Bioproduction of Axenomycins in Batch Cultures of *Streptomyces lisandri*

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Z. Naturforsch. **35 c,** 936–944 (1980); received August 8, 1980

Streptomyces lisandri, Axenomycins, Bioproduction, Control by Nitrogen, Phosphate and Carbon

The influence of various factors such as aeration, pH and size of the inoculum on production of axenomycin A, B, and D and on growth of *Streptomyces lisandri* was studied in batch cultures. An investigation of the nutritional requirements showed that growth and antibiotic production are not necessarily correlated. The yield of acenomycins was increased to 1.7 g per liter medium by repeated selection for a high producing strain. Bioautography showed that these strains produced a hitherto undescribed antibiotic and that all strains tested differed in the total amount of axenomycins produced but not in the composition of the fraction containing antibiotic activity. Addition to the medium of extra amounts of inorganic phosphate and various nitrogen sources showed that both nutritional components selectively inhibited axenomycin formation but did not inhibit growth of *Streptomyces lisandri.* Good growth of *Streptomyces lisandri* was observed in the presence of sucrose and its monomers (glucose, fructose), but whereas sucrose inhibited axenomycin formation almost completely, its monomers did not.

Introduction

Axenomycin A, B and D are a group of antibiotics with anthelmintic, antiprotozoal and antifungal activity [1–3] produced by *Streptomyces lisandri.* They have a molecular weight of ca. 1500 and a complex structure consisting of three main components viz. a macrocyclic lactone ("axenolide"), two sugar residues and a quinonoid chromophore (Fig. 1). In the course of studies of the biosynthesis of these antibiotics a strain of *Streptomyces lisandri* was obtained from the Waksman Institute, which failed to produce any antibiotics although it was grown under conditions previously reported to trigger their production [3]. We therefore investigated the conditions of growth and antibiotic formation in this strain.

Reprint requests to Dr. E. Leistner.

0341-0382/80/1100-0936 $ 01.00/0

Materials and Methods

Organisms

Streptomyces lisandri 3935 was obtained from the Waksman Institute of Microbiology, New Brunswick, New Jersey, USA. *Paecilomyces varioti* as well as 11 different strains of *Saccharomyces* were supplied by the Institut für Mikrobiologie der Universität Münster.

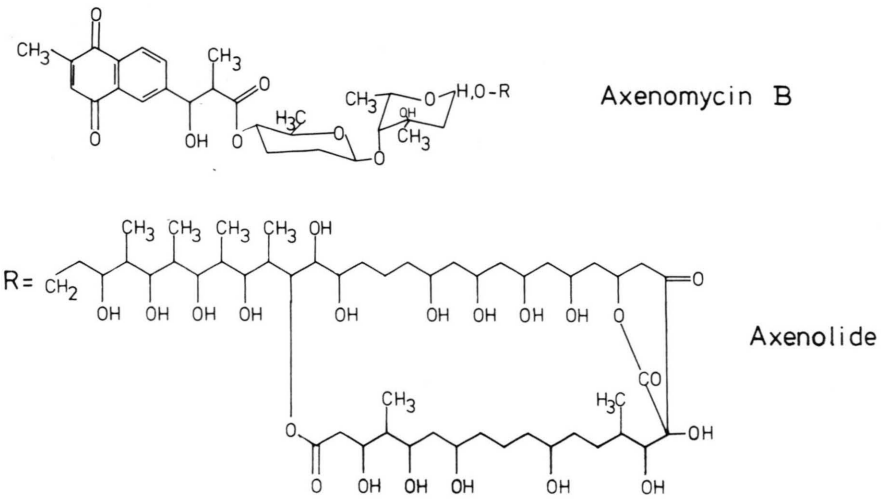

Fig. 1. Structure of axenomycin B.

Media and growth conditions

Streptomyces lisandri was maintained in Petri plates on a medium consisting of yeast extract (4 g/l), malt extract (10 g/l), mannitol (4 g/l) and agar (15 g/l). Before sterilization the pH of the medium was adjusted to 7.2 by addition of NaOH. The inoculated plates were kept at 30 °C for 6 to 7 days and then stored at +4 °C. A good sporulating disk was removed from the agar plate by means of a cork borer and used to inoculate the inoculum medium [3] from which after three days of growth 5 ml suspension was withdrawn in order to inoculate the fermentation medium (30 ml) in an Erlenmeyer flask (300 ml). The inoculum culture was shaken at 30 °C and with 250 rpm. The fermentation medium was shaken at 30 °C and with 290 rpm. Incubation of liquid media were carried out in an Infors gyrotory shaker. The components of the various media are given in Table I. Medium 1 was taken from Ref. [3], medium 12 was personally communicated by Dr. M. Bianchi (Farmitalia, Milan, Italy) whereas medium 15 was taken from Ref. [4]. All other media are variations of these three basic media.

Saccharomyces strains were grown on agar slants containing "Löflund's" malt extract (50 g) and Bacto Difco Agar (15 g) dissolved in water (1 *l*), pH 6.3.

Bioautography

An alcoholic extract of the freeze dried mycelium of *Streptomyces lisandri* from which lipids had been removed with benzene was applied to a precoated silica gel thinlayer plate which was developped in EtAc : EtMeCO : MeOH : H_2O = 60 : 40 : 10 : 5. The plate was covered with a warm (50 °C) suspension of *Paecilomyces varioti* in malt agar (see above) in a sterile chamber. The chamber was kept at 30 °C. Inhibition zones became visible after 16 to 24 h.

Determination of axenomycins

Axenomycins in the culture broth were directly determined by the plate diffusion assay with *Paecilomyces varioti* as the test organism. Axenomycins in the mycelium were extracted for six hours from freeze dried material with ethanol at room temperature. Prior to this extraction lipids were extracted with benzene for three hours at room temperature. The ethanol extract was used directly for determination of axenomycins.

Table I. Components of media used in this investigation. Data are given in gram per liter final volume. Before sterilization (115 °C, 25 min; 1.1 atü) the pH was adjusted (NaOH) to 7.2 unless otherwise given.

Component	Medium																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Glucose	100	100	100	100	100	100	100	100	100	100	100	70	70	70	70	70	70	70
Soya meal	30	30	30	–	–	30	–	–	–	–	–	–	–	–	–	–	–	–
NZ-Amine Type A	–	–	–	–	–	–	–	10	10	–	–	–	–	15	15	–	–	–
Soya peptone	–	–	–	–	–	–	–	–	–	15	15	–	15	–	–	–	–	–
L-Histidine	3	3	3	3	3	3	3	3	3	3	3	–	–	–	–	–	7	–
L-Asparagine	–	–	–	–	–	–	–	–	–	–	–	7	–	–	–	–	–	–
L-Isoleucine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7	–	–
L-Glutamine	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7
Corn-Steep Liquor	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
Yeast extract	–	4	–	–	–	–	–	–	–	–	4	–	–	–	–	–	–	–
Soya oil	5	5	5	5	–	–	5	–	5	5	5	–	–	–	–	–	–	–
$CaCO_3$	10	10	–	10	10	10	–	10	10	10	10	20	20	20	20	20	20	20
NaCl	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	–	–	–	–	–	–	–
KH_2PO_4	–	–	–	–	–	–	–	–	–	–	–	0.1	0.1	0.1	2	0.1	0.1	0.1
$MgSO_4 \times 7H_2O$	–	–	–	–	–	–	–	–	–	–	–	0.5	0.5	0.5	1	0.5	0.5	0.5
KCl	–	–	–	–	–	–	–	–	–	–	–	0.5	0.5	0.5	–	0.5	0.5	0.5
$FeSO_4 \times 7H_2O$	–	–	–	–	–	–	–	–	–	–	–	0.02	0.02	0.02	0.01	0.02	0.02	0.02
$ZnSO_4 \times 7H_2O$	–	–	–	–	–	–	–	–	–	–	–	0.02	0.02	0.02	0.05	0.02	0.02	0.02
$MnSO_4 \times H_2O$	–	–	–	–	–	–	–	–	–	–	–	0.002	0.002	0.002	0.04	0.002	0.002	0.002
$CuSO_4 \times 5H_2O$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.003	–	–	–
$CoCl_2 \times 6H_2O$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.002	–	–	–
$(NH_4)_6Mo_7O_24 \times 4H_2O$	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0.001	–	–	–

Quantitative determination of dextrin in the culture broth

The medium was separated from the mycelium by filtration. One ml of the broth which had been diluted 100 fold was mixed with a solution (2 ml) of iodine (130 g) and potassium iodide (20 g) in water (1000 ml). The extinction was measured at 620 nm in a Perkin Elmer Double Beam Spectrophotometer 124. A calibration curve established with known amounts of dextrin served to calculate the dextrin content of the medium.

Quantitative determination of D-*Glucose*

D-glucose was determined with glucose oxidase and dianisidine hydrochloride according to Bergmeyer [5].

Quantitative determination of amino nitrogen in the medium

Amino nitrogen was determined according to Moore *et al.* [6].

Results and Discussion

Quantitative determination of axenomycins

For the quantitative determination of axenomycins a colour reaction (H.-J. Bauch, forthcoming publication) and an agar diffusion test were worked out. For the agar diffusion test *Paecilomyces varioti* and 11 different species of *Saccharomyces* were checked with respect to their sensitivity against axenomycins and *Paecilomyces varioti* proved to be most sensitive. With the colour reaction 0.3 µmol, and with the agar diffusion test 0.03 µmol of axenomycin D was still detectable. An inhibition zone of 15 mm in diameter was produced by 14 µg of acenomycin A, 0.30 µg axenomycin B and 0.39 µg of axenomycin D.

Selection for a good sporulating mycelium

Streptomyces lisandri was grown on agar plates containing malt extract, yeast extract and mannitol. Since it is often inferred [*e.g.* 7] that sporulation and production of antibiotics are correlated the mycelium was repeatedly selected for good sporulation. After each selection the antibiotic production was checked by inoculation of the preculture medium which in turn was used to start a fermentation culture. Axenomycins were extracted from the freeze dried mycelium with ethanol and axenomycins determined. It was found that by this procedure the yield of antibiotics could be increased to 650 mg per liter medium (Table II).

Nutritional requirements for growth and antibiotic production

Various media were devised to investigate the influence of different components on growth and antibiotic production. Growth of the mycelium (dry weight), pH, amounts of axenomycins in the medium and the mycelium were recorded 72, 96, 120,

Table II. Selection for good sporulating mycelium as related to axenomycin production.

Repeated Selections	Axenomycins * [mg/l]
1	20
2	74
3	181
4	255
5	458
6	663

* Mean of two independent fermentations.

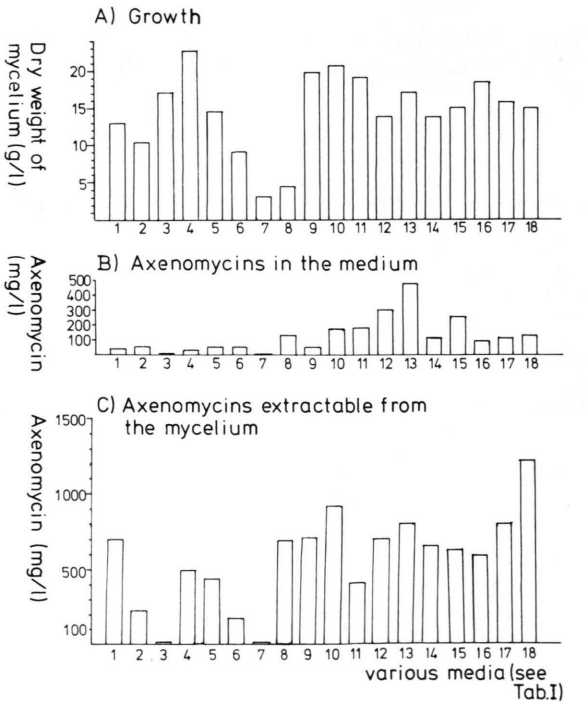

Fig. 2. Growth and axenomycin formation in various media.

140 and 168 h after inoculation of each medium. The data shown in Fig. 2 are maximum values for each individual medium and represent the mean of 2 independent determinations. Addition to medium 1 or to medium 10 of yeast extract which favours sporulation on agar plates, diminished growth and antibiotic production in batch cultures (media 2 and 11). $CaCO_3$ which was omitted from the media turned out to be essential for production of antibiotics (media 3 and 7) but not for growth (medium 3). This can be explained by the observation that in the absence of $CaCO_3$ the pH of the medium decreased to 5.0 whereas in the presence of $CaCO_3$ the pH increased to 8.8. The pH has been found to affect axenomycin production (see later). Alternatively the $CaCO_3$ may precipitate inorganic phosphate present in the corn step liquor. Inorganic phosphate has also been found to influence antibiotic formation (see later). Omission from the medium of soya meal and soya oil resulted in a decrease in growth and axenomycin production (media 4, 5, 6). Soya peptone (medium 10), however, proved to be superior to the casein-derived NZ-amine (medium 9). Medium 12 was a personal communication of Dr. M. Bianchi. Variation of the nitrogen source of this medium (see media 13, 14, 16, 17 and 18) showed that a complex nitrogen source (soya peptone, NZ-amine) could be replaced by amino acids such as L-isoleucin, L-histidin and L-glutamine, of which L-glutamine was most effective (medium 18). Little or no effect was observed when trace elements were changed (compare media 14 and 15).

Data presented in Fig. 2 also show that only minor amounts of axenomycins are detectable in the medium, while the major amount is extractable from the mycelium.

Unless otherwise stated medium 10 was used for all further experiments.

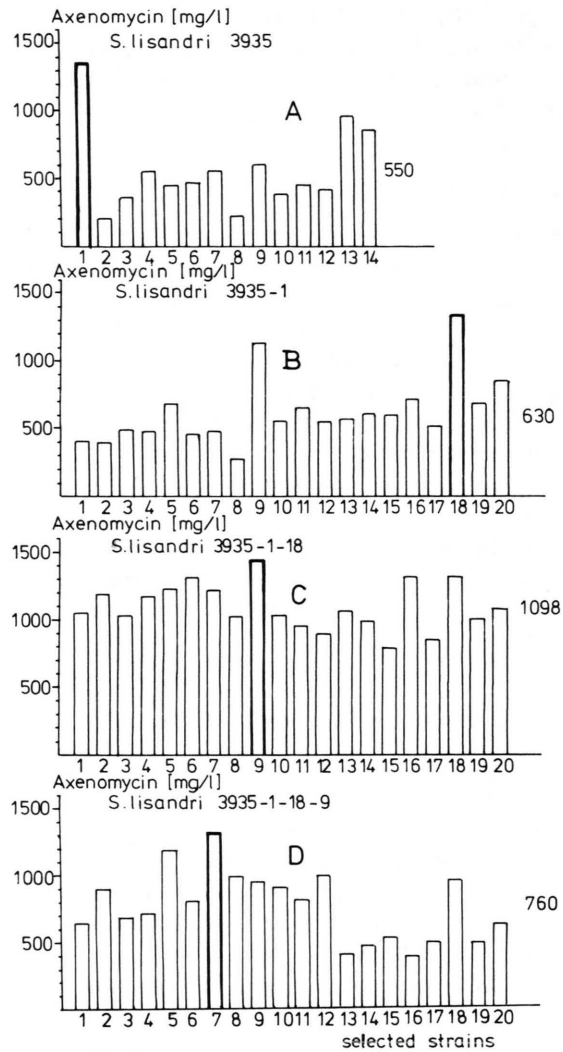

Fig. 3. Variability of different clones (diagram A, B, C and D), repeated selection for a high producing strain (A → B → C) and decrease of axenomycin formation (C → D) after one year of repeated transfer of the high producing strain.

Selection of a high producing strain

Single cell colonies were selected on an agar-medium containing yeast extract, malt extract and mannitol, with preference given to those clones which showed good sporulation. When production of axenomycins in 14 different strains was checked (Fig. 3) the antibiotic production was found to vary between 200 and 1350 mg axenomycin per liter medium. (Average: 550 mg per liter medium.) The highest producing strain was again used for single cell cloning and the average amount of antibiotics produced was thereby increased to 630 mg per liter medium. After a third selection an increase to 1098 mg was attained. The variation in productivity of single clones was, however, markedly reduced in the high producing strain as compared to the wild type strain. The strain with the highest productivity was designated *S. lisandri* 3935-1-18-9 and repeatedly transferred to new agar slants for one year. A subsequent single cell cloning showed that the aver-

age production had decreased to 760 mg per liter medium and that the variability among the new clones now ranged from 350 to 1350 mg per liter medium.

Bioautography

Single cell colonies obtained from strain 3935 and 3935-1 (Fig. 3) were grown in medium 10. Chromatograms of extracts of the mycelium of different strains of *S. lisandri* obtained from these fermentations were covered with a malt agar containing a suspension of the mycelium of the test organism (*Paecilomyces varioti*). After incubation the bioautograms showed that not only axenomycins A, B and D but also another antibiotic with an R_f value of 0.28 had been produced by all strains tested. The bioautograms also showed that while different strains varied in the total amount of axenomycins produced, all four antibiotics were present in each single strain.

pH of the medium and axenomycin formation

During growth in different media the pH of the culture broth rose. From an initial value of 6.5, the pH had increased to 8.8 after 4 days of fermentation. This was not observed, however, in media 3 and 7 in which only negligible amounts of axenomycins were formed because $CaCO_3$ had been omitted. In these media the pH dropped from 6.5 to 4.8 (7 days after inoculation). These observations suggested that the pH of the medium might be influencing axenomycin formation. We therefore tested the influence of different pH values of the culture broth on growth and production of antibiotics. Both were determined 96 h after inoculation when axenomycin formation reached a maximum. As can be seen in Fig. 4 little influence of the initial pH was observed on mycelial growth which was determined as dry weight. A distinct maximum, however, was observed for the production of antibiotics when the initial pH of the culture broth was adjusted to 6.5.

Size of the inoculum and axenomycin formation

Whenever a fermentation culture was started, a cell suspension obtained from a preculture was used for inoculation. Regardless of the volume of the inoculum used, the maximum amount of axenomycin was always detected in the mycelium 96 h after inoculation. When the size of the inoculum was varied from 1 to 10 ml preculture maximum production of axenomycins was detected with an inoculum of 5 to 6 ml per 30 ml fermentation medium. On the other hand, the size of the inoculum influenced growth only slightly (Fig. 5).

It is assumed that the size of the inoculum determines the speed of growth of the organism, in which case these results suggest that the developmental stage of the culture as well as the composition of the medium can be quite different at the time (96 h after inoculation) when maximum accumulation of axenomycins is observed.

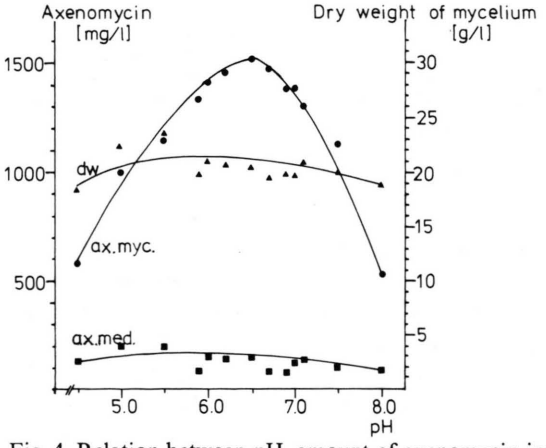

Fig. 4. Relation between pH, amount of axenomycin in the mycelium (ax. myc.) and the medium (ax. med.) and dry weight (dw.) of the mycelium.

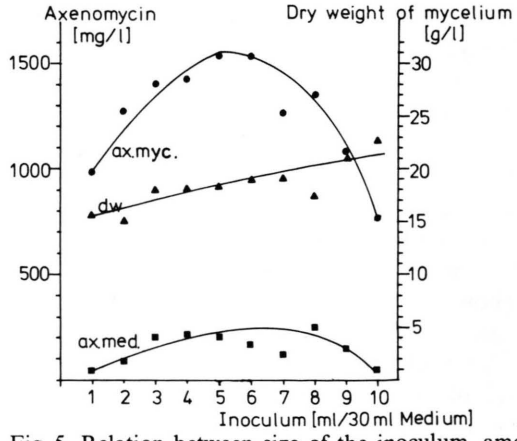

Fig. 5. Relation between size of the inoculum, amount of axenomycin in the mycelium (ax. myc.) and the medium (ax. med.) and dry weight (dw.) of the mycelium.

Table III. Relation between aeration (size of the Erlenmeyer flask), axenomycin production and dry weight of the mycelium. Each flask contained medium amounting to 10% of the volume of the flask.

Volume of flask [ml]	Dry weight of mycelium [g/l]	Axenomycins [mg/l]	
		in mycelium	in medium
100	21.1	1589	173
300	20.8	1533	191
500	23.4	1822	222
1000	20.8	535	81
2000	10.6	189	21
4000	14.5	104	11

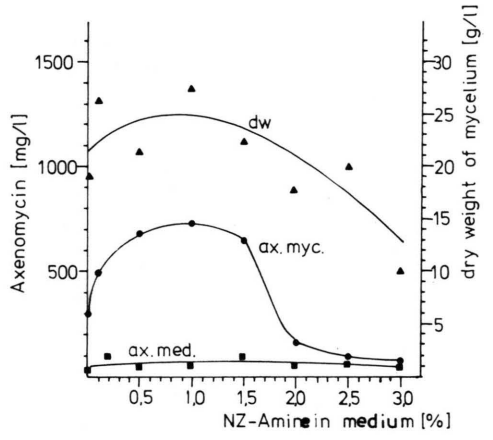

Fig. 6. Relation between NZ-amine in the medium, amount of axenomycin in the mycelium (ax. myc.) and the medium (ax. med.) and dry weight (dw) of the mycelium. The experiment was carried out with strain 3935 ("wild type strain").

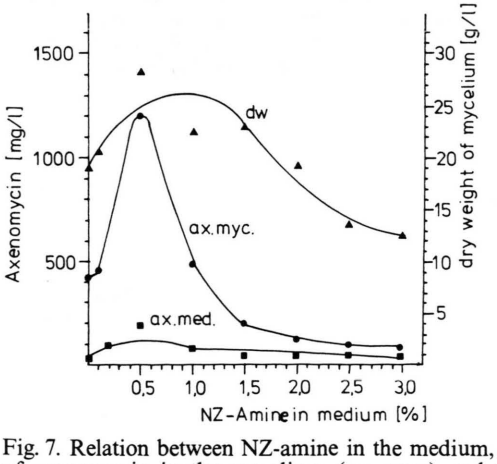

Fig. 7. Relation between NZ-amine in the medium, amount of axenomycin in the mycelium (ax. myc.) and the medium (ax. med.) and dry weight (dw.) of the mycelium. The experiment was carried out with strain 3935-1-18-9 ("high producing strain").

Aeration and formation of axenomycins

Aeration of antibiotic producing cultures is known to be crucial for maximum yields of antibiotics [8]. We have therefore inoculated Erlenmeyer flasks of different sizes each containing medium which amounted to 10% of the volume of the flasks. The flasks were shaken at identical revolutions per minute on a Gyrotory shaker. As can be seen in Table III maximum growth and maximum yield of axenomycins was obtained with 500 ml flasks. All experiments reported herein, however, were carried out with 300 ml flasks.

Nitrogen source and axenomycin formation

It has been repeatedly observed that the nitrogen source influences production of secondary metabolites and especially formation of antibiotics [*e.g.* 9]. The first indication that there is a correlation between the nitrogen source and axenomycin production resulted from two series of experiments (Figs. 6 and 7) in which increasing amounts of NZ-amine were added to cultures of the wild type strain of *Streptomyces lisandri* (selected for good sporulation) and to cultures of the strain (3935-1-18-9) which had been selected for high production of axenomycins (Fig. 3). While no significant influence of the amount of nitrogen on the growth of these two strains was observed, axenomycin formation in the high producing strain (3935-1-18-9) turned out to be much more sensitive to nitrogen supply than in the wild type strain (Figs. 6 and 7). The same was observed when NZ-amine was replaced by soya peptone (data not shown).

When the amount of NZ-amine was doubled in medium 9 (strain 3935-1-18-9) no influence on growth or pH was noted but axenomycin production was reduced by 45% (Fig. 8). Similar observations were made when the amount of soya peptone was increased in medium 10 or asparagine in medium 12 (data not shown). But this effect was not restricted to the routine nitrogen sources. When medium 9 (which contains NZ-amine) or medium 10 (which contains soya peptone) or medium 12 (which contains asparagine) were supplemented with urea, suppression of axenomycin formation was also observed (Fig. 9). Antibiotic production is therefore apparently under control of nitrogen rather than a specific amino acid or peptide present in media 9, 10 or 12.

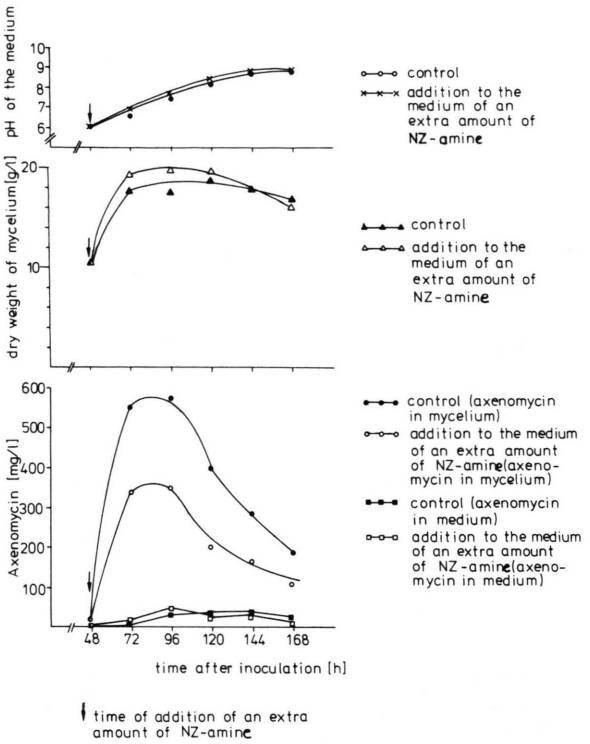

Fig. 8. Time course study of the influence of a "normal" and a two fold amount of NZ-amine on formation of axenomycins, dry weight of the mycelium (dw.) and pH.

Finally it should be noted that addition to the culture of increased amounts of nitrogen does not affect the pH of the medium during fermentation. Thus the nitrogen source does not exhibit its influence via a change of pH.

Inorganic phosphate and axenomycin formation

It is well known that best production of secondary metabolites can be obtained by phosphate concentrations suboptimal for growth [10]. Inorganic phosphate is assumed to influence the energy charge of the cell and the level of glucose-6- P which may in turn control production of secondary metabolites [11] (Fig. 10). Whereas maximum growth of *Streptomyces lisandri* is observed at a phosphate concentration of 0.7 g/liter in medium 12, maximum axenomycin formation occurs when no inorganic phosphate is added to the medium. It is assumed that corn steep liquor, one of the components of medium 12, contains an undetermined amount of inorganic phosphate which is sufficient for good axenomycin formation.

Carbon sources and axenomycin formation

Glucose, fructose, mannitol and sucrose were examined as carbon sources and good growth was observed in each case (Fig. 11). Maximum axenomycin formation, however, occurs at an unusually high glucose concentration (15%).

A fairly good axenomycin production was also observed with fructose, whereas in the presence of sucrose and mannitol it was negligible. The non-

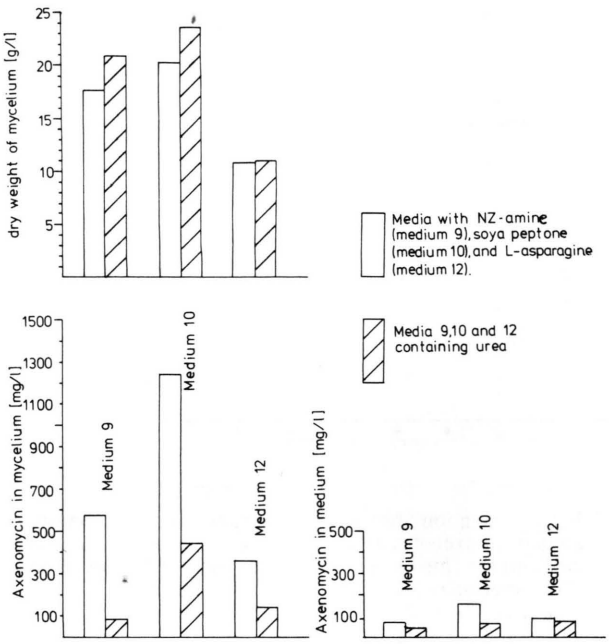

Fig. 9. Dry weight and axenomycin formation as influenced by urea added to different media containing different nitrogen sources such as NZ-amine (medium 9), soya-peptone (medium 10), and asparagine (medium 12).

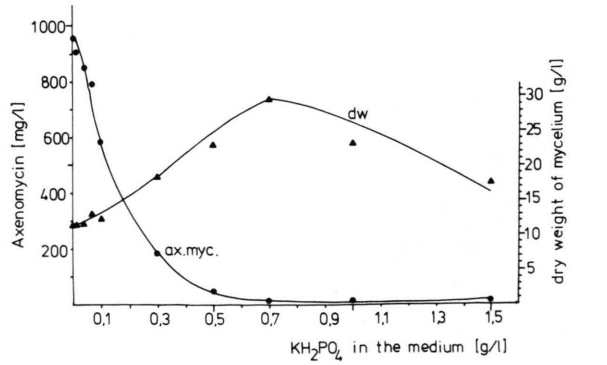

Fig. 10. Dry weight (dw) and axenomycin in the mycelium (ax. myc.) as influenced by increasing amounts of KH_2PO_4 in the medium.

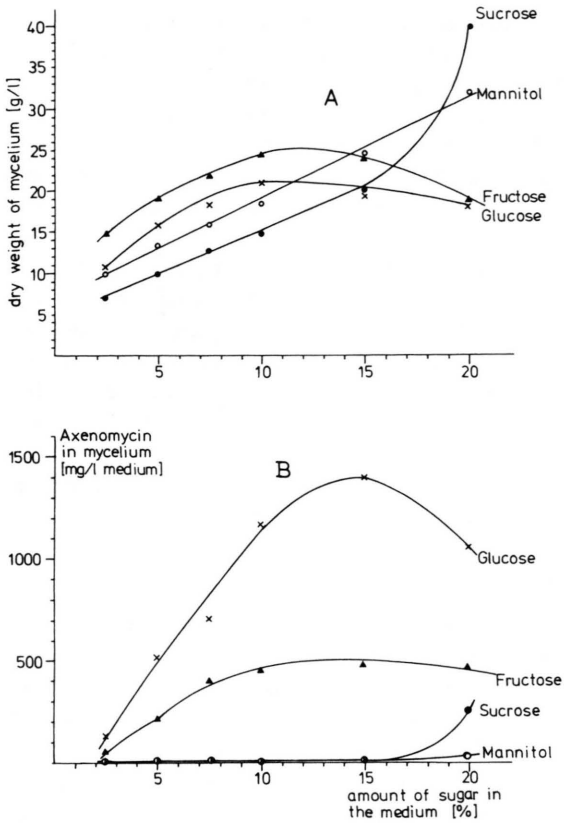

Fig. 11. Relation between increasing amounts of different carbon sources on growth (diagram A) and axenomycin formation (diagram B).

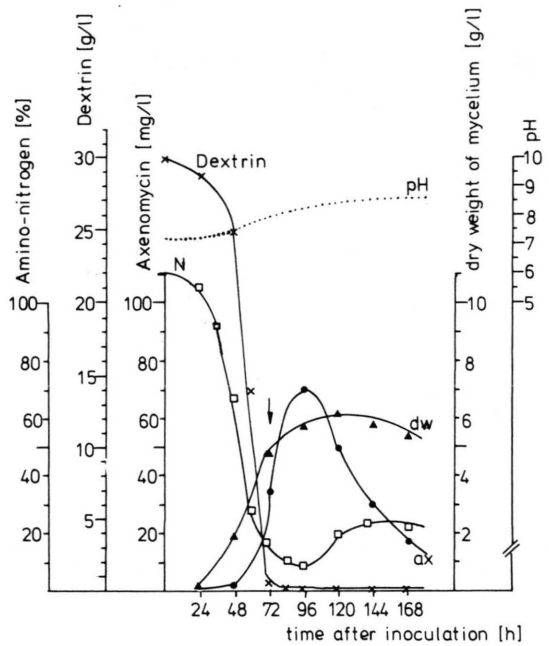

Fig. 12. Time course study of dry weight formation (dw), axenomycin production (ax) in the mycelium, amount of nitrogen (N) and dextrin in the medium and pH of the medium. The experiment was carried out with strain 3935-1-18-9 in the preculture medium.

formation of axenomycin in the sucrose medium is unusual since good production was obtained with the monomers of sucrose (*viz.* glucose and fructose). It is possible that degradation of sucrose results in the preferential utilization of one monomer of sucrose and that an excess of the other monomer accumulates within the cell. This monomer or its phosphorylated derivative might then repress axenomycin formation. *e.g.* Glucose-6- P is known to inhibit tylosine formation in Streptomyces T-59-235 [11].

Time course studies on cultures of Streptomyces lisandri

Time course studies on cultures of *Streptomyces lisandri* were carried out with different media. In a typical experiment, pH, the amount of nitrogen and

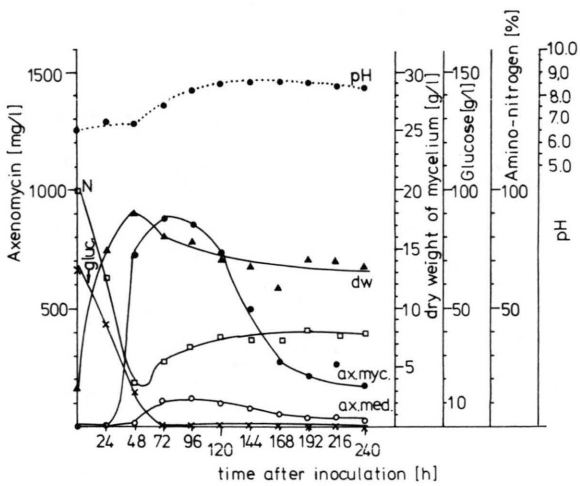

Fig. 13. Time course study of dry weight formation (dw), axenomycin production in the medium (ax. med.) and mycelium (ax. myc.), amount of nitrogen (N) and glucose (gluc) in the medium and pH of the medium. The experiment was carried out with strain 3935-1-18-9 in medium 12.

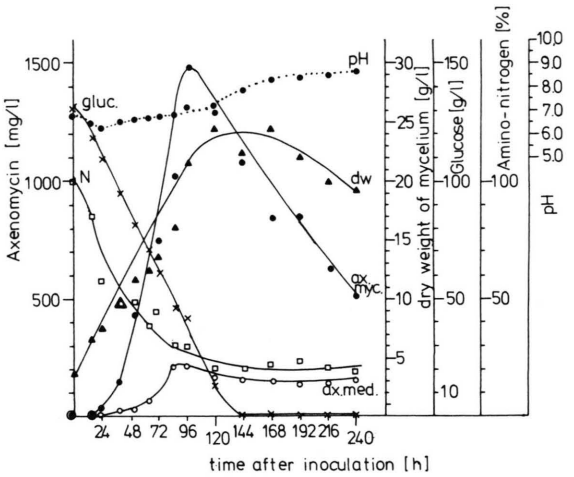

Fig. 14. Time course study of dry weight formation (dw), axenomycin production in the medium (ax. med.) and mycelium (ax. myc.), amount of nitrogen (N) and glucose (gluc.) in the medium and pH of the medium. The experiment was carried out with strain 3935-1-18-9 in medium 10.

carbon in the medium, dry weight of the mycelium and the amount of axenomycin in the medium and the mycelium were recorded. In every case the fermentation cultures were inoculated with a suspension of cells grown in a preculture medium (Fig. 12). There are common characteristics of fermentation runs in different media as exemplified by fermentations in media 10 (Fig. 14) and 12 (Fig. 13). In each case the pH of the medium shifted from 6.5 to 8.8 and only minor amounts of axenomycins were detectable in the medium (compare Fig. 2). As axenomycin production began a considerable amount of the carbon source was still present in the medium. Onset of axenomycin formation coincided in every case, however, with depletion of the nitrogen source (compare Fig. 8). Maximum antibiotic formation was always observed 96 h after inoculation while growth as measured by dry weight, was normally completed 48 h after inoculation (Fig. 13). Medium 10 represents an exception to this rule because growth does not reach the stationary phase before 120 h after inoculation (Fig. 14) *i.e.* trophophase and idiophase are not separated. The sequential appearance of trophophase and idiophase as shown in Fig. 13 is the pattern normally expected and also usually observed in these experiments.

Acknowledgements

Thanks are due to Drs. F. Arcamone, A. Grein, M. Bianchi, and A. Minghetti, Farmitalia, Milan, Italy, for samples of axenomycins A, B and D and for helpful discussions. Appreciation is also expressed to Dr. H. Pape and his coworkers, Institut für Mikrobiologie der Universität Münster, for many helpful suggestions. The technical assistance of Frau M. Winnemöller is gratefully acknowledged. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Forschergruppe "Sekundäre Naturstoffe/Zellkulturen").

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