Formation of Terpenes by Yeasts during Alcoholic Fermentation

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Bildung von Terpenen durch Hefen während der alkoholischen Gärung

Zusammenfassung. Im Rahmen von Untersuchungen über den Einfluß verschiedener Hefearten und -stämme auf die Bildung von Gärungsnebenprodukten wurde die Biosynthesefähigkeit verschiedener Hefen für Terpene und Terpenderivate überprüft. In Modellgärversuchen bildeten *Saccharomyces rosei, Kloeckera apiculata, Metschnikowia pulcherrina* und *Torulopsis stellata* Spuren der Terpenkohlenwasserstoffe β-Myrcen und Limonen sowie der Terpenalkohole Linalool, α-Terpineol und Farnesol. Bei Verwendung von *S. cerevisiae* konnte lediglich die Bildung von Farnesol nachgewiesen werden. Diese Ergebnisse zeigen, daß das in früheren Arbeiten zur Sortencharakterisierung von Weißweinen herangezogene Terpenmuster verschiedener Traubensorten durch eine nach modernen önologischen Methoden durchgeführte alkoholische Gärung nicht beeinflußt wird.

Summary. In the course of studies about the influence of various yeast species and strains on the formation of fermentation by-products, the ability of yeasts to biosynthesize terpenes and terpene derivatives was investigated. In model fermentations, *Saccharomyces rosei, Kloeckera apiculata, Metschnikowia pulcherrima,* and *Torulopsis stellata* formed trace amounts of the terpene hydrocarbons β-myrcene and limonene as well as the terpene alcohols linalool, α-terpineol, and farnesol. With *S. cerevisiae,* only the formation of traces of farnesol could be established. These results clearly demonstrate that terpene patterns of various grape cultivars used in previous work for varietal classification of white wines are not influenced by yeast fermentation carried out according to modern enological methods.

Introduction

Various terpenes and their oxygenated derivatives, well-known constituents of grape aroma [1, 2], play a decisive role in the chemical-analytical classification of wines made from different white grape cultivars [3–5]. For this classification, the premise had be made that during winemaking these substances pass the step of alcoholic fermentation more or less unchanged; i.e. it has to be postulated that wine yeasts are not able to biosynthesize terpenes.

Drawert and Barton [6] could demonstrate the formation of linalool, geraniol and citronellol by *Kluyveromyces lactis,* but this yeast species does not play any role in winemaking. According to studies of Fagan et al. [7] *Saccharomyces fermentati,* a yeast species used in sherry winemaking, is able to produce linalool, (E,E)-farnesol and two isomers of nerolidol. The authors called in question the method of varietal classification of wines by means of the distribution patterns of a number of grape monoterpenes. Therefore, in the course of our model fermentation studies about the influence of various yeast species and strains on the formation of fermentation by-products [12], we included the investigation about the ability of yeasts to biosynthesize terpenes.

Materials and Methods

Yeasts Species and Strains

All yeast species and strains used in this study were obtained from the collection of the Bayerische Landesanstalt für Weinbau und Gartenbau, Würzburg. *Saccharomyces cerevisiae:* 5936/III/10/A/a1; 60/1/10/a8; 1/16/64/a4; 58/MR/a2; H10-36b; 2/27/11. *S. bayanus:* H10-105; H10-110. *S. uvarum:* 77/8/64/a1; 4/12/64/a3. *S. rosei:* 6126/20/a1; 35/1/63/a2. *Kloeckera apiculata:* H10-74; 74/A3/3a. *Metschnikowia pulcherrima:* 73/A0/2; 73/A1/1. *Torulopsis stellata:* 74/A0/22; 74/A3/22. A small amount of cells was transferred from the original culture to a tube with slant culture; incubation at 25 °C; harvesting after 3 days.

Inoculum Preparation

The harvested cells were suspended in physiological saline solution. After appropriate dilution, the number of cells per ml was counted using a Thoma counting-chamber. The original suspension was diluted with physiological saline solution to 8×10^7 cells/ml.

Composition of the Model Solution

In 12 litres of demineralized water were dissolved: 1848 g of D-glucose monohydrate, 1520 g of D-fructose, 112 g of L-malic acid, 48 g

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of L-tartaric acid, 3.2 g of citric acid, 1 082 mg of L-histidine monohydrochloride, 99 mg of L-lysine monohydrochloride, 11 600 mg of L-arginine monohydrochloride, 1 600 mg of L-aspartic acid, 2 560 mg of L-threonine, 5 600 mg of L-serine, 3 200 mg of L-glutamic acid, 4 000 mg of L-proline, 80 mg of glycine, 3 200 mg of L-alanine, 1 200 mg of L-valine, 800 mg of L-methionine, 2 080 mg of L-isoleucine, 2 800 mg of L-leucine, 320 mg of L-tyrosine and 3 200 mg of L-phenylalanine. Minerals, trace elements and growth promotors were added according to the Wickerham medium [8]. Actual acidity was adjusted to pH 3.4 with 20% aqueous potassium hydroxide solution and the solution was diluted with demineralized water to a final volume of 15 litres.

Fermentation Conditions

1 000 ml-Erlenmeyer flasks, charged with 1 g of powdered cellulose (MN 300 for TLC, Macherey & Nagel) and 50 ml of demineralized water, were sterilized (30 min, 1.5 bar). The flasks were each filled with 750 ml of model solution using 0.2 µm-membrane filters, inoculated with 1 ml of standardized cell suspension (8×10^7 cells) and stoppered with special fermentation seals. Fermentation was carried out at 25 °C (control: end of CO_2 formation; no decrease in weight within 2 days). All operations were performed under strictly sterile conditions. After fermentation, yeast cells were removed by centrifuging at 3 000 rpm.

Isolation of Volatiles and Preseparation

After addition of internal standards (dimethyl methylmalonate, 303 µg/l; 2-methylpentan-1-ol, 240 µg/l; γ-undecalactone, 280 µg/l; 2,2-dimethylpentanoic acid, 255 µg/l), liquid-liquid extraction of fermented solutions was carried out using a pentane-dichloromethane mixture (2+1) as described [9]. Acids were removed from the extracts by separation with 5% aqueous $NaHCO_3$ solution, and neutral volatiles were carefully concentrated to 2 ml using a Vigreux column. The concentrates were preseparated into fractions of different polarities by adsorption chromatography on silica gel with a pentane-diethylether gradient (60 ml/h) [10]. Fraction I: 200 ml of pentane; fraction II: 200 ml of pentane+ether (9+1), fraction III: 200 ml of ether. The eluates were carefully concentrated (Vigreux column) to 0.1 ml for HRGC and HRGC-MS analysis.

Capillary Gas Chromatography (HRGC)

Instrument: Carlo Erba Fractovap 4160 fitted with FID and an air-cooled on-column injector. Column: Chrompack CP Wax 57 WSCOT (25 m × 0.33 mm i.d.) (df = 1.2 µm), connected with an uncoated fused silica column 2 m × 0.33 mm (retention gap) [11]. On-column-injection. Temperatur program: 50–250 °C, 5°/min. Carrier gas, He (2 ml/min). Make-up gas, N_2 (30 ml/min). Detector gases, H_2 (30 ml/min); air (300 ml/min). Detector temperature, 220 °C. Sample volumes: 0.5 µl. Data evaluation was carried out using a HP 3388 A laboratory data system.

Capillary Gas Chromatography – Mass Spectrometry (HRGC-MS)

Instrument: Finnigan MAT 44 mass spectrometer coupled by an open-split interface with a Varian Aerograph 1 440 (water-cooled on-column injector). Column: Chrompack CP Wax 57 WSCOT (25 m × 0.33 mm i.d.) (df = 1.2 µm), connected with an uncoated fused silica column 2 m × 0.33 mm (retention gap) [11]. Carrier gas, He (2.3 ml/min). Temperature programme: 60 °C, 2 min isothermal; 60–250 °C, 5°/min. Ionisation: EI, 70 eV. Cathode current: 0.8 mA. Source pressure: 15–20 nbar. Mass range 41-250.

Results and Discussion

Six different strains of *Saccharomyces cerevisiae* and two strains of *S. bayanus, S. uvarum, S. rosei, Kloeckera apiculata, Metschnikowia pulcherrima,* and *Torulopsis stellata,* respectively, were used in a series of model fermentations. Table 1 summarizes the terpene hydrocarbons and alcohols formed in traces (< 10 µg/l) by the activity of the particular yeast species under the specified experimental conditions. The substances were identified by comparing relative gas chromatographic retention and mass-spectrometrical data with those of authentic reference samples. At least one of the isomer farnesols was formed by all strains of *S. cerevisiae* and *S. bayanus* studied in this work as well as by the so-called "wild yeasts", i.e. *S. rosei, K. apiculata, M. pulcherrima,* and *T. stellata,* respectively. *S. uvarum* did not form any of the farnesols. The monoterpenes β-myrcene, limonene, linalool, and α-terpineol could be detected in those model solutions, which were fermented with *S. rosei, M. pulcherrima, K. apiculata* and *T. stellata* yeast strains.

Table 1. Terpene hydrocarbons and alcohols formed in traces (< 10 µg/l) by yeasts in model fermentations and identified by gas chromatography – mass spectrometry in a concentrated extract of fermentation by-products

Compound	Linear ret. index (known)	Linear ret. indices (unknown)						
		S. cer. (6 st.)	*S. bay.* (2 st.)	*S. uv.* (2 st.)	*S. ros.* (2 st.)	*K. ap.* (2 st.)	*M. pu.* (2 st.)	*T. st.* (2 st.)
β-Myrcene	1 174	n.d.	n.d.	n.d.	1 174	1 175	1 176	n.d.
Limonene	1 222	n.d.	n.d.	n.d.	n.d.	1 230	1 230	n.d.
Linalool	1 565	n.d.	n.d.	n.d.	1 570	1 563	1 563	1 572
α-Terpineol	1 744	n.d.	n.d.	n.d.	n.d.	1 740	1 745	n.d.
Farnesol (isomer 1)	2 340	2 346	2 340	n.d.	n.d.	2 350	n.d.	n.d.
Farnesol (isomer 2)	2 414	n.d.	2 405	n.d.	2 410	n.d.	2 420	n.d.
Farnesol (isomer 3)	2 470	n.d.	n.d.	n.d.	n.d.	2 470	n.d.	2 470

n.d. = not detectable; st. = strains; ret. = retention on CP Wax 57; S. cer. = *Saccharomyces cerevisiae*; *S. bay.* = *Saccharomyces bayanus*; *S. uv.* = *Saccharomyces uvarum*; *S. ros.* = *Saccharomyces rosei*; *K. ap.* = *Kloeckera apiculata*; *M. pu.* = *Metschnikowia pulcherrima*; *T. st.* = *Torulopsis stellata*

From a quantitative point of view, these yeasts are dominant in the natural microflora of grape musts [13, 14]. In modern winemaking technology, however, musts are clarified by separators prior to fermentation and re-inoculated with *S. cerevisiae* pure-culture yeasts. Thus, the amount of "wild yeasts" in the fermenting must is drastically reduced and, consequently, the chemical composition of the finished wine cannot be decisively influenced by substances resulting from metabolic activity of these microorganisms.

The results outlined in Table 1 clearly demonstrate that the analytical method to classify white wines by using the distribution pattern of selected grape monoterpenes was based on correct premises: *S. cerevisiae*, the yeast species almost exclusively employed in modern winemaking technology, does not produce any terpene or terpene derivative used for the above mentioned varietal classification [3–5].

Little is known about the metabolic pathways leading to the biosynthesis of terpenes by microorganisms, especially by yeasts. In a study with the fungus *Ceratocystis moniliformis*, using ^{14}C-labelled mevalonic acid, L-leucine and acetate as precursors, Lanza and Palmer [15] pointed out that in this biological system, in analogy to the pathway in higher plants, terpenes were biosynthesized via the well-known mevalonic acid pathway. It can be supposed that this way is the equivalent principle for higher plants as well as for microorganisms. Experimental evidence for the organisms used in this study, however, is still lacking.

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