LABORATORY PRODUCTION AND ^{14}C-LABELLING OF VIOMYCIN

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Conditions established for viomycin production by *Streptomyces griseus* var. *purpureus* in 1-litre flasks were found suitable for production in a 14-litre fermentor, provided that shearing forces were reduced by the use of low initial aeration rates and low agitation rates. The optimum temperature for production was 30°C, no antibiotic being produced at 37°C. ^{14}C-Arginine, and especially ^{14}C-glucose, were satisfactory precursors of ^{14}C-viomycin of moderate specific activity if added 40 hours afer the start of the fermentation. A variety of ^{14}C-amino acids were unsuitable for isotopic labelling since their metabolism led to the release of large amounts of $^{14}CO_2$. ^{14}C-Acetate also was unsuitable for labelling purposes. Viomycin production was complex, the initial production being associated with sporulation and a secondary production being associated with spore germination and subsequent mycelial growth.

Previous studies suggested that the antibiotic viomycin inhibits protein synthesis in bacteria by preventing the transfer of amino acids from charged tRNA to the mRNA-ribosome complex[1]. In addition, it was found[2] that resistance towards this antibiotic can involve the accumulation of a viomycin-binding phospholipid in the external layers of resistant cells. Further studies of these phenomena were hampered by the unavailability of radioactively-labelled viomycin. Since attempted tritiation by a catalytic exchange method resulted in considerable destruction of the drug it was considered necessary to biosynthesize labelled viomycin, although the data on possible biosynthetic precursors were meagre and the possibility of producing high specific activities was poor.

Materials and Methods

Strain: The producing organism was *Streptomyces griseus* var. *purpureus*, strain A 5014 a, obtained from J. EHRLICH (Parke, Davis and Co., Detroit, U.S.A.). It was maintained on RICHARDS' agar (asparagine, 1.0 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeCl_3$, 1.5 mg; dextrose, 5.0 g; agar, 15.0 g; distilled water to 1 litre).

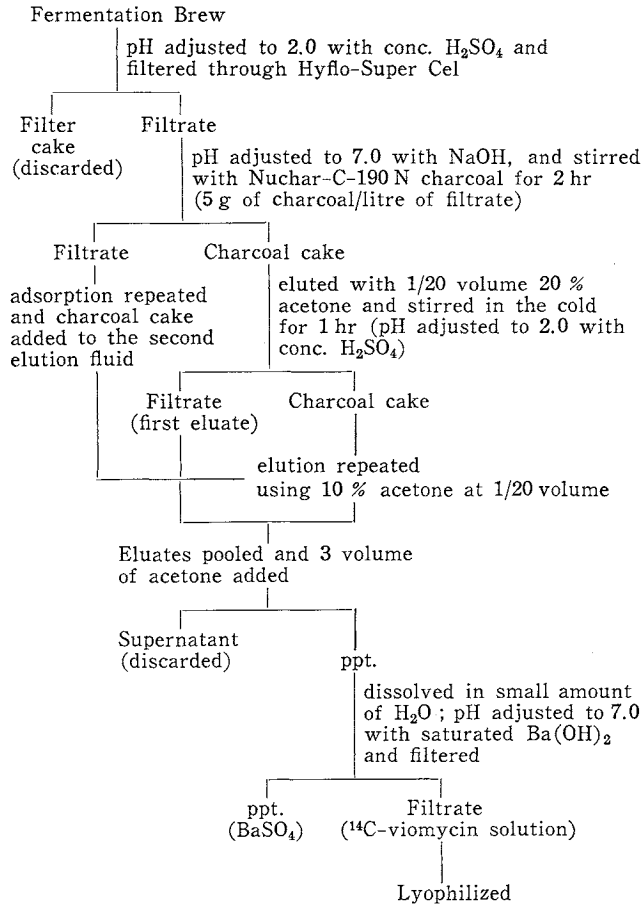
Fermentation: Initial studies involved the use of flask cultures incubated on a reciprocating shaker at a speed of 120 oscillations per minute with a 7.5 cm stroke. To prepare the fermentation medium, various amounts of vitamin-free Casamino acids (Difco) and 0.5 g of NaCl were dissolved in 90 ml of double-distilled water and the pH adjusted to 7.6 with 0.1 N NaOH. Then 0.1 g of $CaCO_3$ was added and the medium dispensed in a 1-litre flask and autoclaved at 121°C for 20 minutes. After cooling, a filter-sterilized glucose solution (of varying concentrations) was added, together with 2.5 ml of a 1/5 dilution of commercial-grade molasses (Domolco brand, Dominion Distributors, Halifax, Canada) to

make a final volume of 100 ml. The molasses solution was sterilized by autoclaving twice at 110 °C for 10 minutes with an intervening 48-hour incubation period. This medium, containing 2 % glucose and 0.5 % Casamino acids, was that previously used by Yu[3].

Later studies involved the use of a 14-litre fermentor (Fermentation Design Inc., Allentown, Pa., U.S.A.), fitted with magnetically-driven turbine impellors and foam control. The antifoam agent was X-foam (S.F. Lawrason and Co., Toronto, Ontario, Canada), an autoclavable material which was tested and found to be non-toxic to the test organism and to a variety of bacteria and yeasts.

Inoculation: After a 24-hour sterility check the medium was inoculated by adding a spore suspension (0.4 % v/v) prepared by mixing the spores from a 7-day, 30°C, slant culture with water (optical density at 420 mμ = 0.17).

Fig. 1. Isolation and purification scheme for viomycin



Analyses: During fermentation samples of broth were collected at 24-hour intervals, filtered through a membrane filter (0.45 μ pore size) and the pH and mycelial dry weight determined. Samples also were plated to detect contamination. Viomycin was assayed by the usual disc method, using *Rhizobium meliloti* R 21, and double-layer plates of Modified Medium 79[4] containing 0.3 % Difco yeast extract. To identify the antibiotic the fermentation filtrate was spotted on filter paper strips, run in a solvent system of butanol-acetic acid-water (50 : 16 : 50), dried, and transferred to double-layer plates seeded with *R. meliloti* for bioautography against a known standard.

Extraction of Viomycin: The method employed (Fig. 1) was that suggested by SCHNEIDER and BARTZ (personal communication). Confirmation of the identity of the final product was made by bioautography and spectrophotometry, the drug showing a characteristic absorption at 286 nm in alkaline and neutral media. In addition to viomycin this organism produced an orange antibiotic, extractable from whole cells with chloroform. Solubility, spectroscopic data and the antimicrobial spectrum coincided with those for griseorhodin A. Fortunately this antibiotic remained with the mycelia and was removed from the fermentation broth prior to viomycin extraction.

Radioactive-labelling: The compounds employed were purchased from Amersham-Searle Corp., Illinois, U.S.A. and added at various times throughout the fermentation, which was terminated 7 days after inoculation. $^{14}CO_2$ produced in experiments a, b, c and d (Table 3) was displaced by a continuous flow of sterile air and swept into vials containing 2 ml of Hyamine-10× hydroxide, which were renewed every 12 hours. ^{14}C was determined by liquid scintillation. The recovered ^{14}C-viomycin was purified, lyophilized

and assayed for ^{14}C.

Microscopy: Samples were Gram-stained for optical microscopy. For electron microscopy the samples were washed in water and examined after negative-staining with 1% uranyl acetate.

Results

Flask Fermentation

A temperature study indicated (Table 1) that, although the maximum growth achieved at 3 different temperatures was similar, no viomycin could be detected at 37°C. At 25°C and 30°C maximum production occurred after 7 days. A temperature of 30°C was used in all further experimentation.

Dissolved oxygen, measured by a probe clamped to the fermentation vessel, decreased from an initial 72% at inoculation to a low of 50% after 24 hours, then gradually increased to 60% at the sixth day as the growth rate diminished. This level was maintained until the fermentation was terminated on the ninth day.

The effects of variations in the basal medium composition are presented in Table 2. The addition of $CoCl_2$, known to stimulate sporulation, did not increase viomycin production, and the addition of a mixture of vitamins reduced production. The maximum level of antibiotic was obtained at a Casamino acids concentration of 0.75%, when the glucose concentration was held at 2%. A fermentation medium containing these particular levels was employed in all future experimentation.

Results of attempts to prepare ^{14}C-viomycin are shown in Table 3. Acetate and all the amino acids except arginine were ineffective as precursors to ^{14}C-viomycin. Much of the substrate ^{14}C was lost from the system as $^{14}CO_2$, the quantity of ^{14}C lost amounting to 19.5, 14.7, 32.7 and 30.6% of that originally possessed by serine, lysine, alanine and the amino acids mixture respectively. ^{14}C-Arginine led to the production of labelled antibiotic but this amino acid was not as effective as glucose, which yielded maximum labelling when added 40 hours after inoculation of the fermentation broth. One μmole of glucose (specific activity 360 mCi/mole) yielded 1.8×10^6 d.p.m. ^{14}C/mg viomycin, whereas an equivalent amount of arginine (336 mCi/mole) yielded 0.65×10^6 d.p.m. ^{14}C/mg viomycin. There

Table 1. Growth and viomycin production in shake flasks at 3 different temperatures

Temperature	Maximum viomycin concentration (mg/litre)	Maximum mycelial weight (mg/ml)
25°C	523	4.6
30°C	657	4.4
37°C	0	5.0

Table 2. Effect of medium composition on growth and viomycin production in shake flasks at 30°C.

Medium variation	Maximum viomycin concentration (mg/litre)	Maximum mycelial weight (mg/ml)
Glucose* 2.00 % (w/v)	530	4.5
4.00	535	5.5
6.00	610	5.8
8.00	540	7.0
Casamino acids** 0.50 % (w/v)	540	4.5
0.75	680	2.0
1.00	600	4.0
plus $CoCl_2$ 0.002 % (w/v)	530	4.3
plus Vitamins*** 1.0 ml	450	4.0

* Concentration of Casamino acids : 0.5% w/v
** Concentration of glucose : 2.0% w/v
*** Stock solution contained (per litre) ; B 12 (0.1 mg) ; nicotinic acid (10.0 mg), *p*-aminobenzoic acid (10.0 mg) ; calcium pantothenate (10.0 mg) ; pyridoxine (10.0 mg) ; choline (200 mg). Filter sterilized.

Table 3. Synthesis of ^{14}C-viomycin from various ^{14}C-sources

Experiment	^{14}C-source*	Specific activity (mCi/mmole)	Radioactivity added (μCi)	Time of addition (hr)	Specific activity of ^{14}C-viomycin (d.p.m./mg)	% Recovery of ^{14}C as ^{14}C-viomycin
a	serine	162	10	0	0	negligible
b	lysine	312	10	0	2	〃
c	alanine	156	10	0	5	〃
d	amino acids		10	72	17	〃
	mixture**	—	10	96	7	〃
e	acetate	58	5	24	10	〃
			5	48	31	〃
			5	72	30	〃
f	glucose	360	100	6	2.33×14^4	0.26
			100	24	5.97×10^4	0.32
			250	40	1.24×10^6	1.39
			100	48	6.85×10^4	0.45
			75	72	2.35×10^4	0.28
			75	96	1.85×10^4	0.18
			100	120	4.43×10^2	0.002
g	arginine	336	50	40	9.8×10^4	0.59
h	arginine	336	50	40	3.18×10^5	0.58
	plus glucose	360	100			

* Uniformly-labelled
** Hydrolysate of *Chlorella* protein (1.49 mCi/mg)

Table 4. Viomycin production at 30°C in a 14-litre fermentor at various agitation and initial aeration rates

Experiment No.	Agitation rate (r.p.m.)	Initial aeration rate* (litres/min.)	Initial dissolved O_2 (%)	Peak viomycin concentration (mg/litre)	Maximum mycelial weight (mg/ml)
		Spore inoculum (Medium volume=10 litres)			
1	200	0.25	40	0	1.7
2	200	0.50	58	0	2.2
3	200	4.60	88	650	3.3
4	200	8.00	100	0	—
5	200	2.00	96	450	5.5
6	300	2.00	98	353	4.9
7	400	2.50	98	0	—
8	400	8.00	100	0	—
9	800	8.00	100	0	—

* Aeration rate increased to 8.6 litres/min. after 17 hr.

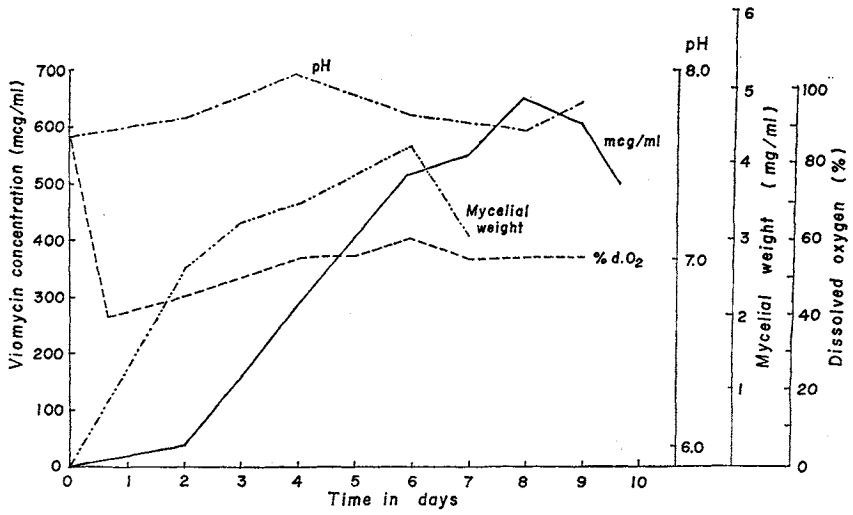
was evidence of a synergistic effect when glucose and arginine were added in combination, as determined by comparing the extent of labelling with that calculated on the basis of equivalent amounts of these 2 compounds added separately.

14-Litre Fermentor

When attempts were made to produce viomycin in the fermentor, using the conditions of medium composition, temperature, pH and dissolved oxygen employed for flask fermentation, no antibiotic was detected, although growth of the organism was good. Viomycin production (Table 4) was found to be dependent on a moderate level of initial aeration, a high final aeration rate and a low agitation rate. Further studies again indicated that 0.75 % Casamino acids was the best level for antibiotic synthesis.

The pattern of fermentation for the optimum production of viomycin under the

Fig. 2. Fermentation pattern for viomycin production in a 14-litre fermentor (see experiment 3, Table 4 for details).



conditions employed is illustrated in Fig. 2.

The work of OWEN and JOHNSON[5] and SENSI and THEIMANN[6] suggested that shifting the incubation temperature during the early growth phase could increase the yield of several antibiotics. In the present investigation lowering the temperature from 30°C to 28°C after 6 days delayed the peak production of viomycin by 3 days but increased the yield by 12%.

Growth of *S. griseus* var. *purpureus* during fermentation could be divided into 4 phases: (i) initial vegetative growth (0~48 th hour), (ii) sporulation and release of free spores (48~120 th hour), (iii) spore germination and late vegetative growth phase (120~168 th hour), and (iv) mycelial degeneration (168 th hour to termination). Active mycelium was Gram-positive; inactive mycelium, as seen in phase (ii) and (iv), was Gram-negative.

Production of antibiotic was complex. Synthesis commenced after 40~48 hours of incubation just after the initial vegetative growth phase had terminated and when sporulation could be observed microscopically. This initial rate of production continued throughout subsequent spore germination and late vegetative growth. Peak production was reached after mycelial lysis had commenced.

Conclusions

Conditions established for viomycin production in 1-litre shake flasks could be employed in fermentors provided that high initial aeration rates and high agitation rates were avoided. An increase in the agitation rate from 200 to 400 r.p.m. completely inhibited viomycin synthesis. The deleterious effects of high initial aeration and high agitation rates may be attributed to the effect of shearing forces on the growth of the producing organism, although an effect on some biochemical event essential to viomycin synthesis cannot be excluded.

Of the radioactive compounds tested, only glucose and arginine yielded significant

labelling of viomycin. The effectiveness of arginine as a suitable substrate strengthens the suggestion of K. RACZYŃSKA-BOJANOWSKA (personal communication) that arginine is a precursor of the viomycidine residue of viomycin. Glucose was more effective than arginine for labelling viomycin under the conditions established, and provided ^{14}C-labelled antibiotic of sufficient specific activity for continued studies on the mode of action of this drug at the subcellular level.

The association between sporulation and the synthesis of peptide antibiotics is not a new finding[7]. SCHATZ and WAKSMAN[8] demonstrated that asporogenous mutants of *S. griseus* and *S.lavendulae* failed to produce streptomycin and streptothricin respectively although the ability to produce these antibiotics was regained in revertant strains. With *S. griseus* var. *purpureus* viomycin synthesis is intimately connected with both sporulation and germination. Little or no viomycin was produced until 48 hours after inoculation, at which time observable sporulation and viomycin production commenced. A continued increased rate of production was noted during spore germination and secondary mycelial growth. These observations suggest that during sporulation an overproduction of spore precursors may result and that such precursors could be involved in the biosynthesis of viomycin. The newly synthesized antibiotic then could be released into the medium along with the free spores and contribute to the first phase of viomycin production. A second production phase may result from the release by the germinating spores of these same precursors. In this connection it has been suggested that bacitracin is a component of the spore coats of *Bacillus subtilis*[9], although a later report is contradictory.[10]

Acknowledgements

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