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Thin-layer chromatography as an aid to production and proper timing of the extraction of an antibiotic complex

Various thin-layer chromatographic procedures for the separation and bio-autography of antibiotic substances exist in the literature[1,2]. An instant thin-layer chromatographic (ITLC)[3] procedure has been much help in assessing the probability of an antibiotic in question being previously isolated or not. These chromatographic procedures combined with chemical studies make it quite easy to assign a proper place to an antibiotic substance in question. The problem is, however, little different when an unknown sample of antimicrobial substance turns out to be a complex of active substances.

While working with a new complex of antibiotic—named cyathin—produced by the fungus *Cyathus helenae*, we encountered difficulties in achieving high yields of

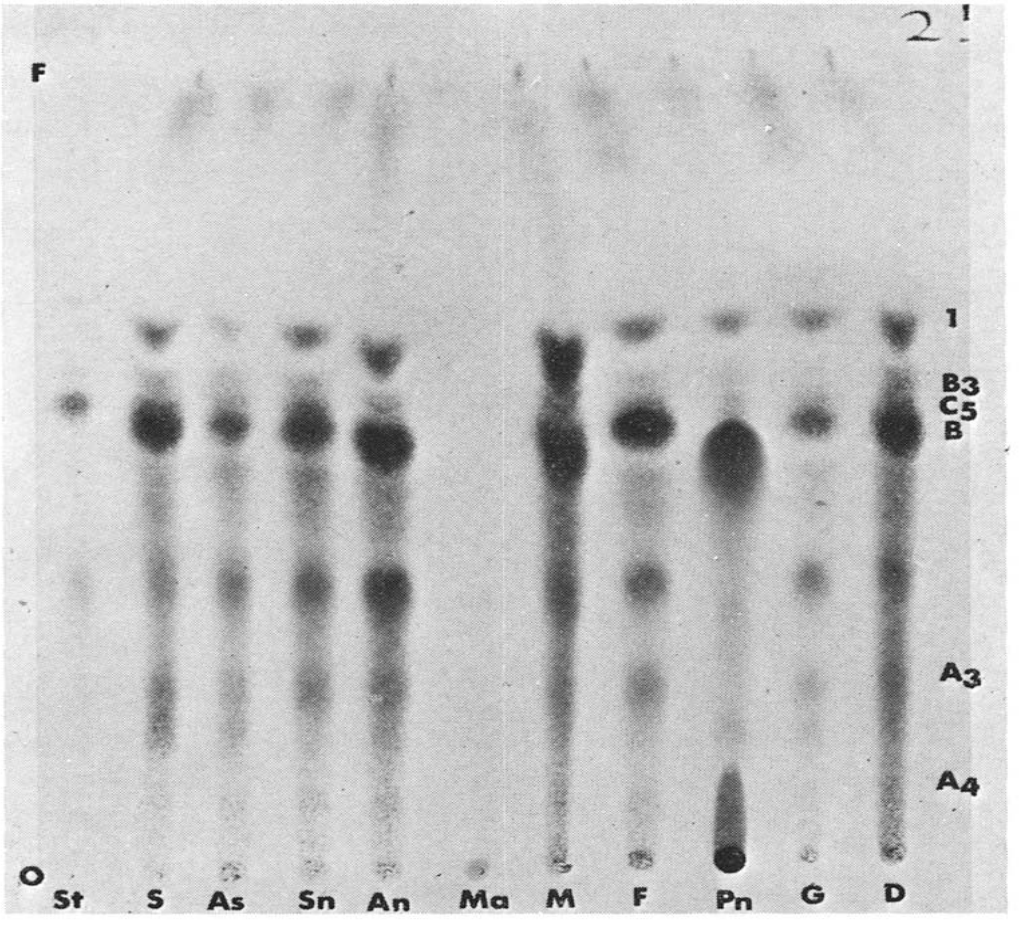

Fig. 1. Thin-layer chromatogram of the cyathin complex produced by the fungus on various C/N sources at 25th day of growth. For procedural details refer to *Methods* section. The letters and numbers on right column refer to various cyathin fractions which have been purified to date. Some of the unnumbered and unmarked spots are yet to be characterized. On the left column O refers to the origin and F to the solvent front of the chromatogram. Bottom line: St = starch; S = sucrose; As = ammonium sulfate; Sn = sodium nitrate; An = ammonium nitrate; Ma = mannitol; M = maltose; F = fructose; Pn = potassium nitrate; G = glycine; D = dextrose.

certain fractions of particular interest to us[4]. Whereas in one batch culture we would get high yield of one fraction, in the other one it may be some other fraction. For single component antibiotics it has been easy to increase the yield of active substance by manipulating the physical and/or chemical environment in which the microorganism is growing; for a multicomponent system, however, such a technique needs further attention because the effect of an environmental change may or may not be reflected upon all the active substances of the complex. During an exhaustive study of the physiology of cyathin production, it was noted that sources of carbon (C) and nitrogen (N) played a deciding role in the changing pattern of the cyathin complex. Therefore, a chromatographic study of the culture filtrate of *Cyathus helenae* was made, to which various combinations of C/N sources had been added. This short note reports on the success achieved by such a technique in the timing of extraction as well as proper selection of a C/N source for maximum production of just a fraction of the cyathin complex.

Materials and methods

The details of the isolation procedure of cyathin and culturing of the fungus have been recently reported[4].

TLC was performed according to STAHL[5]. A Shandon applicator and TLC kit

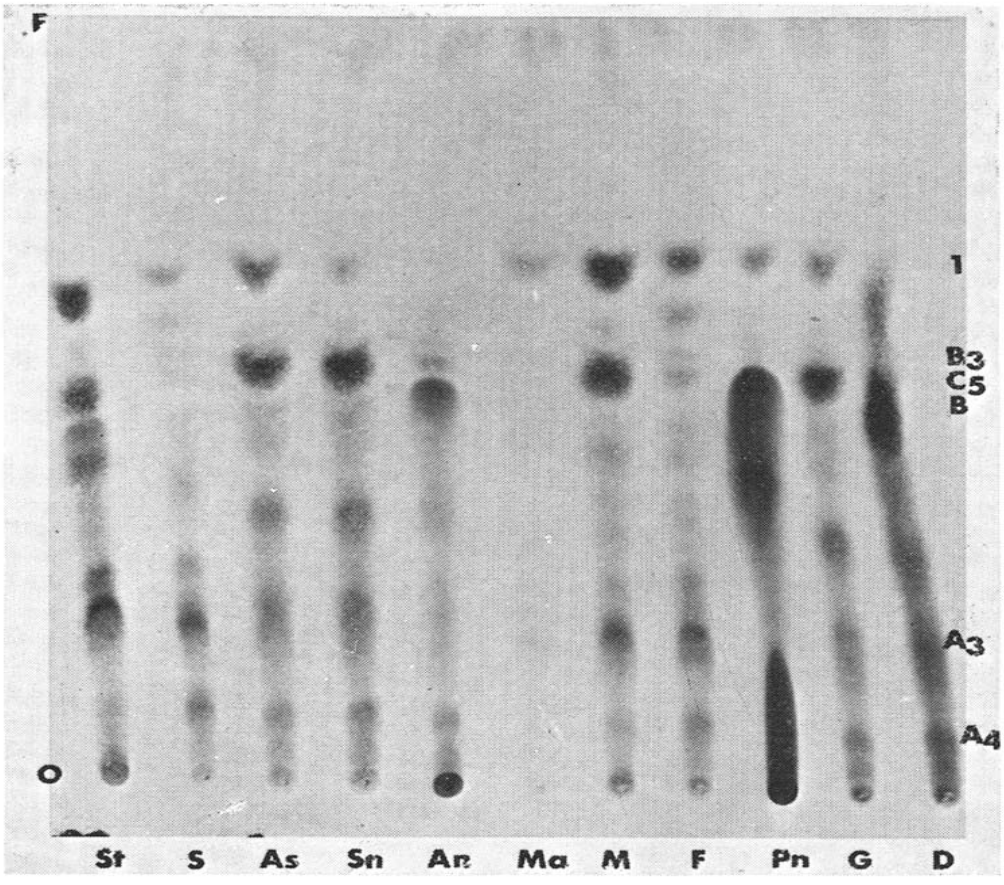

Fig. 2. Thin-layer chromatogram of the cyathin complex produced by *C. helenae* on various C/N sources at 30th day of fungal growth. Details same as for Fig. 1.

was used. Thin-layer plates (20 × 20 cm), 0.5 mm in thickness, were prepared using Silica Gel G (Merck) as the sorbent. The plates were heated at 100° for 30 min before use.

A known volume of the culture filtrate was extracted with ethyl acetate for each combination of C/N source. The organic phase, consisting mainly of the cyathin complex, was reduced under vacuum and the residue was dissolved in sufficient quantity of methanol to provide a concentration of 20 ml crude filtrate for each ml of methanol used. Four such extractions were made, the first at 25th day and the last at 40th day of fungal growth. In all, 11 combinations of C/N sources were tested.

Fifty microliter of concentrated organic phase was spotted onto a thin-layer plate. Benzene–acetone–acetic acid (75:25:1) was used as the solvent system. For routine analysis, known components of the cyathin complex were located on the chromatograms by spraying the plate with a 30% solution of sulphuric acid. These spots had earlier been tested for bioactivity and it was for the convenience only to utilize a quick procedure to identify them without going into the microbiological testing of the plate every time, that sulphuric acid technique was employed. The size and the intensity of the spot for various active fractions was noted to make a rough comparison of the amounts produced at any growth interval.

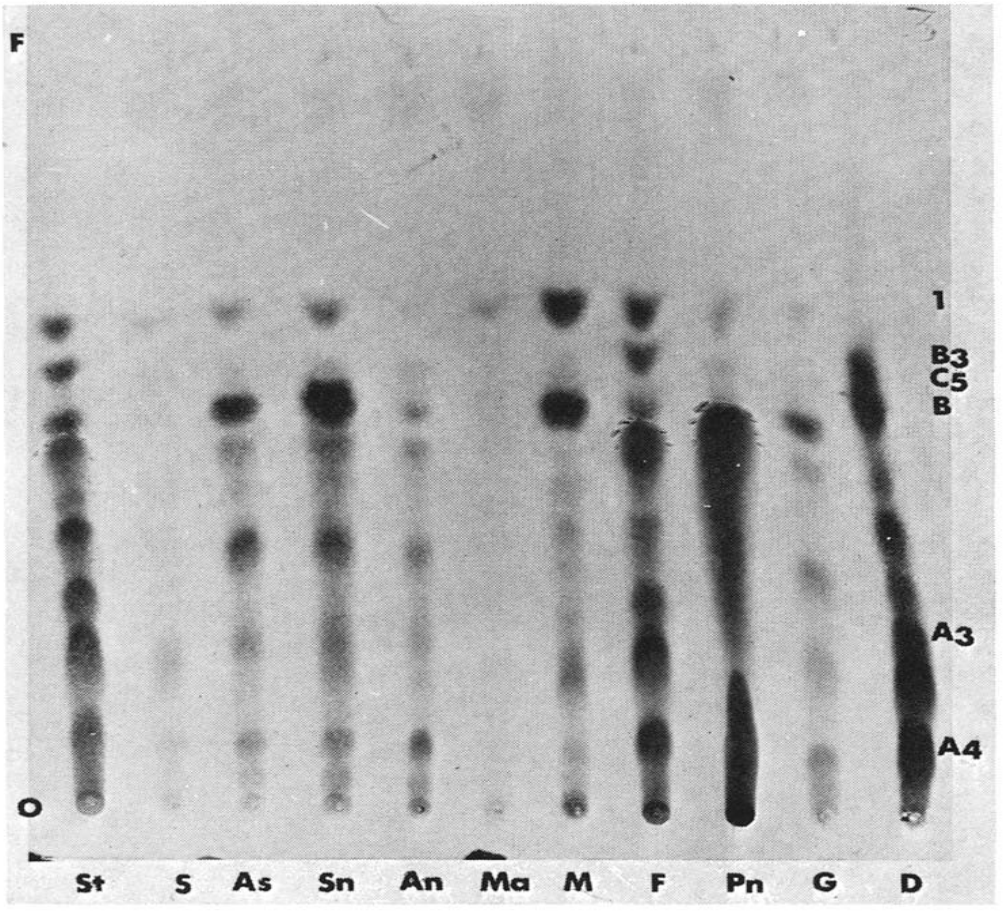

Fig. 3. Thin-layer chromatogram of the cyathin complex produced by *C. helenae* on various C/N sources at 35th day of fungal growth. Details same as for Fig. 1.

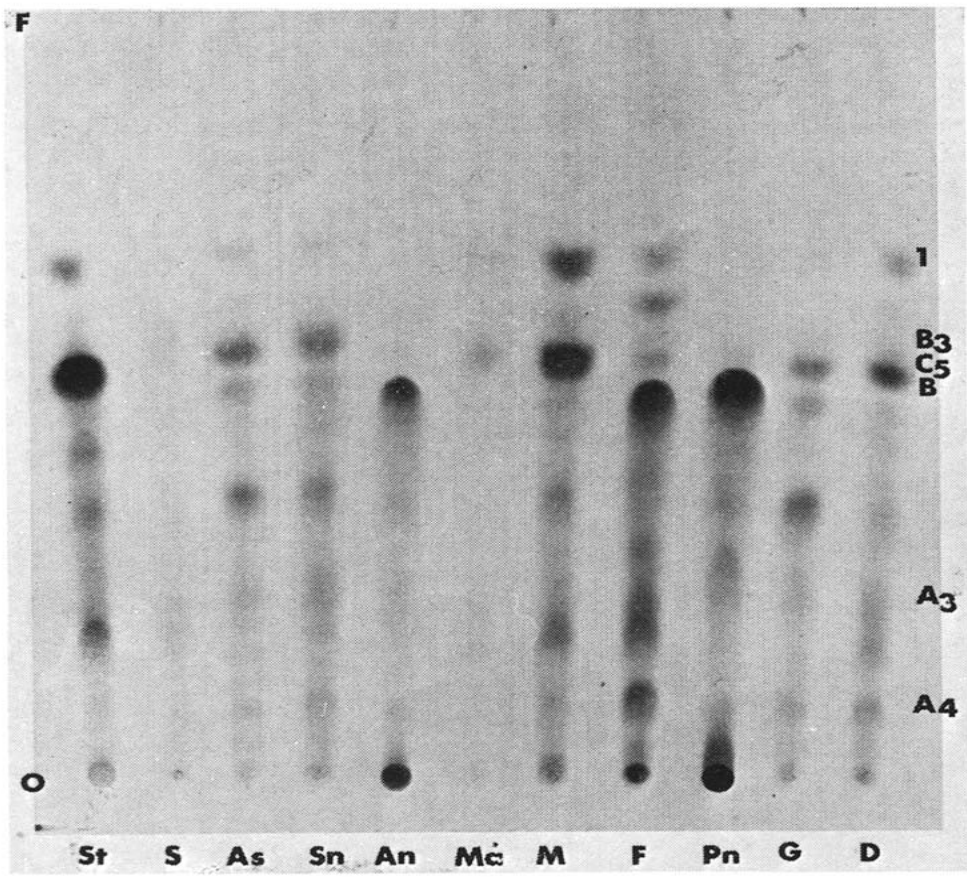

Fig. 4. Thin-layer chromatogram of the cyathin complex produced by *C. helenae* on various C/N sources at 40th day of fungal growth. Details same as for Fig. 1.

Being a complex of active substances, an arbitrary terminology has been adopted to name the active fractions of the cyathin complex. Cyathin No. 1 (67); cyathin B (51); cyathin B_3 (56); cyathin C_5 (54); cyathin A_3 (27); cyathin A_4 (06). The numbers in parentheses are $R_F \times 100$ values in solvent system benzene–acetone–acetic acid (75:25:1).

Results and discussion

The chromatograms of cyathin complex obtained from the culture filtrate of *C. helenae* from various combinations of C/N sources at different intervals are reproduced in Figs. 1–4. The results were tabulated for a quick reference to the amounts of active substances produced under the above mentioned conditions (Table I). Although semiquantitative, the size of the spot and its intensity just after spraying was a rough measure of the concentration of the active substance.

Although cyathin No. 1 was produced by the fungus on a number of C/N sources, sufficient amounts of this component were produced only on ammonium nitrate and maltose. Cyathin C_5 has been found to be a very active substance but insufficient quantities of the compound has always hindered a detailed antimicrobial spectrum. From the chromatographic study of the culture filtrate it became obvious that this fraction apparently disappears just after the 25th day. Maltose with calcium

TABLE I

EVALUATION OF CHROMATOGRAPHIC SPECTRUM OF THE CULTURE FILTRATE FROM CARBON AND NITROGEN SOURCES

For carbon sources, calcium nitrate was used as the basal nitrogen source whereas for different nitrogen sources, dextrose served as the carbon source. —, Absence of a spot for a specific fraction of the cyathin complex. +, Presence of a spot. Comparative amounts of different fractions shown by extra + to denote the larger size and intensity of the spot.

	St	*S*	*As*	*Sn*	*An*	*Ma*	*M*	*F*	*Pn*	*G*	*D*
Cyathin-fractions (25th day)											
No. 1	—	+	+	+	++	—	+++	+	+	+	+
B_3	—	+	+	+	+	—	+++	+	+	+	+++
C_5	+	++	+	++	+++	—	+++	++	—	+	++
B	—	—	—	+	++	—	+	+	+++	—	+
A_3	—	+	+	+	++	—	+	+	—	+	+
A_4	—	—	—	—	—	—	—	—	++	—	+
Cyathin-fractions (30th day)											
No. 1	+	+	+	+	—	—	++	+	+	+	+
B_3	+	—	+	+	—	—	+	+	+	—	++
C_5	—	—	++	++	+	—	++	+	—	+	++
B	—	—	+	+	++	—	—	+	++++	—	+
A_3	—	—	+	+	—	—	—	—	—	+	+
A_4	—	—	+	+	+	—	—	—	++++	+	+
Cyathin-fractions (35th day)											
No. 1	+	—	+	+	—	+	++	+	—	—	+
B_3	++	—	+	+	—	—	+	+	+	—	+++
C_5	—	—	+	++	+	—	++	+	—	+	+
B	—	—	+	+	++	—	—	++	+++++	—	—
A_3	—	—	+	+	—	—	—	+	—	—	+
A_4	—	—	—	+	+	—	—	++	+++	+	+++
Cyathin-fractions (40th day)											
No. 1	+	—	+	+	—	+	+	+	—	—	+
B_3	—	—	—	+	—	—	+	+	—	—	—
C_5	—	—	+	+	—	+	+	+	—	+	+
B	—	—	—	—	++	—	—	++	+++	+	—
A_3	+	—	—	+	—	—	—	+	+	—	—
A_4	—	—	—	—	—	—	—	+	+	—	+

nitrate as the nitrogen source appeared to be a suitable combination for the production of more or less all the non-polar cyathin components.

Cyathin B or chromocyathin was easy to recognize on the TLC plate as it gives an orange brown colour even before spraying with sulphuric acid. This fraction has since been characterized as 2,4,5-trihydroxybenzaldehyde and also chemically synthesized[4]. However, it was interesting to note that when potassium nitrate was used as the nitrogen source along with dextrose, almost all the cyathin complex consisted of cyathin B and cyathin A_4. Production of the latter fraction was also quite marked on a dextrose and calcium nitrate combination. Cyathin A_3 was produced by *C. helenae* on a number of C/N sources but yields were not high on any one of them.

A chromatographic evaluation of the culture filtrate of the fungus from various combinations of C/N sources explained that cyathin components are very variable and that even a small change in the composition of the medium could bring about a drastic change in the pattern of the active complex. This study also indicated the

best combination of C/N source which would aid production of the whole cyathin complex or of just one particular fraction. Last but not the least important, the chromatographic analysis of the culture filtrate also indicated the best time of harvesting the cultures when a particular fraction of the cyathin complex would be present in largest amount.

It is hoped that this study will be of interest to those working with antibiotics or secondary metabolites of microbial origin where often it is essential to do a routine check of the activity and production of the compounds. Such a study is especially useful where antimicrobial products can only be produced biologically.

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