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Identification of Odorous Compounds from Nine Fermentor-Cultivated *Streptomyces* Strains

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ABSTRACT. The chemical composition was determined of odors produced by nine strains of streptomycetes (*Streptomyces aureofaciens*, *S. avermitilis*, *S. cinnamonensis*, *S. coelicolor*, *S. griseus*, *S. lividans*, *S. rimosus*, *S. spectabilis*, *S. virginiae*) cultivated in a fermentor under similar cultivation conditions. GC–MS analysis identified more than twenty noteworthy volatile chemical individuals. The main components of the odor spectrum were geosmin and unique homologues of oxolones (dihydrofuranones), minor compounds included, *e.g*., pyrazine derivatives, acetoin and its homologues, aromatic esters, furan derivatives.

The production of important biologically active compounds by actinomycetes (Shapiro 1989; Běhal 2000) is usually followed by the formation of volatile substances with strong odor. The odors of the soil were subjected to scientific scrutiny for the first time in the 19th century (Berthelot and André 1891). Among the number of soil microorganisms, actinomycetes growing in a pure surface or submerged culture are characterized by the production of a strong and earthy odor. Gerber and Lechevalier (1965) and Gerber (1968) obtained diethyl ether-soluble extracts from actinomycetes that contained a highly concentrated odor, which, after dilution, became earthy-smelling. Rosen *et al.* (1968) described odor production by the odoriferous strain *Streptomyces griseoluteus*. Dougherty *et al*. (1966) studied the volatile metabolites of actinomycetes by NMR and MS spectrometry. The partial chemical structures of the odor components that they obtained (five- or six-membered lactones) were later revised (Gerber 1973). Similar compounds, *i.e*. five- to seven-membered lactones with a branched side chain, but of a slightly different structure, *e.g*., 10-methylundec-2-en-4-olide, 10-methyldodec-2-en-4-olide, 10-methylundec-3-en-4-olide, and 10-methyldodec-3-en-4-olide, have recently been identified in a North Sea *Streptomyces* (Dickschat *et al*. 2005) while a marine streptomycete was found to contain (*R*)-10-methyl-6-undecanolide and (6*R*,10*S*)-10-methyl-6-dodecanolide (Stritzke *et al*. 2004). Six new compounds were also identified in the volatile fractions produced during the submerged cultivation of *S. avermitilis*. By using the GC–MS and other chemical-physical methods, these compounds were determined to be 5(*S*)-methyl-3(*S*)-(methylalkyl)furan-(5*H*)-2-ones. This study thus documented for the first time the existence of volatile lactones with an *anteiso* structure of the side chain (Řezanka and Sigler 2006). Banskota *et al*. (2006) reported on three new 5-alkenyl-3,3(2*H*)-furanones from two *Streptomyces* species.

The most common off-flavor compound and the main source of soil odor produced by microorganisms is geosmin. It is a well-known and widely studied compound (Rosen *et al.* 1968) whose presence in water is recognized by the characteristic earthy smell even at concentrations of 1.3 nmol/L; the smelling water is difficult to drink (*e.g*., fish living in geosmin-contaminated water taste also of mud). Papers published on this topic (Aoyama *et al*. 1991; Bowmer *et al*. 1992) emphasized that geosmin is a main odor component produced by streptomycetes. Enantioselective multidimensional gas chromatography identified a single geosmin enantiomer in pure cultures of *Streptomyces* sp. strain isolated from rotten grapes and revealed that the chiral compound geosmin is in the (–)-form much more odoriferous than in the (+)-one (Darriet *et al*. 2001). Geosmin production was also studied in terms of its genetic determinants (Saadoun and El-Migdadi 1998) and it was found to inhibit plant seed germination (Ogura *et al*. 2000).

As part of an extensive research of physiology of different *Streptomyces* strains, producers of important biologically active compounds, we studied the effect of the type of cultivation on the total odor composition in selected streptomycetes. At present, a total of 67 compounds comprising monoterpenes, hydrocarbons, oxygenated compounds and aromatics have been identified as odor components in batch cultures of different species of *Streptomyces* (Řezanka *et al*. 1994; Řezanka and Votruba 1998*a*,*b*; Jáchymová *et al.* 2002).

The objective of the present study was to analyze the odor substances in pilot plant-fermentor cultures and to evaluate this broad-field analysis.

MATERIALS AND METHODS

Organisms. Nine *Streptomyces* strains from the collection of the *Institute of Microbiology* (Acad. Sci. Czech Rep., Prague) and other collections, *viz*. *S. aureofaciens* Attc 12551, *S. avermitilis* C-18/6, *S. cinnamonensis* C100-5, *S. coelicolor* 165, *S. griseus* IPB 101, *S. lividans* ATTC 69641, *S. rimosus* ATCC 13224, *S. spectabilis* NRRL 2494, *S. virginiae* ATCC 13161 were examined for production of odors.

Cultivation. The strains were cultivated for 1 d in a complex medium containing (%): soybean meal 1.5, yeast extract 0.5, glucose 0.5; fermentation flasks were inoculated with 2 % of the inoculum (1-d old) and cultivated for 2 d. The following synthetic production medium (%) was used for cultivation: glucose 3, $(NH_4)_2SO_4$ 0.2, $CaCO_3$ 0.5, NaCl 0.2, K_2HPO_4 0.05, $MgSO_4 \cdot 7H_2O$ 0.01, $FeSO_4 \cdot 7H_2O$ 0.005, $MnSO_4 \cdot 7H_2O$ 0.005, $ZnSO_4 \cdot 7H_2O$ 0.005. The cultivation was performed in 500-mL round-bottom flasks on a rotary shaker (2.8 Hz) at 28 °C. The contents of two flasks were then transferred into a mechanically stirred fermentor, *i.e*. 7-L MBR *BioReactor A.G*. (Switzerland). The cultivation was performed at the same temperature, aeration rate 0.6 VVM and impeller tip speed 0.5–0.6 m/s. The fermentor was stirred by two similar six-blade Rushton turbines and the vessels had four baffles. The fermentor was equipped with an efficient reflux condenser of volatile compounds contained in outlet air.

Analytical methods. The volatile compounds in liquid medium were determined after steam distillation and extraction by capillary GC–MS (*Jeol* DX 303). The peaks obtained were identified, based on the characteristic splitting in the mass spectrum and/or the spectrum library. The individual volatile, malodorous compounds were quantified by using the total ion current (Řezanka *et al*. 1994). Measured data were processed by the SPSS 15 software (*SPSS Inc*., USA). Each value (Table I) represents the mean of four independent chromatographic analyses.

RESULTS AND DISCUSSION

Pyrazine-derived compounds represented a relatively high proportion of the individual volatile compounds of the streptomycete odor spectrum (Table I). Their formation can be explained by a reaction between the acetoin-type compounds and amino acids and by subsequent oxidation–dehydration aromatization. Among the low-molar-mass aliphatic compounds, oxygen derivatives of butane and hexane dominated. On comparing Table I and data published by Běhal (2000), one can find a relation between the production of secondary metabolites, especially those synthesized in oligoketide pathways, and composition of the odor spectrum. Monensins, tetracyclines and avermectins (representatives of oligoketide-type compounds synthesized *via* condensation of malonyl-CoA and/or other acyl-CoA) were accompanied in *S. cinnamonensis*, *S. aureofaciens* and *S. avermitilis* by high amounts of geosmin (synthesized from 9 units of malonyl-CoA). Likewise, there is a correlation between secondary metabolite production and geosmin formation by *S. avermitilis* (Řezanka and Votruba 1998*b*; Řezanka *et al*. 2007); thus, in these cases, geosmin can be considered to be a 'marker' of overall biosynthetic activity.

No such findings have been reported in most other streptomycetes. In *S. griseus*, which produces streptomycin, where glucose represents the main biosynthetic precursor, the relative content of geosmin in the odor spectrum is low even though the production of the antibiotic is high.

The other important group of identified odor components was represented by oxolone homologues (3-alkyl-5-methyl-3-oxol-2-ones). These compounds were identified and characterized by Gerber (1973) and Řezanka *et al.* (2007). According to MS, we identified another homologous series, *viz*. 3-methyl-, 3-ethyl-, 3-propyl-, 3-butyl-, 3-hexyl-, 3-isohexyl- and 3-heptyl-5-methyl-3-oxol-2-one (Table I); some of the unidentified peaks in the chromatographic profile can be attributed to new and/or as yet unknown oxolone derivatives (*see* Řezanka and Sigler 2006). While the production of geosmin is characterized by earthy smell, the presence of oxolones can be easily identified as a musty or dank smell. Similarly to geosmin, there is a correlation between the formation of some oxolones and secondary metabolite production by streptomycetes. For example, 5 acetate, 7 propionate and 1 butyrate subunits take part in monensin biosynthesis by *S. cinnamonensis*. As the major portion of oxolones in the related odor spectrum is composed of butyl and hexyl oxolones, their synthesis can be assumed to proceed from abundant 'acetate units'. In contrast, the tetracycline overproduction by *S. aureofaciens* is characterized by prior consumption of 'acetate units' (Běhal 2000) and the abundance of unused propionate units can take part in the enhancement of propyl-oxolone formation.

Table I. Odor compounds (relative %) from nine *Streptomyces* strains[a]

Compound	*S. aur.*	*S. ave.*	*S. cin.*	*S. coe.*	*S. gri.*	*S. liv.*	*S. rim.*	*S. spe.*	*S. vir.*
Aliphatic compounds									
4-Hexen-3-one	0.24	0.27	0.27	0.65	0.72	0.65	0.55	0.57	1.56
1-Hexanol	0.21	0.23	0.23	0.74	0.82	0.94	0.81	0.83	0.89
2-Hexenal	0.41	0.45	2.45	2.22	2.46	2.22	2.74	2.83	3.04
3-Nonen-2-one	0.68	0.75	0.75	1.58	1.75	1.58	1.35	0.51	0.55
3-Hydroxy-2-butanone	1.14	1.26	1.26	2.04	1.26	1.27	1.08	1.12	1.20
3-Hydroxy-3-methyl-2-butanone	0.57	0.63	2.63	2.37	2.63	5.37	4.57	4.73	5.07
4-Hydroxy-3-hexanone	0.21	0.23	0.23	0.21	0.23	0.21	0.18	1.06	1.14
4-Methyl-2-hexanone	0.49	0.54	0.54	0.49	0.54	0.49	0.80	1.71	1.83
5-Methyl-3-hexanone	0.32	0.35	0.35	1.22	1.35	1.22	1.04	1.07	0.21
Isopentyl octanoate	0.97	1.07	1.07	0.96	1.07	0.96	0.82	0.84	1.85
Oxolones									
3,5-Dimethyl-3-oxol-2-one	0.31	0.34	0.34	1.21	1.34	1.21	1.03	1.06	2.09
3-Ethyl-5-methyl-3-oxol-2-one	1.17	1.29	1.29	2.96	3.29	2.96	2.52	2.60	2.79
3-Propyl-5-methyl-3-oxol-2-one	6.28	6.94	1.94	2.24	2.49	2.24	1.91	0.21	0.23
3-Butyl-5-methyl-3-oxol-2-one	1.74	1.92	5.92	0.83	0.92	0.83	0.71	0.73	3.62
3-Isohexyl-5-methyl-3-oxol-2-one	0.13	0.14	0.14	0.13	0.14	0.13	0.11	0.11	0.41
3-Hexyl-5-methyl-3-oxol-2-one	2.07	2.29	2.29	2.63	2.92	2.63	2.24	2.31	0.59
3-Heptyl-5-methyl-3-oxol-2-one	0.05	0.06	0.06	0.05	0.06	0.05	0.04	0.04	0.05
Decalones									
Geosmin	41.6	44.6	36.6	33.9	7.59	23.9	28.8	21.0	13.1
Methylgeosmin	0.34	0.38	0.38	2.14	2.38	2.14	1.82	2.12	2.28
Pyrazines									
Pyrazine	2.48	2.74	8.74	7.87	28.7	17.9	15.2	21.0	16.9
Methylpyrazine	1.76	1.95	8.95	9.86	11.0	8.86	11.0	11.3	12.1
2,5-Dimethylpyrazine	2.49	2.75	2.75	1.58	1.75	1.58	1.35	1.39	1.49
Vinylpyrazine	19.5	12.5	2.47	2.23	2.47	2.23	1.90	0.20	0.22
Furanes									
2,3-Dimethyltetrahydrofurane	3.65	4.04	4.04	1.50	1.67	1.50	1.28	1.32	1.42
Methyl 2-hydroxyfurancarboxylate	0.42	0.46	0.46	0.58	0.64	0.58	0.49	0.51	0.55
3,4-Bis(hydroxymethyl)furanecarb-aldehyde	0.42	0.46	0.46	3.12	3.46	4.12	3.51	2.75	4.83
Furfuryl isobutyrate	2.81	3.11	3.11	2.80	3.11	0.80	0.68	0.70	1.70
N-Furfurylpyrrole-2-carbaldehyde	0.34	0.38	1.38	1.24	1.38	1.42	1.21	0.37	1.34
Benzene derivatives									
Methyl benzoate	0.24	0.27	0.27	1.14	1.27	1.14	0.97	0.12	2.02
2-Phenylethanol	1.24	1.37	1.37	2.46	2.73	2.46	2.09	0.40	4.21
Methyl 2-aminobenzoate	1.27	1.42	1.42	1.28	1.42	0.28	1.94	2.01	2.15
2-Phenylethyl isobutyrate	0.24	0.27	0.27	0.78	0.87	0.17	0.14	0.62	0.67
Other heterocyclic compounds									
4-Pyridinecarboxylic acid	2.57	2.84	2.84	2.56	2.84	2.56	2.18	10.20	4.30
5,6-Dimethyltetrahydroxy-2-pyrone	0.65	0.72	1.72	1.55	1.72	2.55	2.17	1.36	3.35
Indole	0.92	1.02	1.02	0.92	1.02	0.92	0.78	0.26	0.27

[a] *S. aur.* *S. aureofaciens*; *S. ave.* *S. avermitilis*; *S. cin.* *S. cinnamonensis*; *S. coe.* *S. coelicolor*; *S. gri.* *S. griseus*; *S. liv.* *S. lividans*; *S. rim.* *S. rimosus*; *S. spe.* *S. spectabilis*; *S. vir.* *S. virginiae*

The furane derivatives (*i.e.* 2,3-dimethyltetrahydrofurane, methyl 2-furancarboxylate, 3,4-bis(hydroxymethyl)furanecarbaldehyde, *N*-furfurylpyrrole-2-carbaldehyde, furfuryl isobutyrate) and 5,6-dimethyltetra-

hydroxy-2-pyrone were identified as another significant component of the odor spectrum; however, not all odor compounds could be identified. The mechanism of synthesis of these compounds and their possible role in the metabolic regulation of secondary metabolite production is not yet known (Shapiro 1989; Běhal 2000, 2001).

Generally, differences in the rate of air ventilation cause the concentrations of gaseous and volatile fermentation products to be higher in shaken flasks and lower in fermentors due to the higher stripping rate (*cf.* Jáchymová *et al*. 2002; Řezanka *et al*. 2007).

Noteworthy is the similarity between the structure of oxolone homologues and regulatory factor A (2-isocapryloyl-3*R*-hydroxymethyl-γ-butyrolactone) (Pliner *et al.* 1975; Yamada and Nihira 1999); considering its role in antibiotic production we can assume a potential role of oxolone derivatives in the regulation of antibiotic production. A similar effect was observed in *S. avermitilis* (Řezanka and Votruba 1998*b*) where the other odorous 'marker' (geosmin) was described to induce the overproduction of avermectins.

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