Folia Microbiol. **52** (1), 26–30 (2007) **http://www.biomed.cas.cz/mbu/folia/**

Relationship between Volatile Odorous Substances and Production of Avermectins by *Streptomyces avermitilis*

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Received 7 December 2006
Revised version 19 February 2007

ABSTRACT. The time course of production of odorous compounds, *i.e*. geosmin and oxolones, and of avermectins was determined during the cultivation of *S. avermitilis* in flasks, 1- and 50-L fermentors. The amount of the antibiotics increased with increasing cultivation time up to more than 2 g/L while the concentration of geosmin rose to more than 4 mg/L. Cultivation without reflux condenser resulted in a lower product formation due to the higher stripping of geosmin. A relatively tight correlation was found between the production of geosmin and the production of avermectins. The production of oxolones peaked on cultivation days 3–5, the sum of oxolones being 60 μg/L. Subsequently, the production dropped below a measurable level. This can be explained as being due to the inhibition of oxolone production by geosmin.

Abbreviations

Avm avermectin(s) Gmn geosmin OX(s) oxolone(s)

Streptomycetes are currently among the microorganisms most intensely used for industrial production of antibiotics and various other biologically active substances (Běhal 2006; Ostash *et al.* 2005; Petrova *et al.* 2006). The production of an antibiotic in a fermentor is often accompanied by the biosynthesis of volatile odorous substances. It is known that experienced technologists can estimate from the intensity of the odor spectrum the type of the streptomyces culture and the phase the given culture is in.

Odorous substances accompanying the cultivation of streptomycetes were first mentioned in the last century (Dougherty *et al*. 1966; Gerber and Lechevalier 1965). These volatile substances were later isolated from cultivation medium and their chemical structure was determined (Gerber 1968, 1973). GC-MS analysis of these substances led to the identification of several tens of different compounds, the most interesting of which were Gmn and the homologous series of OXs (Řezanka *et al*. 1994; Řezanka and Votruba 1998*a*; Jáchymová *et al*. 2002; Řezanka and Sigler 2006). Geosmin production by several species of streptomycetes has been studied with regard to the enzymes participating in the biosynthesis, the influence of carbon source, phosphorus concentration and the presence of micronutrients, as well as the effect of environmental conditions (Schrader and Blevins 1999, 2001; Cane *et al*. 2006).

As found in three cultivations differing in physiological conditions (non-, low- and high-production cultivation), the production phase of biosynthesis of the antibiotic complex of Avm involves a proportional production of Gmn while the production of OXs, which accompanies rather the phase of fast biomass growth, decreases (Řezanka and Votruba 1998*b*).

The sampling intervals during both the flask batch cultivation and the fermentor cultivations of *S. avermitilis* were chosen so as to comply with the previously determined fact (Řezanka *et al*. 1994; Řezanka and Votruba 1998*a*,*b*; Jáchymová *et al*. 2002; Řezanka and Sigler 2006) that a differentiation of the culture to a production or nonproduction mode apparently takes place within the first tens of hours (Janeček *et al.* 1997). The outcome of this differentiation is assumed to be in part due to the interaction of the cells with the external environment. These interactions represent an important factor determining the metabolic type, which can develop during the streptomycete life cycle.

The aim of the experiment described below was to evaluate the time course of production of both Avm and odorous substances, such as Gmn and OXs, and to discuss the interrelationships between the production of the odorous substances and that of Avm.

MATERIALS AND METHODS

All cultivations were performed with the strain *Streptomyces avermitilis* RX 2 from the collection of the *Institute of Microbiology* (Prague). The strain was obtained by mutagenesis from the parent strain X-1-A.

Fermentors. Laboratory fermentor *New Brunswick* (USA) with two Rushton turbines positioned one above the other, with bottom aeration and without reflux condenser contained 1 L fermentation medium. Fermentor *Bioengineering* (Switzerland) with bottom agitation (radial stirrer) and aeration contained 50 L medium.

Analytical apparatus. This included GC-MS *Jeol* DX 303 (Japan) with 60 m capillary column SPB-5 (diameter 0.32 mm; thickness of stationary phase 0.25 μm), with splitless injection, carrier gas helium and ionization energy 11 aJ (70 eV), and *Shimadzu* CS 930 (Japan) densitometer.

Cultivation in flasks. Inoculum was cultivated on a rotary shaker (2.8 Hz) for 1 d at 28 °C in 500 mL flasks with 50 mL inoculation medium. The second generation was inoculated by adding 5 % volume of the inoculation medium from the first generation into 50 mL cultivation medium, with a simultaneous addition of glucose; cultivation on a rotary shaker was carried out for 10 d. Medium composition was (in g/L) soybean flour 15, glucose 5, yeast extract 5, pH adjusted to 7 (inoculation medium) and (in g/L) $CaCO_3$ 5, glucose 3, yeast extract 2, NaCl 2, $(NH_4)_2SO_4$ 2, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.1, $FeSO_4 \cdot 7H_2O$ 0.05, $MnSO_4 \cdot 5H_2O$ 0.05, $ZnSO_4 \cdot 7H_2O$ 0.05, pH adjusted to 7.2 (cultivation medium).

Cultivation in laboratory fermentor (1 L). Cultivation medium (0.5 L) was inoculated by one 50 mL flask with vegetative inoculum; the cultivation was carried out for 9 d. Cultivation medium was the same as with flask cultivation.

Cultivation in semipilot plant fermentor (50 L). Medium (25 L) in a tank was inoculated by 25 inoculation flaks (cultivation medium was the same as in flask cultivation). Cultivation conditions: aeration 2 L/min, radial agitator with 5 Hz, pH 7 (unadjusted), temperature 28 ºC.

Sample collection. The samples were collected after 12 h, then at 12-h intervals for 3 d, and subsequently at 1-d intervals. The cultivation was terminated after 9 d.

Avermectin determination. The concentration of Avm in mycelium samples was determined by TLC. A sample (5 mL) was first extracted with a double volume of ethyl acetate on a reciprocal shaker for 1 h at 28 ºC. An aliquot of the extract (50 or 20 μL) was applied on a Kieselgel 60 F_{254} TLC plate with indicator (*Merck*) and developed in a hexane–ethyl acetate–methanol mixture (9 : 9 : 0.5). The spots were detected in UV light (254 nm). Quantitative determination was performed on a densitometer at 252 nm.

Determination of volatile metabolites. Volatile metabolites were determined by gas chromatography in combination with mass spectrometry (GC-MS). A sample of the medium with the mycelium (20 mL) was first steam-distilled until about 30 % of the volume was distilled away. The distillate was extracted with 1 mL dichloromethane and the extract was analyzed by GC-MS. The injection volume was 1 μL, column temperature was programmed for 2 min isothermally at 50 ºC followed by a temperature gradient of 10 K/min, with a final temperature of 250 ºC.

RESULTS AND DISCUSSION

Avm biosynthesis occurred from the very beginning of the cultivation (Table I). The same holds for the production of OXs, which appeared in significant amounts after 12 h of cultivation. In all three cultivations, the production of Avm increased throughout the cultivation, reaching a maximum at the end of each experiment. In contrast, the production of OXs was higher at the beginning of cultivation and then decreased to a minimum.

In all cultivations, the biosynthesis of Avm correlated with the production of Gmn, increasing from the start of the cultivation without any noticeable maximum being reached. The production of Gmn determined in the 1 L fermentor without reflux condenser was slightly more than ¼ of that found in the 50 L fermentor, obviously due to the stripping of Gmn with air.

In the initial phase of all three cultivations (up to 3 d), the production of Gmn, Avm as well as OXs increased (*see* Table I). Comparison of production kinetics showed that the initial slight drop in the Gmn-to-Avm ratio is followed, in flask and laboratory fermentations, by a remarkable rise between 2 and 4 d of cultivation. In the pilot plant fermentor, this rise takes place 12 h later, obviously due to the larger fermentation volume. These findings fit with previously reported data (Řezanka and Votruba 1998*b*) showing that the amount of synthesized Gmn increases with increasing production of Avm.

Table I. Production[a] of volatile compounds[b] (μg/L) and Avm (μg/mL) in flasks,1- and 50-L fermentors

Sampling	BuOX	PeOX	HeOX	Σ of OX	Gmn	Avm
Flasks						
12 h	1.2 ± 0.2	0.9 ± 0.2	0.5 ± 0.1	2.6 ± 0.4	2 ± 0.4	12 ± 2
24 h	2.7 ± 0.6	1.4 ± 0.4	1.3 ± 0.3	5.4 ± 2.1	5 ± 1	38 ± 5
36 h	3.6 ± 0.9	2.1 ± 0.6	3.4 ± 0.8	9.1 ± 2.0	9 ± 2	75 ± 11
48 h	4.7 ± 1.4	3.5 ± 1.0	5.4 ± 0.9	13.6 ± 4.2	12 ± 4	111 ± 27
60 h	7.4 ± 2.3	5.2 ± 1.1	7.1 ± 1.7	19.7 ± 3.7	26 ± 6	237 ± 39
72 h	11.4 ± 3.0	8.6 ± 2.4	9.6 ± 1.7	29.6 ± 5.2	36 ± 9	425 ± 44
4 d	15.8 ± 3.2	14.7 ± 2.1	11.3 ± 2.9	41.8 ± 7.0	123 ± 12	835 ± 89
5 d	10.1 ± 1.8	17.2 ± 3.3	15.2 ± 3.6	42.5 ± 6.1	275 ± 35	1272 ± 157
6 d	5.3 ± 1.2	10.4 ± 1.6	16.1 ± 2.7	31.8 ± 5.7	413 ± 51	1279 ± 142
7 d	1.8 ± 0.5	3.2 ± 0.7	6.8 ± 1.3	11.8 ± 1.8	963 ± 94	1684 ± 234
8 d	0.6 ± 0.2	1.4 ± 0.5	2.6 ± 0.7	4.6 ± 1.4	1208 ± 108	1832 ± 248
9 d	0.0 ± 0.1	0.3 ± 0.1	0.8 ± 0.2	1.1 ± 0.5	1578 ± 142	2144 ± 407
1-L fermentor						
12 h	3.8 ± 05	2.7 ± 0.3	1.2 ± 0.2	7.7 ± 1.0	1 ± 0.2	5 ± 2
24 h	6.9 ± 1.4	4.1 ± 0.6	3.7 ± 0.4	14.7 ± 1.6	3 ± 1	12 ± 3
36 h	13.4 ± 2.1	7.2 ± 0.9	6.5 ± 0.9	27.1 ± 2.5	7 ± 2	31 ± 6
48 h	18.5 ± 2.8	12.3 ± 2.1	9.5 ± 1.2	40.3 ± 3.1	13 ± 3	97 ± 12
60 h	21.3 ± 3.6	14.5 ± 1.8	11.8 ± 1.4	47.6 ± 2.9	25 ± 3	203 ± 21
72 h	26.8 ± 3.4	16.8 ± 2.4	14.2 ± 1.8	57.8 ± 3.4	37 ± 5	384 ± 37
4 d	29.4 ± 2.8	19.4 ± 0.9	17.5 ± 2.6	66.3 ± 3.8	152 ± 15	912 ± 69
5 d	24.2 ± 2.9	25.6 ± 3.5	19.1 ± 2.7	68.9 ± 4.1	348 ± 67	1186 ± 124
6 d	16.7 ± 1.5	15.0 ± 2.4	19.3 ± 2.2	51.0 ± 4.5	685 ± 82	1312 ± 174
7 d	8.5 ± 1.7	8.2 ± 0.5	12.7 ± 2.0	29.4 ± 3.4	912 ± 103	1625 ± 207
8 d	3.6 ± 0.5	3.4 ± 0.4	6.2 ± 1.2	13.2 ± 2.3	1047 ± 95	1910 ± 264
9 d	0.5 ± 0.1	0.8 ± 0.2	1.4 ± 0.4	2.7 ± 0.5	1109 ± 214	2237 ± 412
50-L fermentor						
12 h	5.3 ± 1.1	2.1 ± 0.5	1.8 ± 0.4	9.2 ± 0.9	1 ± 0.3	2 ± 0.4
24 h	7.1 ± 1.8	3.9 ± 0.9	3.2 ± 0.4	14.2 ± 2.5	7 ± 2	8 ± 2
36 h	9.6 ± 2.4	7.4 ± 1.3	4.8 ± 0.5	21.8 ± 3.8	16 ± 3	23 ± 6
48 h	12.6 ± 2.1	10.4 ± 1.8	6.3 ± 0.5	29.3 ± 3.4	25 ± 9	40 ± 9
60 h	15.7 ± 3.5	10.6 ± 1.4	7.2 ± 0.7	33.5 ± 4.8	42 ± 11	146 ± 14
72 h	18.9 ± 2.8	10.8 ± 1.2	8.1 ± 0.8	37.8 ± 5.9	60 ± 14	239 ± 19
4 d	13.4 ± 2.0	15.8 ± 2.1	8.9 ± 1.6	38.1 ± 4.2	185 ± 21	449 ± 13
5 d	7.2 ± 1.7	12.3 ± 1.4	9.8 ± 1.5	29.3 ± 3.8	910 ± 46	373 ± 23
6 d	1.8 ± 1.0	5.0 ± 0.7	4.5 ± 0.6	11.3 ± 2.7	1860 ± 85	487 ± 45
7 d	1.8 ± 0.7	4.1 ± 0.8	3.2 ± 0.7	9.0 ± 0.8	2420 ± 120	577 ± 56
8 d	0.8 ± 0.2	2.1 ± 0.3	1.7 ± 0.4	4.6 ± 1.1	3380 ± 138	1035 ± 171
9 d	0.4 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	1.7 ± 0.4	4120 ± 141	1176 ± 97

[a]Mean ± SD from three experiments.
[b]Butyloxolone (BuOX), pentyloxolone (PeOX), hexyloxolone (HeOX), and geosmin (Gmn).

The kinetics of production of OXs differs from that of Avm and Gmn (*cf.* Table I). At a Gmn concentration of 400 μg/L the production of OXs was inhibited. A sudden drop in OX production by an order of magnitude occurred at about the same time point when the Gmn production equally sharply rose. These changes in the ratios are conspicuous especially in the flask and laboratory fermentor cultivations.

The content of the most volatile butyl-OX is the highest in laboratory fermentor without reflux condenser in which the volatile substances are stripped by air. This can be explained as being due to the inhibition of OX formation by Gmn. Hence, the ratio of the amount of synthesized OXs to Avm decreases with increasing production of the antibiotic complex and of Gmn. This might imply that, at the beginning of the cultivation, a certain competitive relationship exists between the metabolic pathways leading to these three groups of compounds; these pathways use a common precursor – acetyl-CoA. This competition is at later stages shifted in favor of the biosynthesis of Avm and Gmn and is regulated by the presence of Gmn. The following hypothesis, based on preceding data (Jáchymová *et al*. 2002) and our present results, can then be proposed for the first phase of the cultivation: the first few tens of hours of the cultivation are decisive for

the production of secondary metabolite (Avm) while a given metabolic type of microorganism is differentiated during the first few hours. This metabolic type predestines the given strain for the production of a certain type of substances. At the same time, it is highly probable that the processes taking place during these early stages of culture development determine the availability of primary metabolites during the production phase proper; the formation of selective inhibitors (Gmn) brings about changes in the spectrum of odorous substances.

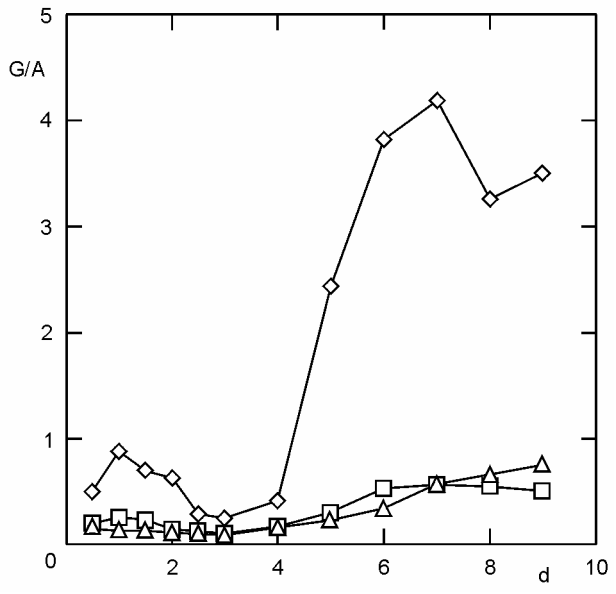

Fig. 1. Geosmin-to-avermectins ratio in flasks (*triangles*), 1-L (*squares*) and 50-L fermentor (*diamonds*).

In the second phase of the cultivation, which occurs between 3 and 9 d, the Gmn/Avm ratio keeps increasing and then becomes constant (*see* Fig. 1). However, the curves illustrating the OXs/Avm ratio in this second cultivation phase are much steeper than those describing the Gmn/Avm ratio, which results in a 1–2 orders of magnitude drop of the OXs/Avm ratio. The possible accidental character of this dependence is excluded by the identical trends in all three cultivation modes.

When judging the differences between the three cultivation types it should be kept in mind that flasks, as well as the two types of fermentors, have different regimes. The exchange of fermentation gases in flasks is much slower than in the fermentors. The concentration of Gmn in the medium in laboratory fermentor without reflux condenser is ¼ of that in both the 50 L fermentor equipped with reflux condenser and in flasks. The observation that the production of Avm closely correlates with Gmn content indicates that the precursors of both compounds may include volatile substances with vapor pressure similar to that of Gmn. Surprising is the increased production of the highly volatile butyl-OX in the 1-L fermentor. Geosmin or some other similar compound with similar vapor pressure apparently inhibits the production of OXs (*see* Fig. 2). This finding provides an entirely new view of the role of aeration in the production of secondary metabolites. An important factor need not be only the concentration of dissolved oxygen in the medium but also the stripping of biologically active volatile substances that regulate the biosynthesis of the product. The scale-up of the process should then involve, in addition to an adjustment of the intensity of stirring and aeration, also the estimation of the correct surface area of the reflux condenser.

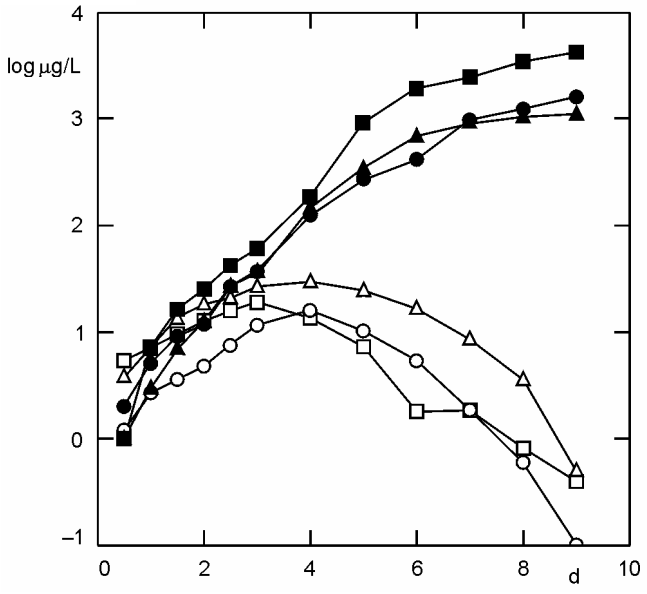

Fig. 2. Production (log μg/L) of butyloxolone (*open symbols*) and geosmin (*closed symbols*) in flasks (*circles*), 1-L (*triangles*) and 50-L (*squares*) fermentor.

The authors are greatly indebted to the prematurely deceased Dr. J. Votruba for performing the large-scale fermentations of *Streptomyces avermitilis*. The work was supported by the *Institutional Research Concept* AV 0Z 5020 0510.

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