JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Symposium Introduction

Cite This: J. Agric. Food Chem. 2019, 67, 13363−13366

pubs.acs.org/JAFC

Bioflavour Conference 2018—Biotechnology for Flavors, Fragrances, and Functional Ingredients

ABSTRACT: The "Bioflavour 2018—Biotechnology of Flavors, Fragrances, and Functional Ingredients" conference was held from September 18th to 21st, 2018 at the DECHEMA house in Frankfurt, Germany. The conference attracted more than 190 participants from over 25 countries, with about 40% share from industry. Particular sessions of Bioflavour 2018 focused on "flavor perception and biotechnology", "microbial cell factories", "novel pathways, enzymes, and biocatalysts", "technological and regulatory aspects of flavor and fragrance biotechnology", "advanced analytics and novel compounds", "plant biosynthesis and plant enzymes", "modern biotechnology in the world of wine", "receptors, flavors, and bioactives", and "bioprocess development and downstream processing". At Bioflavour 2018, both cutting-edge science from renowned academic research groups and current innovation from this modern biotechnology industry were presented and discussed. This special issue highlights a selection of 12 manuscripts from oral presentations and poster contributions.

HISTORY AND REVIVAL OF BIOFLAVOUR CONFERENCE

The term "bioflavour" was coined for the first time by Peter Schreier, who organized the first Bioflavour conference in 1987 in Würzburg, Germany.[1] The motivation in academia and industry during these days was primarily to investigate the potential of enzymes and microbes for the production of natural flavor compounds. Another driver was the steadily improving analytical toolbox, which helped to better understand the biosynthesis of flavors and fragrances and to characterize complex aroma compositions from diverse natural resources. The last Bioflavour conference in the pre-2000s era was held in Dijon, France, in 1995 organized by INRA and focused on "analysis−precursor studies−biotechnology".[2] However, the renowned Wartburg symposia organized by Peter Schieberle and colleagues and the Weurman Symposia constantly offered bioflavour sessions in the "interim period" that allowed the bioflavour community to stay in touch for a vivid scientific exchange, which ultimately led to the revival of a separate Bioflavour conference in 2015. The reason for this "gap" might be a misunderstanding in the late 1990s that the field of flavor and fragrance synthesis by microbes cultivated in the presence of natural raw materials or straightforward enzymatic bioconversion of natural precursors had been comprehensively investigated. Likewise, the production of structurally more demanding targets, such as sesquiterpenes or phenylpropanoids, from simple sugars by use of engineered designer microbes seemed to be far out of reach to ever become competitive to chemical synthesis in the near future. With the advent of modern biotechnology in the early 21st century, the time seemed ready for a revival of the Bioflavour conference series. Rational metabolic engineering, synthetic, and systems biology to tailor biological cells had become reality. Consequently, "Bioflavour—International Conference on Biotechnology of Flavors and Fragrances" was revived in 2015, organized by the DECHEMA Society of Chemical Engineering and Biotechnology together with the DECHEMA Research Institute in Frankfurt, Germany, supported through members of the former European Union (EU) European Cooperation in Science and Technology (COST) Action FA0907 "Bioflavour—Yeast Flavour Production".

The 2015 conference attracted more than 150 participants from over 20 countries worldwide, illustrating the renewed interest in this field of research. About 40% of the attendees came from industry, which was a clear indicator for the steadily increasing relevance of biotechnology in the flavor and fragrance business. With this success, the organizers decided to make Bioflavour a conference series again, which should take place every third year in Frankfurt, Germany. Thus, Bioflavour complements the two highly renowned conferences in aroma sciences, the Weurman and Wartburg Symposia, which themselves are triennial conferences as well.

BIOFLAVOUR 2018

With more than 190 participants from over 25 countries, again with about 40% share from industry, the 2018 conference even surpassed the success of the first Bioflavour in 2015. The conference scope was expanded and also included the biotechnology of functional ingredients. By this means, the steadily increasing importance of functional ingredients for human nutrition and the impact of biotechnology for their production were acknowledged. Furthermore, additional bioactivities of single flavor and fragrance compounds and related natural products together with their often complex chemical structures make their syntheses the perfect "playground" of industrial biotechnology. The 2018 conference became a showcase of the progress made by modern biotechnology, covering a broad spectrum from elucidation and design of biosynthetic pathways to the investigation of molecular mechanisms and biological functions of receptor−ligand interactions in olfaction and taste reception to fermentation and downstream processing. The session titles of Bioflavour 2018 were "flavor perception and biotechnology", "microbial cell factories I, II, and III", "novel pathways, enzymes, and biocatalysts I and II", "technological and regulatory aspects of flavor and fragrance biotechnology", "advanced analytics and novel compounds", "plant biosynthesis and plant enzymes", "modern biotechnology in the world of wine", "receptors, flavors, and bioactives", and "bioprocess

Special Issue: Advances in Bioflavor Research

Published: September 27, 2019

DOI: 10.1021/acs.jafc.9b05192
J. Agric. Food Chem. 2019, 67, 13363−13366

development and downstream processing". At the 2018 conference, not only was cutting-edge science from renowned academic research groups presented but also the current transformation in industry fueled by modern biotechnology. Small enterprises illustrated their proprietary high-throughput technologies for fast strain and bioprocess development; likewise, big aroma houses revealed their view on biotechnology as an increasingly important part of their business toward sustainable processes and to match the demand by consumers for natural products. Thus, the conference was again a perfect place to be for experiencing top science together with an inspiring networking between academia and industry in the field of aroma biotechnology.

The present issue of the American Chemical Society (ACS) *Journal of Agricultural and Food Chemistry* compiles some key topics presented at "Bioflavour 2018—International Conference on Biotechnology of Flavours, Fragrances, and Functional Ingredients" held from September 18 to 21, 2018 in Frankfurt/Main, Germany. Selected authors were asked to submit manuscripts of original work related to their presentations at the conference, which were subsequently peer-reviewed according to the rules of the *Journal of Agricultural and Food Chemistry*. In the following, these papers are briefly introduced within the framework of biotechnology of flavors, fragrances, and functional ingredients.

ELUCIDATION OF FLAVOR BIOSYNTHESIS AND NOVEL PRODUCTS

Surprisingly, a number of aroma-active bicyclic monoterpenoid compounds, including dill ether and wine lactone, have been identified in submerged cultures of the white-rot fungus *Pleurotus sapidus*.[3] Elucidation of the stereochemistry of these compounds by enantioselective-multidimensional gas chromatography (GC) revealed that the fungus forms the rare (3*R*,3a*R*,7a*S*) and (3*S*,3a*R*,7a*S*) stereoisomers of dill ether and wine lactone. These stereoisomers have not been described as constituents of dill and wine, respectively. As indicated by the integration of ^{13}C from 2-^{13}C-labeled D-glucose, the target compounds are formed *de novo* by the fungus. Further supplementation studies with stable isotope-labeled compounds proved limonene and *p*-menth-1-en-9-ol as intermediate metabolites in the fungal biosynthetic pathways.

The key step of the biosynthesis of typical abietane diterpenes (ADs), such as carnosol and carnosic acid, in rosemary and sage (belonging to Lamiaceae) is the oxidation of the precursors at different positions by cytochrome P450 oxidases of the CYP76 family. Bathe et al. reconstituted a CYP76 oxidation network from sage and rosemary to synthesize different abietane diterpenoids using a yeast expression system as an engineering platform.[4] A core module (to produce the diterpene precursors miltiradiene/abietatriene for the downstream CYP activities) was coupled with CYP76AHs and CYP76AK to produce 16 different diterpenoids. A total of 14 of them resulted from the oxidative activity of the expressed CYPs and subsequent spontaneous conversions, and 8 of them had not been reported before. These results expand the portfolio of naturally occurring ADs beyond carnosaldehyde, carnosic acid, carnosol, carnosic acid quinone, pisiferal, and pisiferic acid to a total of 20 compounds.

Green leaf volatiles (GLVs) are aroma-active C_6 or C_9 aldehydes, alcohols, and esters emitting fresh odors reminiscent of cut grass, fruits, and vegetables. The two major pathway enzymes responsible for their formation are lipoxygenases (LOXs) and hydroperoxide lyases (HPLs). While LOXs oxidize polyunsaturated fatty acids, such as linoleic and α-linolenic acids, to the corresponding fatty acid hydroperoxides, HPLs convert the latter into aldehydes and oxo-acids. The available literature on the classification, phylogeny, reaction mechanisms, and three-dimensional structures of these two enzyme classes have been comprehensively reviewed by Stolterfoht et al.[5] This is followed by a critical discussion on the potential of microorganisms, especially yeasts, as whole cell biocatalysts to develop more efficient processes for the production of GLVs in comparison to thus far used plant extracts.

MICROBIAL CELL FACTORIES AND ENZYME CASCADES

Microbial cell factories are increasingly attracting the interest of the aroma industry because designer host strains, such as engineered bacteria and yeasts, can provide an alternative sustainable resource for desired flavor and fragrance compounds. Many aroma molecules, such as those bearing chiral centers or having larger structures, such as sesqui- and diterpenoids or phenylpropanoids, are not easily produced by classical chemical synthesis. Here, biosynthesis by engineered microbes holds much promise to be an economically viable alternative. This is even more obvious for targets derived from different natural product classes, such as prenylated flavonoids, where terpene and flavonoid biosyntheses meet and which are very interesting bioactive natural products useful as antioxidants, antimicrobials, or phytohormones, among others. Here, the paper by Levisson et al. shows a promising proof-of-concept using recombinant yeast cells.[6] With the introduction of a flavonoid prenyltransferase SfFPT from *Sophora flavescens* in a yeast harboring a heterologous pathway for naringenin biosynthesis, the authors were able to produce 0.12 mg/L 8-prenylnaringenin, a phytoestrogen found in few plants in relatively low concentrations. At the same time, the yeast strain produced about 100 mg/L naringenin as the immediate flavonoid precursor, indicating a far higher product yield potential of the producer strain. The authors suggest the combination of different strategies, e.g., downregulation of the endogenous FPP synthase, deregulation of the hydroxymethylglutaryl-CoA reductase (HMGR), and cellular co-localization of the collaborating naringenin and terpene biosynthesis enzymes with prenyltransferase. A complementary approach is described by Zhang et al. in their paper in this issue.[7] They investigated the *in vitro* biosynthesis of naringenin from *p*-coumaric acid using heterologously expressed 4-coumaroyl-CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI). The authors found 4CL and CHS to be the critical enzymes of the reaction resulting in an optimized *in vitro* enzyme cascade with a loading ratio 4CL/CHS/CHI of 10:10:1. Additionally, the co-substrate of CHS, malonyl-CoA, was identified as the limiting factor, as identified previously during fermentations. These findings of *in vivo* and *in vitro* investigations of flavonoid and prenylflavonoid biosynthesis will certainly be of mutual benefit and further push biotechnological production in this field.

The third paper presented in this chapter deals with the production of the sought-after sweet and violet-like aroma compound α-ionone in a recombinant *Escherichia coli* strain. Chen et al. addressed typical problems limiting the yields of synthetic pathways based on heterologous enzymes, namely, substrate promiscuity of key enzymes, leading to unwanted

DOI: 10.1021/acs.jafc.9b05192
J. Agric. Food Chem. 2019, 67, 13363−13366

byproducts and the loss of precursors as a result of competing side reactions in the host.[8] The key enzyme of α-ionone synthesis is the carotenoid cleavage dioxygenase 1 (CCD1) from *Osmanthus fragrans*. Enzyme engineering by site-directed mutagenesis together with substrate channeling by creating a fusion of CCD1 and the enzyme catalyzing the previous reaction step, the lycopene ε-cyclase from *Lactuca sativa*, led to a 2.5-fold improvement of the α-ionone concentration. In combination with further metabolic engineering measures, such as ribosome binding site optimization, a specific yield of ∼3.5 mg L^{-1} OD^{-1} of ∼100% enantiopure α-ionone was achieved. This would correlate with product concentrations in the g/L range if typical *E. coli* high-cell density cultivations would be performed, which makes the microbial cell factory approach for α-ionone production an economically attractive scenario.

ADVANCED ANALYTICS FOR NATURAL PRODUCT IDENTIFICATION AND AUTHENTICATION

To evaluate the contribution of individual aroma compounds to the overall flavor of a food, aroma extract dilution analysis is commonly performed. Unfortunately, especially highly volatile compounds may be lost during the extraction process and/or the concentration of the organic extract.[9] Therefore, a novel concept of flavor dilution analysis using headspace solid-phase microextraction (HS-SPME) and stir bar sorptive extraction (SBSE) has been employed by Rigling et al. for the analysis of the aroma of Chios mastic gum (*Pistacia lentiscus* var. *Chia*).[10] In this concept, aroma dilution is achieved by increasing the GC inlet and cold injection system split ratios, respectively. High aroma dilution factors of ≥256 were observed for perillene, β-linalool, and α-pinene. The chiral compounds β-linalool and α-pinene were present in Chios mastic gum in high enantiomeric excess ratios of >91 and >99%, respectively. Quantitation of the key aroma compounds by multiple HS-SPME (using four consecutive extractions of the headspace) allowed for calculation of aroma values, and recombination and omission experiments finally proved the major contribution of the five aroma compounds β-myrcene, limonene, perillene, β-linalool, and α-pinene to the overall aroma of Chios mastic gum.

Because natural flavors typically achieve far higher market prices compared to their synthetic counterparts, robust and efficient analytical methods are required to identify potential adulterations with petrochemical-derived nature-identical compounds. In a case study on garlic oil, Ramos et al. employed gas chromatography−mass spectrometry (GC−MS) analyses, isotope ratio mass spectrometry (IRMS), and accelerator mass spectrometry carbon-14 analyses for the authentication of several commercial garlic oil samples.[11] Especially the results on the percentage of bio-based carbon as a fraction of the total carbon (determined by carbon-14 analyses) impressively demonstrated the high degree of food fraud in the field of natural aroma extracts. Three of four commercial samples analyzed were shown to be adulterated, although all of the samples had been labeled as "100% natural".

An interesting investigation on the spatial distribution of bioactive secondary metabolites in plant materials has been presented by Bednarz et al.[12] They employed matrix-assisted laser desorption/ionization−mass spectrometry imaging (MALDI−MSI) for the analysis of, among others, soladulcine, solasodine, tomatidine, and tomatidenol together with their corresponding glycoalkaloids in a variety of nightshades. The localization of the alkaloids was found to be tissue-specific and depended strongly upon the ripeness and developmental stage of the plants. These data may be extremely helpful for the construction of cDNA libraries and, thus, the identification of the enzymes involved in the biosynthetic pathways toward glycosylated alkaloids.

Wines have a highly complex chemical composition, which is subject to continuous changes after winemaking and bottling until consumption. 1,1,6-Trimethyl-1,2-dihydronapthalene (TDN) is an off-flavor, giving wines a petrol or kerosene note. It is formed especially in Riesling wines over years of bottle-aging. It would thus be beneficial for producers and consumers to predict the potential of TDN synthesis. Unfortunately, an accurate prediction is difficult as a result of differences in chemical reactions under harsh hydrolytic conditions and biological pathways during bottle-aging over long periods. In their paper about "Understanding Yeast Impact on 1,1,6-Trimethyl-1,2-dihydronaphthalene Formation in Riesling Wine through a Formation-Pathway-Informed Hydrolytic Assay", Grebneva et al. optimized the conditions of a "total" TDN hydrolytic assay with respect to yeast-derived formation pathways in a first step by applying a Box−Behnken design experiment.[13] Validation of the developed assay was performed with grape juices, young wines, and bottle-aged wines of Australian and German origin. Additionally, the impact of commercial yeasts as well as novel hybrid strains on total TDN concentrations was evaluated. This allowed for the assessment of yeast-derived changes in metabolite formation in the context of light exposure, a known driver for TDN formation in wine. The yeast-derived differences in total TDN in German Riesling wine as observed under the assay conditions were negligible when compared to the consequences of defoliation-based vineyard management practices.

BIOPROCESSING

Considering the huge amounts of culture broth arising from industrial fermentation processes, reutilization of these side streams for extraction of natural flavor compounds is an alluring idea. Riboflavin is industrially produced by fermentation processes with the fungus *Ashbya gossypii*, and the culture broth emits (after removal of the riboflavin-containing mycelium) an appealing nutty, floral, and fruity smell.[14] A number of intensely smelling saturated and unsaturated lactones along with some Ehrlich pathway products were identified to shape the overall flavor of the culture supernatant. An aroma extract dilution analysis proved 2-phenylethan-1-ol and γ-(Z)-dodec-6-enlactone to be the main contributors. About 170 mg/L of six different lactones were accumulated in the culture broth in total. To elucidate the positions of the double bonds of the unsaturated lactones, the authors introduced an interesting photochemical method into the field of flavor analysis. Paternò−Büchi functionalization was performed prior to tandem mass spectrometric analysis, and collision-induced dissociation experiments and evaluation of the fragmentation patterns allowed for identification of γ-dec-7-enlactone, γ-dec-5-enlactone, and γ-dodec-6-enlactone. Additional nuclear magnetic resonance (NMR) and multidimensional gas chromatographic analyses allowed for an unambiguous assignment of the configuration of the double bonds and the enantiomeric excess values.

α-Ionone, a key flavor compound of raspberries and blackberries, represents a highly sought-after natural flavor compound. As discussed above, it is formed via CCD-catalyzed

DOI: 10.1021/acs.jafc.9b05192
J. Agric. Food Chem. 2019, 67, 13363−13366

oxidative cleavage of the precursor carotenoids α-carotene or ε-carotene. However, only very low amounts are synthesized in the plants, and extraction is not commercially viable. Lukin et al. report on a novel production process for enantiopure (*R*)-α-ionone on a 10 000 L scale based on the engineered *E. coli* strain EcPHY-G81.[15] The authors developed a recovery process comprising a solid–liquid extraction step from the biomass and an additional adsorption step from the aqueous supernatant, followed by desorption with *n*-hexane. By combination of the optimized production of enantiopure (*R*)-α-ionone and yield-maximized recovery from the biomass and culture supernatant, a purity of >95% of the target compound was achieved.

SHORT OUTLOOK: THE FUTURE OF "BIOFLAVOUR"

The concept of joining bioflavors with further functional food ingredients in a holistic conference has attracted excellent international scientists from academia and industry and created a climate of vivid discussions and exchange. Especially the close interaction between natural scientists and engineers was of mutual benefit and turned the conference into a real think tank. Motivated by the successful 2018 conference and the positive feedback from many participants, planning for "Bioflavour 2021" has already been started. The conference will take place from September 28th to October 1st, 2021 in Frankfurt, Germany, and we are looking forward to meeting you there!

Jens Schrader

Industrial Biotechnology, DECHEMA Research Institute, Theodor-Heuss-Allee 25, 60486 Frankfurt am Main, Germany

Holger Zorn*

Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Heinrich-Buff-Ring 17, 35392 Giessen, Germany

Fraunhofer Institute for Molecular Biology and Applied Ecology, Winchesterstraße 2, 35394 Gießen, Germany

Christian von Wallbrunn

Institute for Microbiology and Biochemistry, Hochschule Geisenheim University, Von-Lade-Straße 1, 65366 Geisenheim, Germany

AUTHOR INFORMATION

ORCID

Jens Schrader: 0000-0003-2235-6553

Holger Zorn: 0000-0002-8383-8196

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Drawert, F. Bioflavour—What does it mean? In *Bioflavour'87—Analysis—Biochemistry—Biotechnology*; Schreier, P., Ed.; Walter de Gruyter: Berlin, Germany, 1988.

(2) *Bioflavour'95*; Etievant, P., Schreier, P., Eds.; INRA Edition: Paris, France, 1995.

(3) Trapp, T.; Kirchner, T.; Birk, F.; Fraatz, M. A.; Zorn, H. Biosynthesis of Stereoisomers of Dill Ether and Wine Lactone by *Pleurotus sapidus*. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.8b07263.

(4) Bathe, U.; Frolov, A.; Porzel, A.; Tissier, A. CYP76 Oxidation Network of Abietane Diterpenes in Lamiaceae Reconstituted in Yeast. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b00714.

(5) Stolterfoht, H.; Rinnofner, C.; Pichler, H. Recombinant Lipoxygenases and Hydroperoxide Lyases for the Synthesis of Green Leaf Volatiles. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b02690.

(6) Levisson, M.; Araya-Cloutier, C.; de Bruijn, W. J. C.; van der Heide, M.; Salvador López, J. M.; Daran, J.-M.; Vincken, J.-P.; Beekwilder, J. Toward Developing a Yeast Cell Factory for the Production of Prenylated Flavonoids. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b01367.

(7) Zang, Y.; Zha, J.; Wu, X.; Zheng, Z.; Ouyang, J.; Koffas, M. A. G. *In Vitro* Naringenin Biosynthesis from *p*-Coumaric Acid Using Recombinant Enzymes. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b00413.

(8) Chen, X.; Shukal, S.; Zhang, C. Integrating Enzyme and Metabolic Engineering Tools for Enhanced Ionone Production. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b00860.

(9) Zhang, Y.; Fraatz, M. A.; Horlamus, F.; Quitmann, H.; Zorn, H. Identification of potent odorants in a novel nonalcoholic beverage produced by fermentation of wort with shiitake (*Lentinula edodes*). *J. Agric. Food Chem.* **2014**, *62* (18), 4195–4203.

(10) Rigling, M.; Fraatz, M. A.; Trögel, S.; Sun, J.; Zorn, H.; Zhang, Y. Aroma Investigation of Chios Mastic Gum (*Pistacia lentiscus* Variety *Chia*) Using Headspace Gas Chromatography Combined with Olfactory Detection and Chiral Analysis. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b00143.

(11) Gershon, H.; Lykkeberg, A.; Goren, F.; Mason, S. Identifying Fraudulent Natural Products: A Perspective on the Application of Carbon-14 Analysis. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b01821.

(12) Bednarz, H.; Roloff, N.; Niehaus, K. Mass Spectrometry Imaging of the Spatial and Temporal Localization of Alkaloids in Nightshades. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b01155.

(13) Grebneva, Y.; Bellon, J. R.; Herderich, M. J.; Rauhut, D.; Stoll, M.; Hixson, J. L. Understanding Yeast Impact on 1,1,6-Trimethyl-1,2-dihydronaphthalene Formation in Riesling Wine through a Formation-Pathway-Informed Hydrolytic Assay. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b03228.

(14) Birk, F.; Fraatz, M. A.; Esch, P.; Heiles, S.; Pelzer, R.; Zorn, H. Industrial Riboflavin Fermentation Broths Represent a Diverse Source of Natural Saturated and Unsaturated Lactones. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.9b01154.

(15) Lukin, I.; Jach, G.; Wingartz, I.; Welters, P.; Schembecker, G. Recovery of Natural α-Ionone from Fermentation Broth. *J. Agric. Food Chem.* **2019**, DOI: 10.1021/acs.jafc.8b07270.

DOI: 10.1021/acs.jafc.9b05192
J. Agric. Food Chem. 2019, 67, 13363–13366