

After supplementation of the medium with U-$^{13}$C-L-isoleucine, the mass spectrum of methyl 2-methylbutanoate showed a molecular ion with $m/z = 121$ (Fig. 4). Compared to the non-labeled reference compound with $m/z = 116$ the maximum number of five $^{13}$C-atoms was incorporated. The ratio of the abundance of the labeled transformation

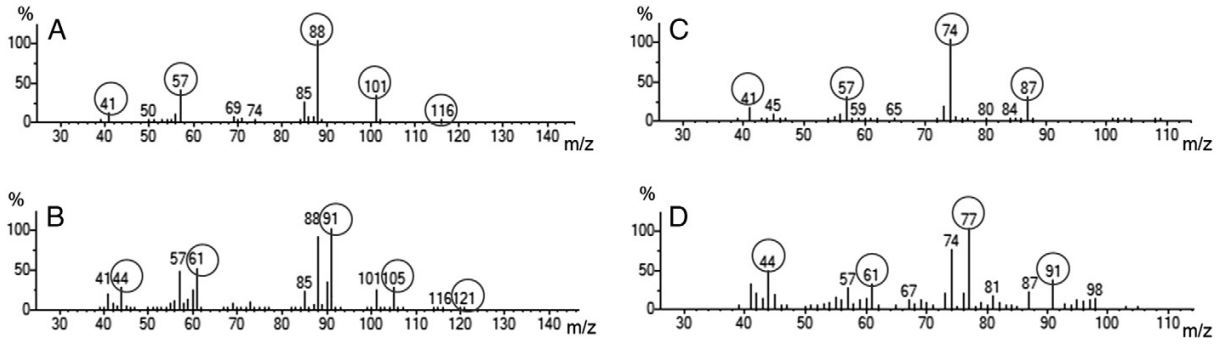


**Fig. 4.** Mass spectra of non-labeled methyl 2-methylbutanoate (A) and 2-methylbutanoic acid (C) in comparison to the mass spectra of methyl 2-methylbutanoate (B) and 2-methylbutanoic acid (D) after supplementation of wort with L-isoleucine (U-$^{13}$C).

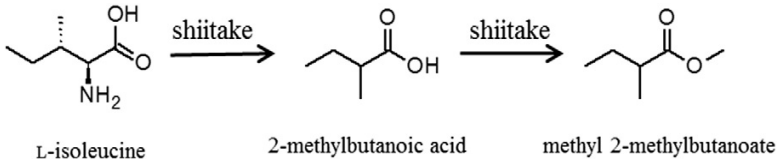


**Fig. 5.** Biosynthetic pathway of methyl 2-methylbutanoate in shiitake.

product's base peak ($m/z = 91$) to the corresponding peak of the non-labeled reference compound ($m/z = 88$) was 50/50. Therefore, in addition to naturally available precursors in the wort, shiitake transformed the supplemented isotopic labeled L-isoleucine to methyl 2-methylbutanoate. The $^{13}C$ labels were also detected in 2-methylbutanoic acid. The similar mass spectrum of 2-methylbutanoic acid is distinguished by a fragment ion resulting from McLafferty rearrangement at $m/z = 77$ (non-labeled counter-part: $m/z = 74$) and by another fragment ion at $m/z = 91$ (non-labeled counter-part: $m/z = 87$) resulting from the loss of the branched methyl group. It is assumed that 2-methylbutanoic acid was an intermediate in the biogenetic pathway of methyl 2-methylbutanoate (Fig. 5). Rowan, Allen, Fielder, and Hunt (1999) confirmed a similar pathway in apples, where L-isoleucine was considered to be a biosynthetic precursor of 2-methylbutanoic acid and its corresponding ester. A second pathway towards saturated methyl branched fruity esters starting from unsaturated precursors like ethyl tiglate was observed in apple (Matich & Rowan, 2007). The existence of such a pathway in shiitake should be verified in a future study by determination of the optical purity of methyl 2-methylbutanoate by chiral GC analysis. Methyl esters have already been identified in many different basidiomycetes species (Cho, Choi, & Kim, 2006; Kim et al., 2012). However, there are only few studies on their biosynthetic pathways (Wat & Towers, 1975) and, to the best of our knowledge, the biosynthesis pathway of methyl 2-methylbutanoate formation by shiitake is reported here for the first time.

## 4. Conclusion

The standard addition method in combination with HS-SPME–GC–MS/MS–O analysis was appropriate and efficient for extraction and quantification of the aroma compounds of a novel non-alcoholic fermented beverage. The aroma model, consisting of the twelve key odorants (methyl 2-methylbutanoate, methional, *o*-aminoacetophenone, 2-phenylethanol, β-damascenone, 2-acetylthiazole, (*E*)-methyl cinnamate, 2-acetylpyrrole, 2,5-dimethylpyrazine, methyl hexadecanoate, 2-phenylethyl acetate, and (*Z*)-3-nonenol), well represented the typical odor of the beverage. Methyl 2-methylbutanoate was synthesized by shiitake and showed the highest OAV in the beverage. Its biosynthesis by transformation of L-isoleucine and 2-methylbutanoic acid by shiitake was confirmed for the first time using an isotopic labeling experiment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.01.019.

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Supplementary material Table 1 Preparations of standard mixture solutions.

| | Compound | Mass (g) | Purity (%) | Ethanol solution (mL) | Volume of ethanol solution transferred to the final aqueous solution | Concentration (mg/L water) |
|---|---|---|---|---|---|---|
| standard mixture solution 1 | methyl 2-methylbutanoate | 0.0585 | 98 | 50 | 250 µL ad 100 mL | 2.866 |
| | (*E*)-methyl cinnamate | 0.0130 | 99 | | | 0.644 |
| | (*Z*)-3-nonenol | 0.0133 | 95 | | | 0.632 |
| | 2-phenylethanol | 3.2099 | 99 | | | 158.890 |
| standard mixture solution 2 | *o*-aminoacetophenone | 0.1506 | 98 | 50 | 250 µL ad 1 L | 0.738 |
| | *ß*-damascenone | 0.1029 | 1.2 | | | 0.00617 |
| | 2,5-dimethylpyrazine | 0.2981 | 98 | | | 1.461 |
| | methyl hexadecanoate | 0.0217 | 99 | | | 0.107 |
| | 2-phenylethyl acetate | 0.1970 | 99 | | | 0.975 |
| individual standard solution | 2-acetylpyrrole | 0.9966 | 98 | 10 | 250 µL ad 1 L | 24.417 |
| | 2-acetylthiazole | 0.0368 | 99 | 25 | 250 µL ad 1 L | 0.364 |
| | methional | 0.1106 | 98 | 50 | 500 µL ad 1 L | 1.084 |

Supplementary material Table 2 Correlation between supplementation of wort with L-isoleucine and sensory evaluation.

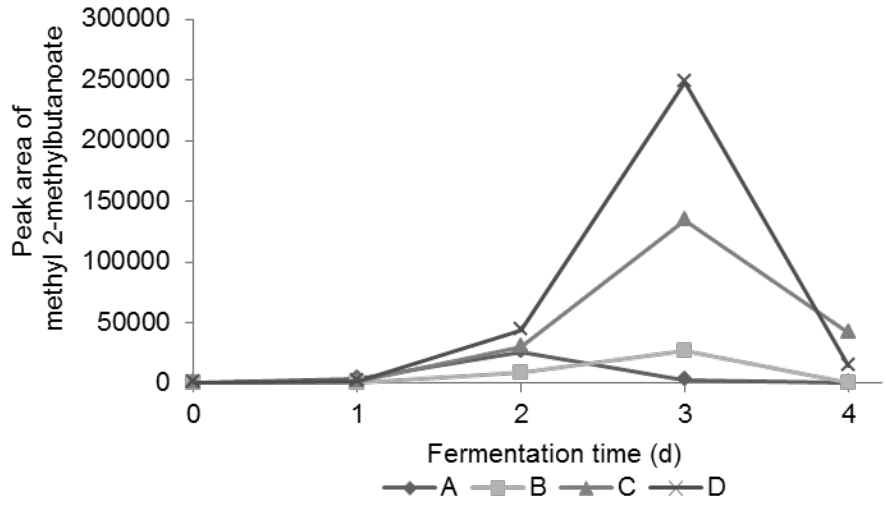
| Sample | Fermentation time (d) | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| A | Odor impression | malty | malty, sweetish | malty, sweetish, sourish | slightly sweetish, almost odorless |
| | Intensity[a] | 2 | 1 - 2 | 2 | < 1 |
| B | Odor impression | malty, sweetish | fresh, malty, sweetish | malty, sweetish, sourish | slightly malty, almost odorless |
| | Intensity | 1 - 2 | 1 | 1 | < 1 |
| C | Odor impression | malty, sweetish | fruity, malty, sweetish,sourish | fruity, sweetish | slightly malty, almost odorless |
| | Intensity | 1 - 2 | 1 - 2 | 1 | < 1 |
| D | Odor impression | sweetish, malty, honey-like | fruity, malty, sweetish | fresh, fruity, sweetish | odorless |
| | Intensity | 2 | 2 | 2 | < 1 |

A: fermented wort. B: wort supplemented with L-isoleucine (100 mg/L wort) before fermentation. C: wort supplemented with L-isoleucine (350 mg/L wort) before fermentation. D: wort supplemented with L-isoleucine (600 mg/L wort) before fermentation. [a]: the odor intensity was rated on a scale of 1 – 5 (1, low intensity; 5, intense, strong odor).

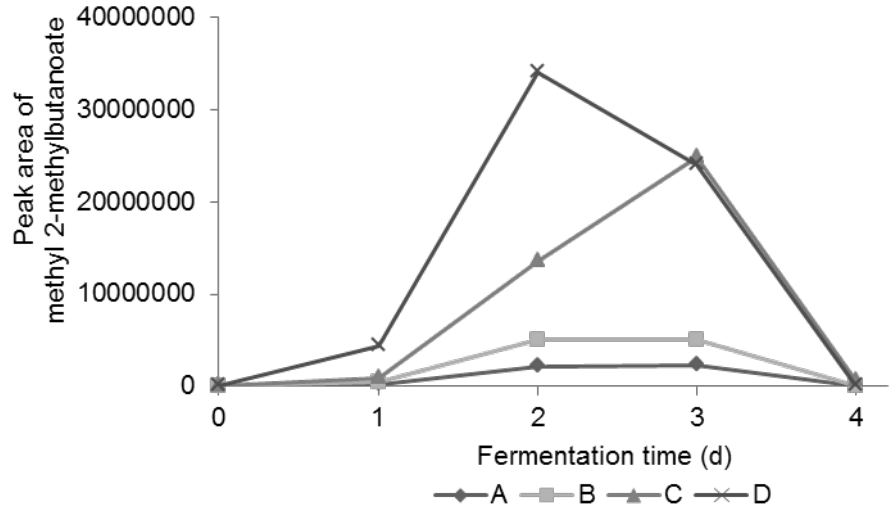
Supplementary material Table 3 Correlation between supplementation of wort with 2-methylbutanoic acid and sensory evaluation.

| Sample | Fermentation time (d) | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| A | Odor impression | malty | malty, sweetish | malty, sweetish, sourish | slightly sweetish, almost odorless |
| | Intensity[a] | 2 | 2 - 3 | 2 | 1 |
| B | Odor impression | malty, sweetish | fresh, malty, sweetish | malty, sweetish, sourish | slightly malty, almost odorless |
| | Intensity | 2 | 2 - 3 | 2 | 1 |
| C | Odor impression | malty, sweetish | fruity, malty, sweetish, sourish | fruity, malty, sweetish | slightly malty, almost odorless |
| | Intensity | 1 - 2 | 1 - 2 | 2 | 1 |
| D | Odor impression | sweetish, less malty | fruity, sweetish | fruity, malty, sweetish | odorless |
| | Intensity | 1 - 2 | 2 | 2 - 3 | < 1 |

A: fermented wort. B: wort supplemented with 2-methylbutanoic acid (1 µL/L wort) before fermentation. C: wort supplemented with 2-methylbutanoic acid (4 µL/L wort) before fermentation. D: wort supplemented with 2-methylbutanoic acid (8 µL/L wort) before fermentation. [a]: the odor intensity was rated on a scale of 1 – 5 (1, low intensity; 5, intense, strong odor).




Supplementary material Fig. 1. Relative concentration of methyl 2-methylbutanoate in samples supplemented with L-isoleucine as a function of fermentation time (A: fermented wort. B: wort supplemented with L-isoleucine (100 mg/L wort) before fermentation. C: wort supplemented with L-isoleucine (350 mg/L wort) before fermentation. D: wort supplemented with L-isoleucine (600 mg/L wort) before fermentation)




Supplementary material Fig. 2. Relative concentration of methyl 2-methylbutanoate in samples supplemented with 2-methylbutanoic acid as a function of fermentation time (A: fermented wort. B: wort supplemented with 2-methylbutanoic acid (1 µL/L wort) before fermentation. C: wort supplemented with 2-methylbutanoic acid (4 µL/L wort) before fermentation. D: wort supplemented with 2-methylbutanoic acid (8 µL/L wort) before fermentation)

# Aroma Characterization and Safety Assessment of a Beverage Fermented by *Trametes versicolor*

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# Aroma Characterization and Safety Assessment of a Beverage Fermented by *Trametes versicolor*

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Supporting Information

**ABSTRACT:** A cereal-based beverage was developed by fermentation of wort with the basidiomycete *Trametes versicolor*. The beverage possessed a fruity, fresh, and slightly floral aroma. The volatiles of the beverage were isolated by liquid−liquid extraction (LLE) and additionally by headspace solid phase microextraction (HS-SPME). The aroma compounds were analyzed by a gas chromatography system equipped with a tandem mass spectrometer and an olfactory detection port (GC-MS/MS-O) followed by aroma (extract) dilution analysis. Thirty-four different odor impressions were perceived, and 27 corresponding compounds were identified. Fifteen key odorants with flavor dilution (FD) factors ranging from 8 to 128 were quantitated, and their respective odor activity values (OAVs) were calculated. Six key odorants were synthesized de novo by *T. versicolor*. Furthermore, quantitative changes during the fermentation process were analyzed. To prepare for the market introduction of the beverage, a comprehensive safety assessment was performed.

**KEYWORDS:** *flavor, Trametes versicolor, wort, fermented cereal beverage, safety assessment*

## INTRODUCTION

Cereals, as a major source of human diet, are rich in carbohydrates, proteins, vitamins, minerals, and fiber. On the other hand, they are deficient in certain essential amino acids (e.g., lysine), show a limited availability of the starch fraction, and may contain certain antinutrients (e.g., phytic acid) or toxic compounds (e.g., oxalic acid).[1] Fermentation is known as an efficient and economical way to improve a product's nutritional value, its sensory properties, and its functional qualities.[2] To improve the nutritional value of cereal-based products, beverages fermented by lactic acid bacteria, such as Boza, Pozol, and Sobia, have been developed.[3−5] However, the spectrum of aroma compounds that impart the characteristic sourish flavor of the majority of these traditional beverages is rather limited.[1]

Different from bacteria, yeasts, and lower fungi, which are usually used for the production of nonalcoholic fermented beverages,[1] basidiomycetes possess a diverse repertoire of extra- and intracellular enzymes for producing a broad spectrum of natural flavor compounds.[6] To satisfy the consumers' preference for pleasant natural flavors, novel fermentation systems based on basidiomycetes were successfully developed.[7,8] Among the screened fungi, *Trametes versicolor* produced a very pleasant aroma profile within a fermentation time of 38 h.[7] Additionally, *T. versicolor* has been consumed in the Far East as part of the traditional diet and medicine for centuries because of its potential health-promoting, disease-preventing, and medicinal qualities.[9] A variety of promising dietary supplements, such as protein-bound polysaccharides, have been produced by *T. versicolor* and marketed in the form of capsules, tablets, or powder recently.[10−12] Therefore, *T. versicolor* was chosen as a good candidate for producing a novel nonalcoholic fermented cereal-based beverage.

To the best of our knowledge, this is the first comprehensive study on the fermentation of wort by *T. versicolor*. Therefore, the basic nutritional parameters, flavor, and safety of the fermented beverage were systematically evaluated in this work.

## MATERIALS AND METHODS

**Materials and Chemicals.** *T. versicolor* (480.63) was obtained from the Centraalbureau voor Schimmelcultures (CBS; Utrecht, The Netherlands).

Methional (98%), 2-methylbutanoic acid (98%), 2-methylpropanoic acid (99%), and 2-phenylacetaldehyde (95%) were obtained from Alfa Aesar (Karlsruhe, Germany). Benzaldehyde (99%) was bought from Applichem GmbH (Darmstadt, Germany) and ethanol (99.5%) from Carl Roth (Karlsruhe, Germany). Linalool (97%), (*R*)-(−)-linalool (95%), (*S*)-(+)-2-methylbutanoic acid (98%), and 2-phenylethanol (99%) were purchased from Fisher Scientific GmbH (Schwerte, Germany). 2,3-Butanedione (97%) was from Merck-Schuchardt OHG (Hohenbrunn, Germany). 2-Acetylthiazole (99%), ethyl 2-methylpropanoate (99%), *β*-damascenone (1.1−1.3 wt % in 190 proof ethanol), 2,5-dimethylpyrazine (98%), and 2-phenylacetic acid (99%) were purchased from Sigma-Aldrich (Steinheim, Germany). Saponine for molecular biology was from Sigma-Aldrich (Taufkirchen, Germany). Ethyl 2-methylbutanoate (98%), 3-methylbutanoic acid (99%), and thymol (99%) were bought from TCI (Eschborn, Germany). For gas chromatography (GC) helium (5.0) and nitrogen (5.0) were used.

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**Fermentation of Wort by *T. versicolor*.** Wort (Kölsch type, 13 °Plato) was provided by the University of Applied Sciences (Giessen, Germany).[7] Erlenmeyer flasks containing standard nutrition solution (100 mL/250 mL, medium volume/flask volume) were inoculated with fungal mycelia as reported previously.[6] The precultures were incubated on a rotary shaker (24 °C, 150 rpm, 25 mm shaking diameter) for 5 days in the dark. The mycelium of 10 mL of preculture broth was precipitated by centrifugation (2150*g*, 10 min, 20 °C) and washed three times with sterile water. The fungal pellets were resuspended in 10 mL of sterile wort. The suspension was added into an Erlenmeyer flask (250 mL) containing 100 mL of wort, and fermentation was carried out at 24 °C for 38 h under aerobic conditions on a rotary shaker (150 rpm). Subsequently, the mycelium was removed by centrifugation (2150*g*, 10 min, 4 °C), and the fermented beverage was used for the determination of nutritionally relevant parameters, for flavor analysis, and for safety assessment.

**Determination of Nutritionally Relevant Parameters.** The glucose content was determined with a D-glucose kit from R-Biopharm AG (Darmstadt, Germany). The total amount of reducing sugars was analyzed according to the method of Luff-Schoorl, and crude protein was quantified by Kjeldahl analysis. Oxalic acid was determined by ion exclusion chromatography with direct conductivity detection (883 basic IEC system, Metrohm AG, Filderstadt, Germany) as described by Marten.[13] Quantitation of ethanol was carried out by gas chromatography combined with headspace solid phase microextraction (HS-SPME) using standard addition.[14]

**Liquid–Liquid Extraction (LLE) and Headspace Solid Phase Microextraction.** For LLE, 90 mL of wort or fermented beverage was used as described previously.[7]

For HS-SPME, a CAR/PDMS fiber (75 μm carboxen/polydimethylsiloxane, fiber length = 1 cm) (Supelco, Steinheim, Germany) in combination with an MPS 2 XL multipurpose sampler (GERSTEL, Mülheim an der Ruhr, Germany) was used according to the method of Zhang et al.[8]

**Aroma (Extract) Dilution Analysis.** For LLE, the flavor dilution (FD) factors were determined by aroma extract dilution analysis (AEDA).[15] The organic extracts were diluted with pentane/diethyl ether (1:1.12, v/v) in a series of 1:1 dilutions. For HS-SPME, aroma dilution analysis (ADA) was performed by increasing the GC inlet split ratios.[7] After extraction, the volatiles of the beverage were diluted stepwise by controlling the split ratio as follows: splitless (splitless time = 2 min), 2:1, 4:1, 8:1, 16:1, 32:1, 64:1, 128:1, and 256:1. After dilution, each sample was sniffed twice until no odorants were perceived at the ODP 3 olfactory detection port (GERSTEL) of the GC-MS/MS-O system.

**GC-MS/MS-O Analysis.** GC-MS/MS-O analysis was based on the method described by Zhang et al.[7,8]

**Multidimensional Gas Chromatography–Mass Spectrometry (MDGC-MS).** Chiral separations were performed on an MDGC-MS system (Shimadzu Europa GmbH, Duisburg, Germany), which consists of two Shimadzu GC-2010 Plus gas chromatographs (GC 1 and GC 2), equipped with a multi Dean's switch (MDS), a Shimadzu QP2010 Ultra mass spectrometer, and a Shimadzu AOC-20i autoinjector.

*GC 1.* Helium (5.0) was used as carrier gas. A polar Agilent J&W VF-WAXms column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) (Agilent Technologies, Waldbronn, Germany) was used for initial separation. The operational conditions were as follows: constant inlet pressure, 208.1 kPa; inlet temperature, 250 °C; splitless, 2 min; initial linear velocity, 25 cm/s; temperature program, 40 °C (3 min), raised at 10 °C/min to 200 °C (6 min). The FID (250 °C; $H_2$ flow, 40 mL/min; air flow, 400 mL/min; makeup gas $N_2$ (5.0), 30 mL/min) was connected via a stainless steel retention gap to the MDS.

*GC 2.* A Hydrodex β-TBDAc column (heptakis-(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin; 25 m × 0.25 mm i.d.; Macherey-Nagel, Düren, Germany) was applied for the analysis of linalool. The oven temperature program was held at 40 °C for 1 min, ramped at 10 °C/min to 80 °C, held for 50 min, increased at 20 °C/min to 200 °C, and finally held for 4 min. An Astec Chiraldex B-DM column ((2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin; 30 m × 0.25 mm i.d. × 0.12 μm film thickness; Sigma-Aldrich, Taufkirchen, Germany) was applied for the analysis of 2-methylbutanoic acid. The oven temperature program for the analysis of 2-methylbutanoic acid was held at 40 °C for 1 min, ramped at 10 °C/min to 65 °C, held for 45 min, increased at 20 °C/min to 200 °C, and finally held for 4 min. Further conditions were as follows: transfer line temperature between GC 1 and GC 2, 200 °C; initial linear velocity, 47.2 cm/s; switching pressure, 129.1 kPa; MS modes, selected ion monitoring (*m*/*z* 57, 74, 87) for 2-methylbutanoic acid and scan mode (*m*/*z* 33–300) for linalool; electron ionization energy, 70 eV; source temperature, 200 °C; quadrupole temperature, 150 °C; MS transfer line temperature, 200 °C. All data were collected using Shimadzu MDGC solution software 1.01.

**Compound Identification.** The aroma compounds were identified by comparison of their respective odor impressions, their retention indices (RI) on two columns of different polarity (VF-WAXms and DB-5MS), and their mass spectra with those of authentic standards and published data.

**Quantitative Analysis and Calculation of Odor Activity Values (OAVs).** The concentrations of aroma compounds extracted by LLE were estimated via internal standard (IS) method (100 μL of IS; 888 mg thymol/L pentane/diethyl ether). The corresponding response factors (Rf) were calculated for each analyte according to eq 1

$$\mathrm{Rf} = \frac{A_{\mathrm{IS}} m_{\mathrm{a}}}{m_{\mathrm{IS}} A_{\mathrm{a}}} \quad (1)$$

where *A* = peak area, *m* = mass (μg), IS = internal standard, and a = analyte.

The aroma compounds extracted by HS-SPME were quantitated by standard addition.[8] A standard solution mixture was prepared containing the target compounds in their respective concentrations.

The quantifier and qualifier ions of each compound are listed in Table 1.

**Table 1. *m*/*z* Fragments (Quantifier and Qualifier Ions) Used for Quantitative Analysis**

| compound | MS mode[a] | quantifier ion (*m*/*z*) | qualifier ion (*m*/*z*) |
|---|---|---|---|
| ethyl 2-methylpropanoate[b] | SIM | 71 | 116 |
| 2,3-butanedione[b] | SIM | 86 | 53 |
| ethyl 2-methylbutanoate[b] | SIM | 102 | 57 |
| 2,5-dimethylpyrazine[b] | SIM | 108 | 42 |
| methional[b] | SIM | 104 | 76 |
| benzaldehyde[b] | SIM | 106 | 51 |
| linalool[b] | SIM | 71 | 93 |
| 2-phenylacetaldehyde[b] | SIM | 120 | 91 |
| 2-acetylthiazole[b] | SIM | 99 | 43 |
| β-damascenone[b] | SIM | 69 | 190 |
| thymol[b] | SIM | 135 | 150 |
| 2-methylbutanoic acid[c] | scan (EIC) | 74 | |
| 3-methylbutanoic acid[c] | scan (EIC) | 60 | |
| 2-methylpropanoic acid[c] | scan (TIC) | 33–300 | |
| 2-phenylacetic acid[c] | scan (TIC) | 33–300 | |
| 2-phenylethanol[c] | scan (TIC) | 33–300 | |
| thymol[c] | scan (EIC/TIC) | 135/33–300 | 150/– |

[a]EIC, extracted ion chromatogram; SIM, single ion monitoring; TIC, total ion current. [b]Aroma compounds extracted by HS-SPME were quantified by means of standard addition. [c]Aroma compounds extracted by LLE were quantified by means of an internal standard.



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**Table 2. Nutritionally Relevant Parameters of the Beverage Fermented by *T. versicolor* in Comparison to Nonfermented Wort**

| | total reducing sugars (g/L) | glucose (g/L) | crude protein (g/L) | oxalic acid (mg/L) | ethanol (%, v/v) | pH |
|---|---|---|---|---|---|---|
| nonfermented wort | 57.2 ± 0.19 | 10.5 ± 0.12 | 5.5 ± 0.07 | 28 ± 2 | –[a] | 5.39 ± 0.03 |
| fermented wort | 45.3 ± 0.74 | 6.0 ± 0.05 | 5.2 ± 0.14 | 23 ± 2 | 0.385 ± 0.005 | 5.33 ± 0.05 |

[a]No distinct MS signal.

**Table 3. Aroma Compounds Identified in the Beverage by Means of GC-MS/MS-O after LLE and HS-SPME**

| | RI[a] | | | | | FD factor[b] | |
|---|---|---|---|---|---|---|---|
| no. | VF-WAXms | DB-5MS | compound | odor impression | identification | LLE | HS-SPME |
| 1 | 929 | <800 | 3-methylbutanal | green | MS, RI, odor | –[c] | 1 |
| 2 | 977 | <800 | ethyl 2-methylpropanoate | fruity | MS, RI, odor | – | 8 |
| 3 | 994 | <800 | 2,3-butanedione | buttery | MS, RI, odor | – | 16 |
| 4 | 1024 | <800 | methyl 2-methylbutanoate | fruity | MS, RI, odor | – | 2 |
| 5 | 1052 | 800 | ethyl butanoate | fruity | MS, RI, odor | – | 1 |
| 6 | 1064 | 853 | ethyl 2-methylbutanoate | fruity | MS, RI, odor | – | 32 |
| 7 | 1085 | <800 | hexanal | green | MS, RI, odor | – | 1 |
| 8 | 1121 | <800 | 2-pentanol | green | MS, RI, odor | 1 | – |
| 9[d] | 1240 | <800 | 2-methylbutanol | fresh, sourish | MS, RI, odor | – | 1 |
| | | <800 | 3-methylbutanol | | | | |
| 10[e] | 1271 | nd[f] | unknown | rubber | | – | 2 |
| 11[e,g] | 1299 | nd | 1-octen-3-one | mushroom | RI, odor | – | 8 |
| 12 | 1348 | 914 | 2,5-dimethylpyrazine | baked, nutty | MS, RI, odor | – | 64 |
| 13[e] | 1426 | nd | unknown | caramel | | 1 | – |
| 14 | 1456 | <800 | acetic acid | sourish, vinegar | MS, RI, odor | 4 | – |
| 15 | 1478 | 908 | methional | boiled potato | MS, RI, odor | – | 128 |
| 16[e] | 1499 | nd | unknown | green | | 32 | 1 |
| 17 | 1534 | 965 | benzaldehyde | almond, cereal | MS, RI, odor | – | 32 |
| 18 | 1562 | 1099 | linalool | floral | MS, RI, odor | – | 64 |
| 19 | 1569 | <800 | 2-methylpropanoic acid | stinky, sourish | MS, RI, odor | 16 | – |
| 20[e] | 1645 | nd | unknown | caramel | | 1 | – |
| 21 | 1652 | 1048 | 2-phenylacetaldehyde | floral | MS, RI, odor | – | 16 |
| 22 | 1655 | 1066 | 2-acetylthiazole | toasted, sweetish | MS, RI, odor | – | 16 |
| 23[d] | 1678 | 861 | 2-methylbutanoic acid | cheesy, stinky, sourish | MS, RI, odor | 128 | 4 |
| | | 857 | 3-methylbutanoic acid | | | | |
| 24[e] | 1733 | nd | unknown | burnt | | 32 | 4 |
| 25 | 1823 | 1379 | β-damascenone | fruity, like pear | MS, RI, odor | 4 | 128 |
| 26 | 1920 | 1113 | 2-phenylethanol | rose | MS, RI, odor | 128 | 4 |
| 27[e] | 1933 | nd | unknown | burnt | | 2 | – |
| 28 | 1960 | 1109 | maltol | caramel | MS, RI, odor | 4 | – |
| 29 | 1990 | 1019 | 2-acetylpyrrole | burnt, sweetish | MS, RI, odor | – | 4 |
| 30[h] | 2048 | 1363 | γ-nonalactone | caramel | MS, RI, odor | 4 | 4 |
| 31[e] | 2189 | nd | unknown | sweetish | | 1 | - |
| 32 | 2242 | 1301 | o-aminoacetophenone | grape | MS, RI, odor | 4 | 4 |
| 33[e] | 2308 | nd | unknown | sweetish | | 8 | – |
| 34 | 2557 | 1254 | 2-phenylacetic acid | toasted, pungent, sweetish | MS, RI, odor | 128 | – |

[a]Mean of RI values from LLE and SPME. [b]Flavor dilution (FD) factor of the corresponding compound. [c]–, odor was not perceived. [d]Odor corresponded to two coeluting compounds. [e]Retention indices (RI) were calculated on the basis of the retention time of the odor impression perceived at the ODP. [f]nd, no distinct MS spectrum on the DB-5MS column. [g]The peak area was below the limit of detection; the compound was tentatively identified on the basis of RI, odor impression, and literature data.[25] [h]MS spectrum was obtained from wort.

The OAVs of the key aroma compounds were calculated as the ratios of the concentrations in the beverage to their respective odor thresholds in water.

**Concentrations of Key Aroma Compounds during the Fermentation Process.** The concentrations of the key aroma compounds were quantitated after 0 (before fermentation) and 38 h (after fermentation) by means of LLE and HS-SPME and GC-MS/MS-O analysis. Quantitative changes were expressed by the relative peak areas of each target compound. Each sample was analyzed in duplicate.

**Cytotoxicity in Rat Hepatocytes in Primary Culture.** Cytotoxicity of the beverage and of the nonfermented wort was determined in rat hepatocytes in primary culture by means of the resazurin reduction assay and the lactate dehydrogenase leakage assay. Before use, the pH of the beverage was adjusted to neutral.

Rat livers were perfused according to the two-step method of Seglen to isolate primary rat hepatocytes.[16] The viability of cell preparations always exceeded 90%. The procedure of hepatocyte preparation was authorized by the Ethical Committee of the State of Rhineland-Palatinate. Hepatocytes were treated with the beverage or wort at concentrations of 0.1, 1, and 10% (v/v) in culture medium and incubated at 37 °C and 5% $CO_2$. Addition of 0.1% (w/v) saponine (added as aqueous solution) served as a positive control, culture medium as a negative control.



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For the resazurin reduction assay,[17] cells were seeded in collagenated 24-well plates at a density of 100,000 cells/cm$^2$. After 48 h of treatment, cells were incubated with medium containing resazurin for 90 min. The fluorescent metabolite resorufin was measured in a fluorescence plate reader.

For the lactate dehydrogenase (LDH) leakage assay,[18] cells were seeded in collagenated 60 mm dishes at a density of 100,000 cells/cm$^2$. After 6 h of treatment, the integrity of cell membranes was determined by measuring the activity of the cytosolic enzyme LDH leaked out of cells into the culture medium in comparison to the total amount of LDH activity (medium + cell pellet). LDH activity was measured by the fluorescence of metabolized NADH.

**Mutagenicity Testing in Ames Fluctuation Assay.** The mutagenicity of the beverage was tested according to the method of Maron and Ames in *Salmonella typhimurium* in a variation of the Ames fluctuation test (ISO 11350, 2012).[19,20] Strain TA98 was used to detect frameshift mutations and strain TA100 to detect base pair substitutions. The tests were performed with and without metabolic activation by rat hepatic S9 mix.

Hepatic S9 mix was prepared from the livers of rats treated with Aroclor 1254 according to the method of Maron and Ames.[19] The procedure of S9 preparation was authorized by the Ethical Committee of the State of Rhineland-Palatinate.

Bacteria suspensions were treated with the beverage at concentrations of 0.1, 1, and 10% (v/v) in histidine-deficient medium and incubated at 37 °C. 4-Nitro-*o*-phenylenediamine (for strain TA 98 without S9), nitrofurantoin (for strain TA 100 without S9), and 2-aminoanthracene (for both strains with S9) served as positive controls and medium, water, and DMSO as negative controls.

The growth of revertants in histidine-deficient medium was measured in 384-well plates by means of the pH indicator dye bromocresol purple, and the percentage of positive wells was calculated accordingly.

**Statistical Analysis of Cytotoxicity and Mutagenicity Results.** Statistical values (means and standard deviations) and data used in charts (bar graphs) were calculated by use of the software package Origin 9.1 (OriginLab, Northampton, MA, USA). Statistical significance was determined by ANOVA and Dunnett post test with the software InStat 3.0 (GraphPad, La Jolla, CA, USA). All experiments were performed in triplicate.

## RESULTS AND DISCUSSION

**Nutritionally Relevant Parameters.** Due to the steadily declining consumption of beer and to satisfy market needs for non- or low-alcoholic alternatives, new fermentation systems have been developed. In the past, predominantly yeasts and lactic acid bacteria have been utilized to ferment wort.[21,22] In our study, wort was fermented by the basidiomycete *T. versicolor* for the first time.

During the fermentation, the concentrations of total reducing sugars and glucose decreased significantly by 21 and 43%, respectively (Table 2). The protein concentration decreased by about 5%, and the concentration of oxalic acid, which is typically present in wort, was reduced from 28 to 23 mg/L. Oxalic acid might be converted to formic acid and carbon dioxide by oxalate decarboxylase produced by *T. versicolor*.[23] Oxalic acid may cause haze-forming and gushing of beer. In the fermented beverage the concentration of ethanol was determined to be 0.39% (v/v), whereas no ethanol was detected in wort. An alcohol dehydrogenase of *T. versicolor* has been described previously by Kudahettige et al.[24] In most of the European Union countries and the United States, an upper limit of 0.5% alcohol by volume has been defined for alcohol-free beer.[21] Therefore, the novel beverage may still be considered a nonalcoholic refreshment.

**Flavor Analysis of the Novel Fermented Beverage.** The odor of the fermented beverage was perceived as fruity, fresh, sweetish, and slightly floral. It was strikingly different from the typical odor of wort and most cereal beverages fermented by lactic acid bacteria, yeasts, or lower fungi. To understand the formation of the unique aroma, the flavor of the beverage was analyzed.

*Extraction and Identification of Key Aroma Compounds.* Two different extraction methods were used together with GC-MS/MS-O analysis to comprehensively analyze the beverage's aroma. Previous studies have shown that fibers containing porous carbon in their stationary phase were highly effective in extracting various types of volatile compounds.[7] Therefore, a CAR/PDMS fiber was chosen for extracting the volatile compounds.

By GC-MS/MS-O analysis, 17 and 24 odor-active regions from the fermented beverage were detected at the ODP after LLE and HS-SPME, respectively (Table 3). Only seven of them were detected by both methods. Thus, in total 34 different odor impressions were perceived. Twenty-seven aroma compounds including inter alia acids, alcohols, aldehydes, ketones, and esters/lactones were identified. Most odor-active regions were attributed to a single compound. Only two odor impressions (no. 9 and 23) originated from coeluting compounds (Table 3). The mixture of 2-methylbutanol and 3-methylbutanol imparted a fresh and sourish odor (no. 9). A cheesy, stinky, and sourish odor was perceived from 2-methylbutanoic acid accompanied by 3-methylbutanoic acid (no. 23).

By performing an A(E)DA for each extraction method (Table 3), the key aroma compounds of the beverage were identified (FD factors from 8 to 128 for at least one of the two extraction techniques). A compound assigned to a high FD factor after LLE might exhibit a low FD factor after HS-SPME and vice versa (cf. 2-/3-methylbutanoic acid, 2-phenylethanol, 2-phenylacetic acid, methional, linalool, 2,3-butanedione, and ethyl 2-methylbutanoate). Compared to HS-SPME, LLE performed better in trapping organic acids and higher molecular weight alcohols and showed lower sensitivities for highly volatile compounds as well as for some trace compounds. By combining the two different extraction techniques, the aroma compounds of the beverage were trapped in a wide range.

Several of the main odor-active compounds of the beverage, such as 2-methylbutanoic acid (FD 128) and linalool (FD 64), are chiral. The enantiomeric purities of both odorants were determined by means of an MDGC-MS system. Both components showed high enantiomeric excess (ee) values of 91.5% for (*R*)-linalool and >95% for (*S*)-2-methylbutanoic acid. The aroma of (*R*)-linalool is described as floral, whereas the (*S*)-enantiomer imparts an intense green note.[34] The chiral analysis supported the perceived "floral" odor impression (Table 3). The aroma of (*S*)-2-methylbutanoic acid is perceived as sweetish and fruity.[35] However, 2-methylbutanoic acid coeluted with 3-methylbutanoic acid, which imparts a sourish and sweaty odor. Therefore, it seems reasonable that the corresponding odor impression (no. 23) was perceived as cheesy, stinky, and sourish (Table 3). As methyl 2-methylbutanoate (FD 2), 2-methylbutanol (FD 1), and γ-nonalactone (FD 4) showed only low flavor dilution factors, their ee values were not determined. No authentic enantiopure standard of ethyl 2-methylbutanoate (FD 32) was commercially available, and the absolute configuration of this compound could thus not be assigned.

*Quantification and Calculation of the OAVs of Key Aroma Compounds.* To evaluate the contribution of a single



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compound to the overall aroma, OAVs of the key aroma compounds of the beverage were calculated. Therefore, the concentrations of 15 odorants with FD factors of 8−128 were quantified. 2-Methylbutanoic acid, 3-methylbutanoic acid, 2-methylpropanoic acid, 2-phenylacetic acid, and 2-phenylethanol were quantified in the organic extract according to the internal standard thymol. The other compounds were quantified by HS-SPME using standard addition.

2-Phenylacetaldehyde, ethyl 2-methylpropanoate, linalool, 2,3-butanedione, and methional showed high OAVs (≥10) (Table 4). Additionally, 3-methylbutanoic acid, β-damascenone, 2-methylpropanoic acid, 2-methylbutanoic acid, and ethyl 2-methylbutanoate presumably contributed to the overall aroma.

**Table 4. Quantitative Data, Odor Thresholds, and Odor Activity Values (OAVs)**

| compound | concentration in the beverage (μg/L) | odor threshold (μg/L) | OAV |
|---|---|---|---|
| ethyl 2-methylpropanoate | 0.6 ± 0.16 | 0.02[26] | 31 |
| 2,3-butanedione | 30 ± 7.4 | 3[27] | 10 |
| ethyl 2-methylbutanoate | 0.18 ± 0.039 | 0.15[27] | 1.2 |
| 2,5-dimethylpyrazine | 2.68 ± 0.034 | 1700[28] | <0.1 |
| methional | 14 ± 1.1 | 1.4[29] | 10 |
| benzaldehyde | 4.8 ± 0.66 | 25[26] | 0.2 |
| linalool | 2.30 ± 0.011 | 0.14[30] | 16 |
| 2-phenylacetaldehyde | 162 ± 28 | 4[28] | 40 |
| 2-acetylthiazole | 2.68 ± 0.22 | 10[31] | 0.3 |
| β-damascenone | 0.13 ± 0.013 | 0.056[29] | 2.3 |
| 2-methylbutanoic acid | 205 ± 12 | 100[32] | 2.0 |
| 3-methylbutanoic acid | 154 ± 20 | 22[26] | 7.0 |
| 2-methylpropanoic acid | 432 ± 15 | 190[33] | 2.3 |
| 2-phenylacetic acid | 84.2 ± 0.77 | 360[26] | 0.2 |
| 2-phenylethanol | 31 ± 2.0 | 390[29] | 0.1 |

*Changes of Key Aroma Compounds during the Fermentation Process.* To control the quality of the beverage in a future industrial process, relative changes in the concentrations of the key aroma compounds were investigated before and after the fermentation process (Figure 1). Whereas

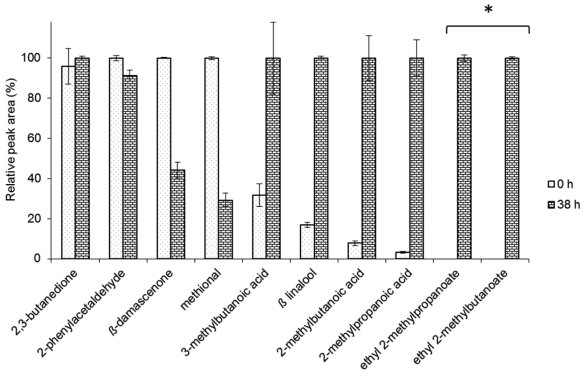


**Figure 1.** Relative changes of the concentrations of the key aroma compounds in the beverage during the fermentation (0 vs 38 h) (*, compounds detected only in the fermented beverage).

most of the odorants were already present in the substrate wort (0 h), the fruity aroma compounds ethyl 2-methylpropanoate and ethyl 2-methylbutanoate were synthesized de novo by *T. versicolor*. The concentrations of β-damascenone and methional were reduced, whereas those of linalool, 2-methylbutanoic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid increased during the fermentation. The degradation of β-damascenone and methional and/or adsorption to the cell wall have been reported for yeasts.[36,37] Linalool with its floral odor is a known constituent of the aroma of fruiting bodies of *T. versicolor* as well as of many other fungi (e.g., *Clitocybe odora*, *Hydnum repandum*, *Lactarius salmonicolor*, *Lepista nuda*, *Mycena rosea*, and *Tricholoma sulfureum*).[38,39] Although basidiomycetes have a huge potential for producing terpenoids, relatively few biosynthetic pathways of fungal terpenoids have been described.[40] The formation of 2-methylbutanoic acid, 3-methylbutanoic acid, and 2-methylpropanoic acid might be related to the transformation of L-isoleucine, L-leucine, or L-valine present in wort.[41] The biotransformation of branched-chain amino acids by shiitake to volatiles has been described recently by Zhang et al.[9]

The odor qualities of ethyl 2-methylpropanoate and ethyl 2-methylbutanoate have been described as "fruity, strawberry"- and "fruity, green apple"-like. To our knowledge, this is the first time that these two ethyl esters have been detected in cultures of *T. versicolor*. Ethyl 2-methylpropanoate has been reported as a flavor compound formed by *Tuber melanosporum*, *Tuber indicum*, *Ceratocystis fimbriata*, yeasts, and fruits.[42−45] Ethyl 2-methylbutanoate was identified in the pine-mushroom (*Tricholoma matsutake* Sing.).[46] Comprehensive information on the pathways and control mechanisms of the biogenesis and accumulation of these two ethyl esters in basidiomycetes is not available yet. However, it is well-known that branched-chain amino acids, branched-chain fatty acids, and branched-chain alcohols serve as precursors of fruity esters in yeasts and fruits (e.g., apple, banana, and melon).[47−50] The relative concentrations of 2-methylpropanoic acid and 2-methylbutanoic acid increased significantly during fermentation.

**Cytotoxicity and Mutagenicity of the Beverage and Nonfermented Wort.** *Cytotoxicity in Rat Hepatocytes in Primary Culture.* Compared to the negative control, neither in the resazurin reduction assay nor in the LDH leakage assay was a statistically significant decrease in metabolic activity and membrane integrity, respectively, observed after treatment of the cells with the beverage or nonfermented wort. The functionality of both assays was validated by negative and positive controls.

*Mutagenicity in the Ames Fluctuation Test.* Neither in *Salmonella typhimurium* strain TA 98 used for screening for frameshift mutations nor in strain TA 100 employed for base pair substitutions were statistically significant mutagenic events detected compared to the negative control after treatment of bacteria suspensions with the beverage or nonfermented wort with or without metabolic activation by S9 mix. The functionality of the assay was validated by negative and positive controls.

In conclusion, a novel fermented beverage was developed by incubation of wort with *T. versicolor*. Due to the fermentation, changes in the concentrations of carbohydrates, proteins, and oxalic acid were observed. Its aroma was perceived as fruity, fresh, sweetish, and floral, which was significantly different from the sourish odor of traditional nonalcoholic fermented cereal beverages. Ten key aroma compounds significantly contributed



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to the overall aroma of the beverage. Most of them originated from wort, but their concentrations changed during the fermentation process. The fruity odorants ethyl 2-methylbutanoate and ethyl 2-methylpropanoate were detectable only in the fermented beverage and thus synthesized by *T. versicolor*. Comprehensive toxicological studies indicated no cytotoxicity and mutagenicity activity.

Taken together, a beverage fermented by *T. versicolor* may present an interesting option for novel cereal-based products. Prior to the final product development, that is, up-scaling and control of the product quality, reconstitution and omission experiments should be performed in further studies.

## ■ ASSOCIATED CONTENT

### Supporting Information

Table S1, preparation of standard mixture solutions; Table S2, parameters of calibration curves for the quantitation of key aroma compounds; Figure S1, cytotoxicity (resazurin assay) in rat hepatocytes in primary culture; and Figure S2, mutagenicity in the Ames fluctuation assay. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.5b02167.

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### Notes

The authors declare no competing financial interest.

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Supporting Information

Table S1. Preparation of Standard Mixture Solutions

| | Compound | Mass (g) | Purity (%) | Ethanol (mL) | Volume of ethanolic solution transferred to the final aqueous solution | Concentration (mg/L water) |
|---|---|---|---|---|---|---|
| standard solution 1 | ethyl 2-methylpropanoate | 0.0378 | 99 | 50 | 100 µL ad 1 L | 0.0748 |
| | ethyl 2-methylbutanoate | 0.0112 | 98 | | | 0.0220 |
| standard solution 2 | 2,3-butanedione | 0.3710 | 97 | 50 | 1 mL ad 1 L | 7.197 |
| | 2,5-dimethylpyrazine | 0.0148 | 98 | | | 0.290 |
| | methional | 0.0609 | 98 | | | 1.194 |
| | benzaldehyde | 0.0435 | 99 | | | 0.861 |
| | linalool | 0.0211 | 97 | | | 0.409 |
| | 2-acetylthiazole | 0.0207 | 99 | | | 0.410 |
| | *β*-damascenone | 0.0645 | 1.2 | | | 0.0155 |
| standard solution 3 | 2-phenylacetaldehyde | 1.4925 | 99 | 50 | 250 µL ad 100 mL | 73.879 |
| internal standard solution | thymol | 0.0444 | 99 | 50 | 1 mL ad 100 mL | 8.791 |

Table S2. Parameters of Calibration Curves for the Quantification of Key Aroma Compounds

| Compound | Slope | Intercept | $s_m$ | $s_b$ | $R^2$ | Calibration range (µg/L) |
|---|---|---|---|---|---|---|
| ethyl 2-methylpropanoate | 14005 | 8915 | 1760 | 3367 | 0.962 | 0.08 - 1.50 |
| 2,3-butanedione | 438 | 12903 | 5 | 3077 | 0.969 | 7.2 - 143.8 |
| ethyl 2-methylbutanoate | 116330 | 16642 | 12634 | 1893 | 0.996 | 0.02 - 0.44 |
| 2,5-dimethylpyrazine | 19045 | 51148 | 1895 | 5687 | 0.992 | 0.29 - 5.86 |
| methional | 394 | 5589 | 73 | 612 | 0.954 | 1.2 - 23.9 |
| benzaldehyde | 298792 | 1425432 | 16829 | 117813 | 0.985 | 0.9 - 17.2 |
| linalool | 113692 | 261518 | 9510 | 20634 | 0.993 | 0.4 - 8.2 |
| 2-phenylacetaldehyde | 3824 | 579658 | 1391 | 118678 | 0.981 | 74 - 1478 |
| 2-acetylthiazole | 8276 | 21990 | 962 | 730 | 0.994 | 0.4 - 8.2 |
| $\beta$-damascenone | 320758 | 40674 | 40132 | 960 | 0.992 | 0.02 - 0.31 |

$s_m$, standard deviation (SD) of slope. $s_b$, SD of intercept.

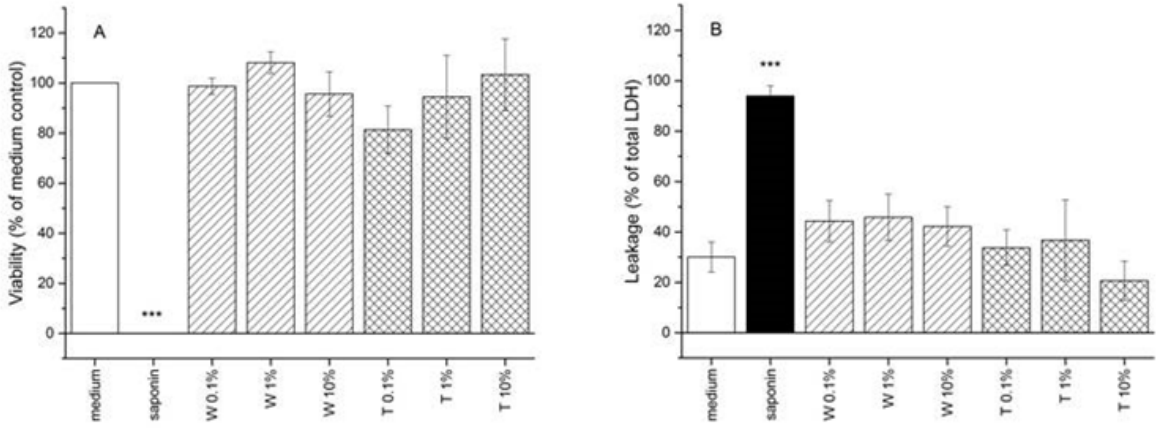


Figure S1. Cytotoxicity (resazurin assay) in rat hepatocytes in primary culture.

W = wort T = *T. versicolor* *** = $p < 0.001$

A: Resazurin assay after 48 h.

B: LDH leakage assay after 6 h.

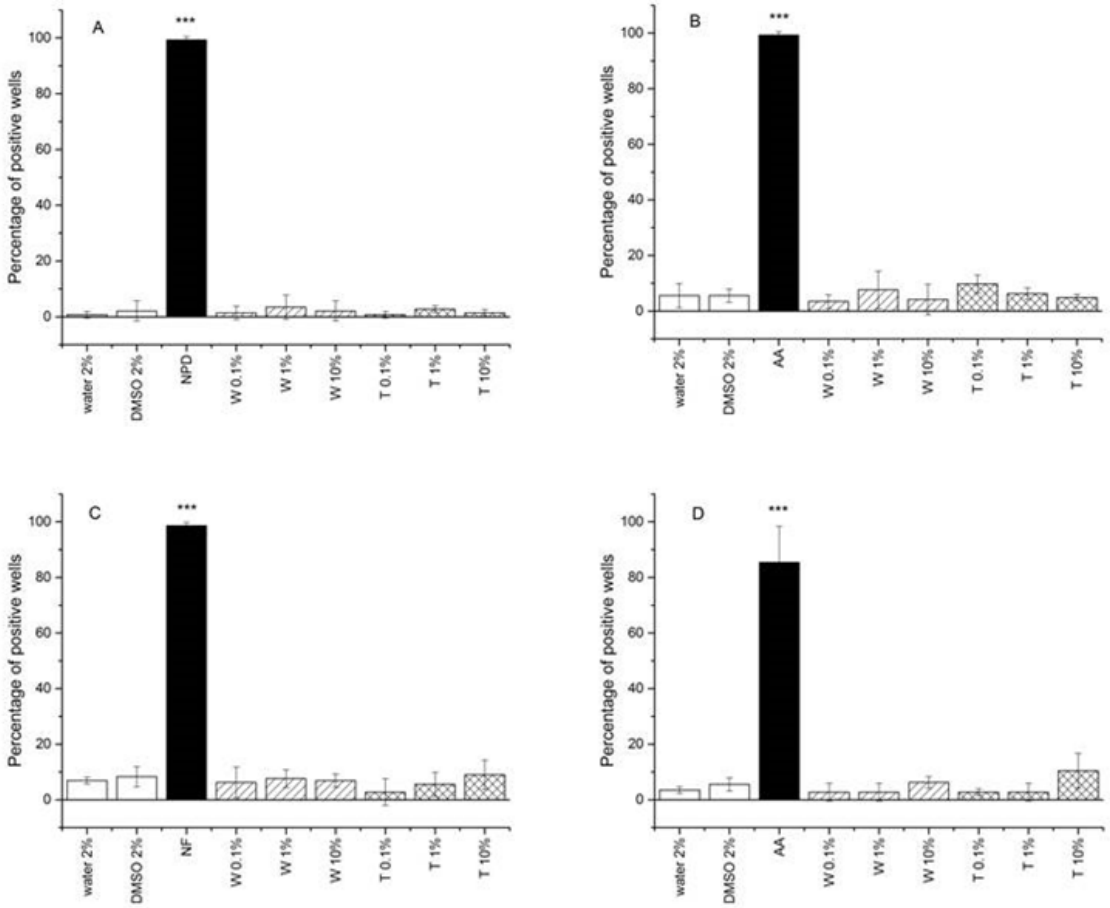


Figure S2. Mutagenicity in the Ames fluctuation assay.

W = wort T = *T. versicolor* *** = $p < 0.001$

A: TA 98 without S9. NPD = 4-nitro-*o*-phenylenediamine (10 µg/mL)

B: TA 98 with S9. AA = 2-aminoanthracene (0.1 µg/mL)

C: TA 100 without S9. NF = nitrofuantoin (250 ng/mL)

D: TA 100 with S9. AA = 2-aminoanthracene (1.6 µg/mL)