Continuous collection of volatiles produced by *Streptomyces* grown on oatmeal agar by headspace extraction and GC-MS

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Abstract

Volatile organic compounds (VOCs) produced by bacteria are known to play a significant role in interactions among many organisms, but VOCs also impart aroma to many food products. *Streptomyces* bacteria include a large group of organisms that produce a wide range of secondary volatile metabolites with potential for clinical and industrial applications. VOC profiles of bacteria are typically analyzed in liquid media. Yet, many bacteria also grow well on solid media and may here produce different VOCs than in liquid media. However, it is challenging to capture and measure VOCs from bacteria grown on solid media, and such limitations could bias measured VOCs profiles from bacteria. In this study, a special approach was applied to examine VOC production by *Streptomyces* when grown on oatmeal agar in a large gas-washing flask with Tenax-TA traps attached 96 hours of incubation. The obtained VOC profiles of two *Streptomyces* species show presence of geosmin and 2-MIB along with a total of 110 compounds, including 51 terpenes.

Introduction

Bacterial volatile organic compounds (VOCs) may play a significant role in inter- and intraspecies relations in ecosystems [1], but they also add flavor to many food products [2]. Bacteria belonging to the genus *Streptomyces* are known for their production of the off-flavors geosmin and 2-methylisoborneol (2-MIB), but they produce a wide range of other volatile metabolites with potential clinical and industrial applications [3]. Much attention has been given to geosmin and 2-MIB because these two VOCs are commonly occurring in many aquatic environments. Geosmin and 2-MIB are reported to spoil the quality of fish in aquaculture systems due accumulation in the flesh, and to add unattractive flavor to drinking water that is produced from surface water reservoirs.

The production of volatiles by bacteria is influenced by growth conditions and the metabolism of the organisms [4, 5]. Typically, in studies of VOCs production by microorganisms, bacteria have been grown in liquid media [4], probably because the determination of VOC production from cells grown on solid media is challenging. Liquid media may not provide optimum growth conditions for all microorganisms and may also underestimate the production of volatiles. Thus, in some bacterial species like *Streptomyces* and fungi, the metabolite production is stimulated by growth on surfaces, as compared to liquid media [5].

The objective of the present study was to establish a method for measuring volatiles produced by bacteria when cultured on solid media. Two species of *Streptomyces* were grown on oatmeal agar in gas-washing flasks attached with Tenax traps to facilitate continuous adsorption of volatiles upon equilibration in headspace. Geosmin, 2-MIB and other volatiles produced by *Streptomyces* were monitored for four days by collection of

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VOCs in the headspace onto the Tenax traps by flushing with N_2 every 24 hours. The volatiles were quantified by GC-MS analysis.

Experimental

The two species included in the study are *Streptomyces* 2R (isolated from a Danish aquaculture pond) and *Streptomyces diastatochromogenes* (SD) (from DSMZ, Germany). Thirty ml of oatmeal agar was added to 500 ml sterile gas-washing flasks and inoculated with conidia (spores) to a number of 2×10^7 per flask of strain 2R or SD. The flasks were sealed with a purge-head attached to a Tenax-TA trap and incubated at 29°C. Volatiles in the headspace were collected by purging with N_2 at 200 ml/min for 15 min. Headspace was collected every 24 h until 96 h and analyzed by GC-MS settings as described in Podduturi et al. [2]. In brief, volatiles from the Tenax traps were analyzed by an automatic thermal desorption unit (ATD 400, PerkinElmer, Norwalk, USA) in combination with gas chromatograph mass spectrometer (GC-MS, 7890A GC-system interfaced with a 5975C VL MSD and a Triple-Axis detector from Agilent Technologies, Palo Alto, California). Separation of the volatiles was carried out on a DB-Wax capillary column (30 m length × 0.25 mm internal diameter and 0.5 μm film thickness) using H_2 as carrier gas with an initial flow rate of 1.0 mL/min. The column temperature program was held at 40°C for 10 min, then raised to 240°C at the rate of 8°C/min and finally at 240°C for 5 min. The mass spectrometer was operated in electron ionization mode at 70 eV. Mass-to-charge (m/z) ratios between 15 and 300 were scanned. Tenax-TA traps were changed every 24 h to avoid overloading of the traps. Parallel setups were used to collect cell biomass without interrupting the VOC production. Biomass of the bacteria was estimated using a DNA-based assay after staining of the cells with SYBR green I.

Results and discussion

The obtained VOC profiles from cultivation of *Streptomyces* showed the presence of geosmin and 2-MIB along with several other terpenes.

Geosmin and 2-MIB production

Production of geosmin and 2-MIB was detected by both strains throughout the 96 h growth period. *Streptomyces* 2R produced rather similar amounts of both compounds, while *Streptomyces* SD produced a higher amount of 2-MIB (800 ng per flask) as compared to geosmin (70 ng per flask) after 96 h (Figure 1). A large increase in the production of geosmin and 2-MIB occurred from 24 to 48 h for both strains. After 48 h, no major changes in production of the metabolites were found, but the biomass of both strains still increased after 48 h. In strain 2R, the geosmin and 2-MIB production rate increased 2- to 3-fold between 24 and 48 h and then declined to the initial 0 to 24 h rate. In strain SD, a high production of 2-MIB (4×10^{-18} g/cell) occurred from 24 to 48 h, while the geosmin production was lower. From 24 to 48 h, the geosmin and 2-MIB production rate increased almost 200-fold, followed by decline in the production rate, as also observed for 2R. *Streptomyces* 2R's geosmin production rate (0.28×10^{-18} g/cell) observed in this study is in similar range as previously reported, *S. albidoflavus* (0.3×10^{-18} g/cell) grown on solid media [6] and *S. citreus* (0.28×10^{-18} g/cell) grown in submerged culture during active growth stage [7].

Species of *Streptomyces* are known to produce several secondary metabolites during transition from compartmentalized mycelium to aerial mycelium, simultaneous with initiation of the sporulation [8]. Among metabolites produced in this phase are geosmin [9]. According to Yagüe et al. [8], *Streptomyces* grown on agar media begin forming

aerial mycelium after about 24 h. This agrees with the observed formation of a dense layer of white spores on the agar surface at bottom of the present culture flasks. The spore formation coincided with the increased geosmin and 2-MIB production by 48 h, relative to 24 h, and resulted in the highest metabolite production rate during 24-48 h.

Table 1: Terpenoid compounds found in headspace extracts of *Streptomyces* 2R and *Streptomyces diastatochromogenes*

	Calculated RI(DB-Wax)	*Suggested Compound*		*Calculated RI*	*Suggested Compound*
1	1064	2-Methylenebornane[a]	18	1688	Humulene [b]
2	1105	β-Pinene[b]	19	1705	γ-Muurolene[a]
3	1176	β-Myrcene[b]	20	1721	γ-Gurjunene[a]
4	1197	D-Limonene[b]	21	1728	Germacrene D[a]
5	1467	α-Cubebene[a]	22	1739	Eremophilene[a]
6	1503	α-Copaene[a]	23	1741	α-Muurolene[a]
7	1531	β-Bourbonene[a]	24	1752	β-Dihydroagarofuran[a]
8	1552	β-Cubebene[a]	25	1763	β-Bisabolene[a]
9	1584	(-)-Aristolene[a]	26	1773	δ-Cadinene[a]
10	1589	β-Copaene[a]	27	1778	γ-Cadinene[a]
11	1602	β-Elemene[a]	28	1800	Cadina-1(2),4-diene[a]
12	1614	2-Methylisoborneol [b]	29	1853	Calamenene[a]
13	1637	Allo-Aromadendrene[a]	30	1854	Geosmin [b]
14	1642	α-Himachalene[a]	31	2084	Caryophyllenyl-alcohol[a]
15	1649	cis-β-Guaiene[a]	32	2094	Cubenol[a]
16	1658	α-Guaiene[a]	33	2177	γ-Eudesmol[a]
17	1683	Viridiflorene[a]			

[a] Compounds identified by NIST MS library and RI from literature
[b] Compounds identified by NIST MS library and RI of pure compounds

Total VOC profile

A total of 110 different VOCs were found in headspace of strain 2R, while 108 different compounds were identified for strain SD. The VOC composition of both strains was dominated by 51 terpenoids (mono and sesquiterpenes and their derivatives) and 23 hydrocarbon compounds. The list of terpenoids identified with standards and tentatively identified with NIST library similarity and RI values from literature are shown in Table 1. The identity of 12 of the detected 51 terpenoids was unknown. The total number compounds found in this study are considerably higher than the *Streptomyces* grown in liquid media [7].

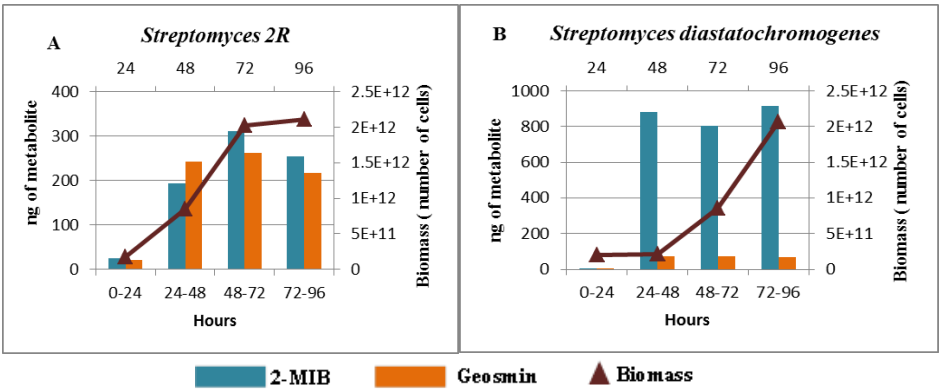

Figure 1: Production of geosmin and 2-MIB as well as biomass by *Streptomyces 2R* (A) and *Streptomyces diastatchromogenes* (B) over 96 hours

The headspace composition of various volatiles produced by the *Streptomyces* isolates in this study (dominance of mono and sesquiterpenes) resemblances the volatile profiles by other geosmin-producing *Streptomyces* [3, 10]. Most of the compounds listed in Table 1, including 1, 2, 4, 5, 6, 8, 11, 12, 18, 21, 23, 24, 25, 30 and 32, have previously reported to be produced by various *Streptomyces* strains [3, 10].

Conclusion

The experimental setup demonstrates that volatile metabolites produced by *Streptomyces*, such as geosmin and 2-MIB, can be detected and identified by cells growing on solid media. The current study also shows that *Streptomyces* grown on solid media produces higher number of metabolites compared to submerged culture with almost similar rate of production. The present approach can be applied for detection and quantification of VOCs produced by bacteria, but the experimental setup could also be useful in studying dynamics and kinetics of volatiles and metabolites produced by bacteria.

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