# ANTIMYCIN A FERMENTATION

## II. FERMENTATION IN AERATED-AGITATED FERMENTERS

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Fermentation characteristics, previously studied in shake flasks, were reproduced in aerated-agitated fermenters, using three strains of *Streptomyces* sp. which had been selected for their high antimycin A productivity in shake flasks. Fermentation in fermenters was run in three stages. The medium consisted of soy flour, glucose, ammonium sulfate and calcium carbonate; initial pH was 7.2~7.5, and temperature 25°C.

The course of fermentation was then modified to encourage maximal growth and eliminate the intermediate lag period observed in shake flasks. Useful corrections included continuous addition of soybean oil at 1.25%/day and maintenance of pH at 6 by addition of ammonium hydroxide on demand. The ammonium hydroxide added also served as a rapidly utilized nitrogen source and could not be replaced by NaOH or KOH. Under optimal conditions antimycin A was produced at constant rate from the second to the sixth day, when maximum yields of more than 9 g/liter were attained. A procedure for antimycin A extraction is described.

Optimization of antimycin A fermentation in shake flasks has been reported[14]. Maximal productivity of strains selected after mutagenic treatment was expressed, when environmental conditions were adjusted to meet their individual requirements. Critical factors were absence of phosphate and organic salts, proper choice of the organic nitrogen source and ratio organic/inorganic nitrogen, addition of calcium carbonate, and presence of a rapidly utilizable carbon source, glucose. Addition of a slowly utilizable carbon source, lactose, and a non-carbohydrate carbon source, soybean oil, was highly beneficial. The rapid utilization of glucose entailed a sharp pH drop which curtailed growth and antibiotic production until the organism could metabolize the acids produced and excrete enough ammonia into the medium to restore pH conditions appropriate for growth and further antibiotic production. Control of pH should contribute to eliminate this intermediate, unproductive period, and shorten the fermentation time.

The purpose of this work is three-fold: scale-up of antimycin A fermentation, which had been optimized in shake flasks[14], to aerated-agitated fermenters[1]; comparison of improved strains selected for productivity in shake flasks; and corrections of the fermentation parameters to obtain maximum yields in the shortest time.

### Materials and Methods

Microorganisms. *Streptomyces* sp. strains AY M-306, M-506 and M-606 were used[14]. Preservation, maintenance and propagation methods, as well as preparation of the spore inoculum for fermentation, were the same as before[14].

Media. The inoculum medium consisted of (g/liter): Special X soy flour (Archer Daniels

Midland Co., Minneapolis, Minn.), 40; "Cerelose" (a pharmaceutical grade of glucose, Corn Products Corporation, New York), 20; $(NH_4)_2SO_4$, 3; $CaCO_3$ U.S.P. (Baker), 1.5; lard oil ("Larex No. 1", Swift, Montréal), 1 ml; pH 7.0~7.2; the medium was completed to volume with tap water. The medium was dispensed into 500-ml Erlenmeyer flasks, 12-liter Florence flasks, 5-liter fermenters (model FS-305, New Brunswick Scientific Co., New Brunswick, N.J.), or 14-liter fermenters (model CFS-314), at the rate of 50 ml, 1.6 liters, 3 liters or 9 liters respectively, and sterilized at 121°C for 20 minutes, 45 minutes, 1 hour or 2 hours, respectively. Occasionally, 130-liter fermenters (model CF-130) equipped with automatic antifoam addition and pH control systems were used: each fermenter was filled with 80 liters of inoculum medium, and sterilized at 121°C for 45 minutes (150 rev/min) by circulating steam in the jacket.

Two production media were used: initially the production medium was the same as the inoculum medium above; it is referred to as medium A in the text. Medium B had the following composition (g/liter tap water): Nutrisoy 220 soy flour (Archer Daniels Midland Co., Minneapolis, Minn.), 60; "Cerelose", 20; $(NH_4)_2SO_4$, 6; $CaCO_3$ U.S.P., 3; lard oil, 2 ml; pH 7.1~7.2. The production media were dispensed into 250-liter fermenters (model F-250) equipped with automatic antifoam addition systems, and pH recorders-controllers. Sterilization was at 121°C for 45 minutes (150 rev/min) by circulating steam in the jacket. The role of lard oil was to prevent excessive foaming during sterilization; the amount added was rapidlyutilized after inoculation.

Inoculum. Fermentation was generally carried out in three stages. The first-stage inoculum was prepared in shake flasks: 500-ml Erlenmeyer flasks (50 ml of inoculum medium) were inoculated with 1% spore suspension and incubated for 24 hours, at 25°C and 70% relative humidity, on a New Brunswick Scientific gyrotory shaker, model G-53, at 240 rev/min (2″-stroke). The second-stage inoculum was generally prepared in 12-liter Florence flasks containing 1.6 liter of inoculum medium; flasks were inoculated with 1% of the first-stage inoculum, and incubated for 18 hours, at 25°C and 70% R.H., on a reciprocating, model R-82, shaker set at 75 rev/min (2″-stroke). Occasionally, the second-stage inoculum was prepared in 5-liter and 14-liter fermenters: the fermenters were inoculated with 1% of the first-stage inoculum, and incubated fours 18 hours at 25°C; agitation was 400 rev/min and aeration 0.5 vol air/vol medium/min. DF-143 PX antifoam agent (Mazer Chemicals, Inc., Gurnee, Illinois) was added automatically on demand. When 130-liter fermenters were used, the conditions were the same as above, except for agitation which was set at 300 rev/min.

Production. The production stage was carried out in 250-liter fermenters inoculated with 1~5% of the second stage inoculum. Temperature was 25 or 30°C, agitation 250 rev/min, and aeration 0.25~1.0 vol/vol/min. Foaming was controlled automatically by addition of DF-143 PX antifoam agent on demand. For pH control a Leeds & Northrup, model 7678, recorder-controller dual system was used, which consists of steam-sterilizable electrodes and acid/base reservoir assemblies, and a potentiometer. pH was automatically controlled by addition of 10 N $NH_4OH$ or 5 N $H_2SO_4$. Sulfuric acid was available as a precaution, but was generally not used. When continuous addition of nutrients was required, a peristaltic pump was used which operated at preset speed necessary to deliver a given volume of a 40% solution of "Cerelose" or of crude soybean oil SB-1 (Canada Linseed Oil Ltd., Montréal) per time unit: the delivery is expressed in percent sugar or oil/day. The "Cerelose" solution was sterilized at 121°C for 45 minutes; soybean oil was sterilized at 140°C for 16 hours (dry heat).

Antimycin A determination. Antimycin A in fermentation broth was determined spectrophotofluorometrically[11,12].

Mycelial mass determination. Inoculum and production media contained particulate matter, and the mycelial mass could not be determined accurately. However, an approximate value was obtained by the method previously described[14].

Glucose estimation. To determine glucose in fermentation broth filtrates HUNTER's[8] automated assay was used.

Non-protein nitrogen estimation. Trichloracetic acid was added to fermentation broth filtrates to precipitate proteins; the non-protein nitrogen content was determined in the supernate, using the Technicon automated assay[2].

## Results

### Influence of Aeration and Agitation

The first goal was to establish in 5-liter fermenters the conditions required to reproduce the yields observed in shake flasks[14]. *Streptomyces* sp. M-306 was grown in medium A in two stages, and 2% inoculum was used at each stage. The first stage was run in shake flasks and the second stage either in shake flasks or in 5-liter fermenters. The results are reported in

Table 1. Influence of agitation and aeration on antimycin A production by *Streptomyces* sp. M-306.

| Type of vessel[a] | Agitation (rev/min) | Aeration (vol/vol/min) | Antimycin A[b] ($\mu$g/ml) |
|---|---|---|---|
| 5-liter fermenters (two-stage fermentation) | 250 | 0.5 | <700 |
| | | 0.75 | <700 |
| | | 1.0 | <700 |
| | 350 | 0.5 | 1,110 |
| | | 0.75 | 1,320 |
| | | 1.0 | 1,800 |
| | 450 | 0.5 | 1,930 |
| | | 0.75 | 1,940 |
| | | 1.0 | 1,950 |
| Shake flasks, 500-ml Erlenmeyer (two-stage fermentation) | Rotry shaker, 240 rev/min, 2″-stroke | | 2,190 |

[a] Production medium: medium A; 2% inoculum.
[b] 70-hour fermentation; final pH 8.2.

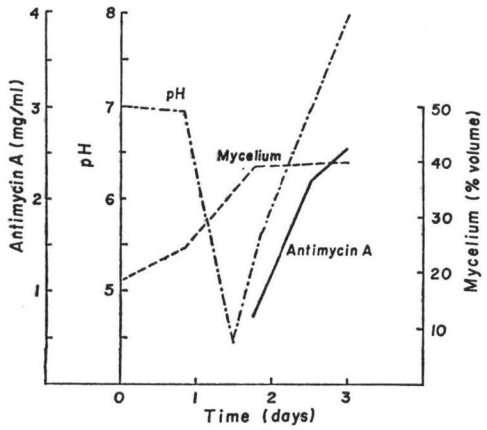
Table I. In fermenters, the conditions that most closely matched those for shake flasks were an aeration of 0.5~1.0 vol air/vol/min, and agitation of 450 rev/min. Maximum yield was attained after 70 hours of incubation. When the production stage was run in 14-liter and 130-liter fermenters, the results were essentially the same, except that in 130-liter fermenters the production peak occurred 12 hours later. The same yields were obtained in 250-liter fermenters operated at 250 rev/min and 0.5 vol air/vol/min: under these conditions the power input was 1.4 HP/100 gallons.

### Influence of Inoculum Size and Age

In shake flasks, highest yields were observed when 1% spore suspension was used to inoculate the first-stage inoculum, 1% of the latter to inoculate the second-stage inoculum, and 1% of the second-stage inoculum to inoculate the production stage; the age of the first-stage inoculum was 18~24 hours, but the second-stage inoculum had to be 18-hour old; a 24-hour old inoculum was much less productive. This effect may result from the selection of less active variant cells developing in the older inoculum. A similar effect was observed in fermenters: no difference was noticed when 1 or 2% inocula were used, but yields were decreased by 10%

Fig. 1. Time course of antimycin A fermentation in 5-liter fermenters.

Medium, A, two-stage fermentation. *Streptomyces* sp. M-306.




accordance with a trophophase-idiophase pattern[8] which is typical of many antibiotic fermentations (see DEMAIN[5]).

when the production stage was inoculated with 5% of second-stage inoculum. Here again, the best inoculum age was 18 hours; a 40-hour old second-stage inoculum depressed yields by 90%. DF-143 PX antifoam agent was added on demand.

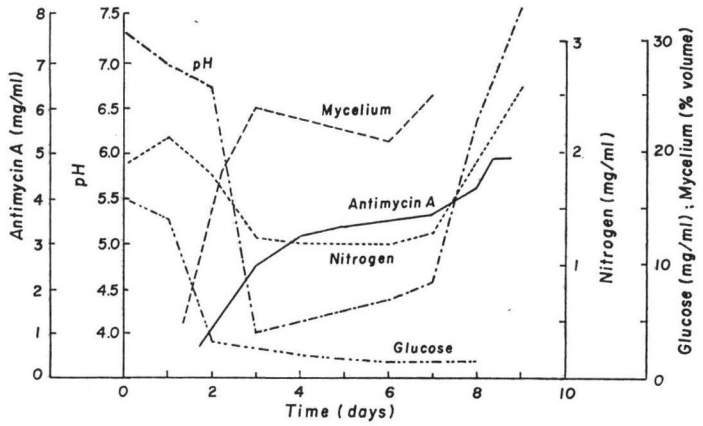
A typical time course of antimycin A fermentation by *Streptomyces* sp. M-306 in medium A is illustrated in Fig. 1. Mycelial mass increased steadily and pH dropped sharply during the first 40 hours of incubation. Then, pH climbed rapidly to 8.2 after 72 hours. Antimycin A was produced linearly from the 40th to the 72nd hour when it abruptly attained a plateau. This fermentation is in

## Comparison of Strains M-306 and M-506

When medium B became available[14], the influence of a large number of antifoam agents was studied. All agents, except lard oil and DF-143 PX, were found toxic when added before sterilization. Lard oil added at 0.2% before sterilization controlled excessive foaming during the process; its effect lasted up to 8~12 hours after inoculation, after which it had no value probably because, like other oils (sperm oil, soybean oil, peanut oil), it was utilized very rapidly. During fermentation, DF-143 PX was the least toxic and most efficient agent tested: only 0.3~0.4% were needed during the course of fermentation. A three-stage fermentation (1st stage in 500-ml flasks, 2nd stage in Florence flasks) with strain M-306 in medium B (250 rev/min; 1 vol air/vol/min) yielded 3,400 $\mu$g/ml after 6 days of fermentation; under the same conditions strain M-506 yielded 3,800 $\mu$g/ml. When 0.5% lactose and 1.5% soybean oil were added to medium B, yields of 6,200 and 6,750 $\mu$g/ml were obtained with strains M-306 and M-506 respectively.

A typical time course of antimycin A fermentation by strain M-506 in enriched medium B is illustrated in Fig. 2; it is very similar to that previously observed in shake flasks[14]. Initially, the organism grew very rapidly, while glucose was utilized and became nearly exhausted at 48-hour incubation; pH dropped sharply to reach a value of 4 at 72 hours, and antimycin A accumulated significantly. From the 3rd to the 6th days, pH, mycelial mass, non-protein nitrogen and glucose remained constant, and antimycin A was produced very slowly. On the 6th day, growth was resumed, and from the 7th day on, pH rose steadily, as well as non-protein nitrogen excreted, and a second cycle of rapid antimycin A production was observed. Fermentation was terminated on the 9th day, when pH had reached 8.2 and antimycin A concentration was 5,000 $\mu$g/ml. Under the same conditions strain M-306 yielded only 3,500 $\mu$g/ml and the fermentation lasted 11 days. During these fermentations the effect of increasing the concentration of the carbon sources by the addition of lactose and oil at the beginning of fermentation was to increase growth and accumulation of the antibiotic in the first days of

Fig. 2. Time course of antimycin A fermentation in 250-liter fermenters.
Medium B; enriched with 0.5% lactose and 1.5% soybean oil. Three-stage fermentation.
*Streptomyces* sp. M-506.




fermentation, and to keep pH at very low values until most of the carbon source was utilized. During this period the organism could not grow, but could metabolize and eventually utilize the acids previously formed and excrete sufficient ammonia nitrogen to increase the pH to a value that allowed further growth and antibiotic production. Another effect was to double the fermentation time.

## Continuous Addition of Glucose

JOHNSON and his collaborators[4,7,13] reported that lactose, in penicillin fermentation, could be replaced by more rapidly assimilated sugars, such as glucose and sucrose, if these were added continuously at rates of the same order as that at which lactose disappeared. To test this effect in antimycin A fermentation strain M-506 was inoculated in medium B, which contained neither lactose nor oil; continuous addition of glucose at 0.7%/day was started 30 hours after inoculation, when 50% of the sugar originally present in the medium had been utilized; this precaution was dictated by the previous observation[14] that the organism cannot tolerate more than 2% glucose and 0.5% lactose at inoculation time. As expected, pH dropped rapidly to 4.1 after 72 hours; antimycin A was produced from the 2nd to the 5th days, when the yield observed was 4,000 $\mu$g/ml. pH remained stationary and antimycin A constant until the 12th day when glucose addition was stopped. Then, the pH rose slowly to reach 7.6 on the 15th day, and antimycin A was produced to reach 5,900 $\mu$g/ml on the same day. Thus, the beneficial effect of lactose and oil in enriched medium B could be reproduced by continuous addition of glucose to medium B, but fermentation time was increased by 6~7 days, a very un-economical consequence[10].

## Effect of pH Control

From the previous experiment in fermenters and results in shake flasks[14], it appears that the organism neither grows nor produces the antibiotic once pH has fallen below 5. On the other hand, it has been shown in fermenters and shake flasks[14] that satisfactory fermentation was always associated with a sharp drop of pH during the first days of fermentation. To determine the effect of pH control, strain M-506 was inoculated in medium B, and glucose was fed continuously as before (0.7%/day); pH dropped to 7 in 24 hours and was maintained

Fig. 3. Time course of antimycin A fermentation in 250-liter fermenters.
Medium B; continuous addition of glucose at 0.7 %/day; pH controlled at 7 by automatic addition of 10 N $NH_4OH$.
Three-stage fermentation. *Streptomyces* sp. M-506.

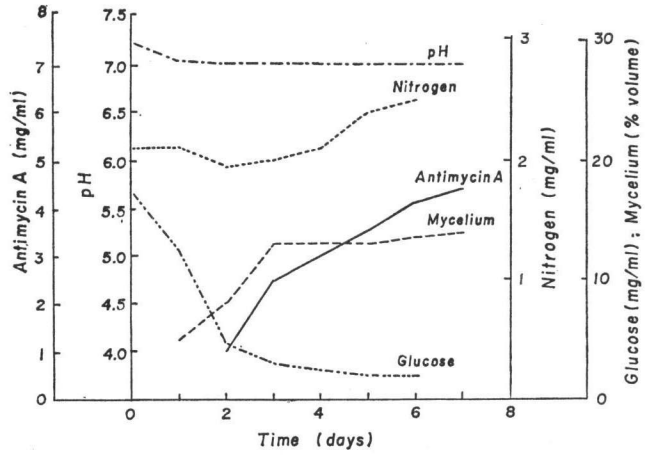
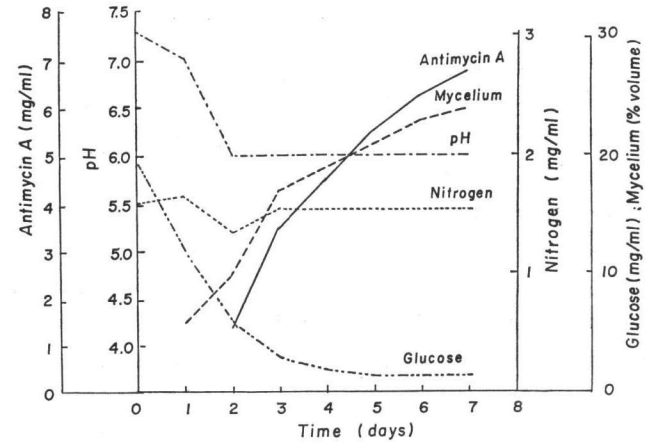



Fig. 4. Time course of antimycin A fermentation in 250-liter fermenters.
Medium B; continuous addition of glucose at 0.7 %/day; pH controlled at 6 by automatic addition of 10 N $NH_4OH$.
Three-stage fermentation. *Streptomyces* sp. M-506.




at this value through automatic addition of 10 N $NH_4OH$. The time course of this fermentation run is illustrated in Fig. 3. Glucose was utilized very rapidly. Maximum yield of antimycin A (4,200 $\mu$g/ml) was reached in 7 days, a time economy of 11 days over the previous experimental run. It should also be noted that non-protein nitrogen remained at a high level throughout the fermentation course (compare Figs. 2 and 3). In subsequent experiments pH was controlled at several other values, but the most beneficial effect of pH was found for a value of 6. This is shown in Fig. 4, where a maximum yield of 6,800 $\mu$g/ml was reached in 7 days.

In a separate experiment defatted Special X soy flour (50 % protein, 6~8 % fat) was substituted for Nutrisoy 220 soy flour (43 % protein, 22~23 % fat). pH was controlled at 6, and glucose fed continuously at 0.7 and even 1.4 % to compensate for the loss of energy source

resulting from the substitution of the soy flour. Maximum yields of only 4,500 $\mu$g/ml were obtained in 6 days. These results gave the indication that addition of oil to the medium might be desirable, as it was previously shown for many antibiotic fermentations.[8,9]

### Effect of Continuous addition of Soybean Oil

Strain M-506 was inoculated in Medium B. At 30 hours pH had dropped to 6 and was automatically maintained at this value (10 N $NH_4OH$) till the end of fermentation; soybean oil was added continuously at the rate of 0.7 and 1.25 % per day, starting on the 30th hour. Oil

Table 2. Influence of addition rate of soybean oil on growth and antimycin A production by *Streptomyces* sp. M-506.

| Addition rate[a] (% per day) | Fermentation time | | | | | |
|---|---|---|---|---|---|---|
| | 2 days | | 3 days | | 4 days | |
| | Mycelium (%) | Antimycin A (mg/ml) | Mycelium (%) | Antimycin A (mg/ml) | Mycelium (%) | Antimycin A (mg/ml) |
| 0.7 | 14 | 0.68 | 17 | 2.7 | 19 | 4.36 |
| 1.25 | 16 | 1.39 | 22 | 3.52 | 28 | 5.89 |

| Addition rate[a] (% per day) | Fermentation time | | | |
|---|---|---|---|---|
| | 5 days | | 6 days | |
| | Mycelium (%) | Antimycin A (mg/ml) | Mycelium (%) | Antimycin A (mg/ml) |
| 0.7 | 24 | 5.15 | 28 | 5.57 |
| 1.25 | 29 | 7.51 | 30 | 8.94 |

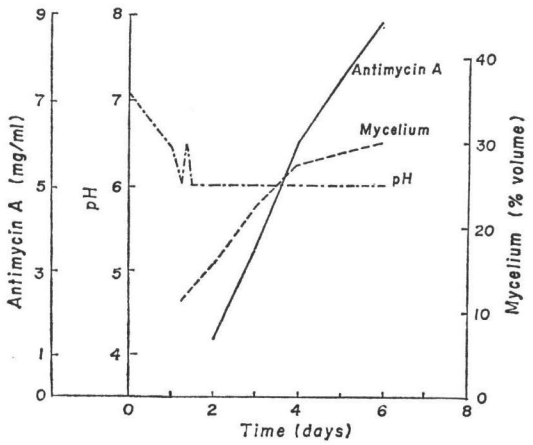
[a] Medium B, 250-liter fermenters; pH controlled at 6 by continuous addition of 10 N $NH_4OH$; three-stage fermentation.

addition was stopped when pH started to rise above 6, at which time antimycin A did not accumulate further; pH increase was irreversible and pH control using $H_2SO_4$ was useless: the productivity could not be restored, and the organism had probably reached a stage of autolysis, although no morphological changes could be observed. The effect of addition rates of soybean oil is reported in Table 2. In subsequent experiments addition rates were varied, and 1.25 %/day was found to be optimal. Higher rates led to accumulation of oil and complicated the assay and extraction procedures. A typical time course is shown in Fig. 5: antimycin A accumulated almost linearly from the second day to reach a maximum of 9,000 $\mu$g/ml in 6 days. With strain M-606, maximum yields were much

Fig. 5. Time course of antimycin A fermentation in 250-liter fermenters.

Medium B; continuous addition of soybean oil at 1.25 %/day; pH controlled at 6 by automatic addition of 10 N $NH_4OH$.

Three-stage fermentation. *Streptomyces* sp. M-506.

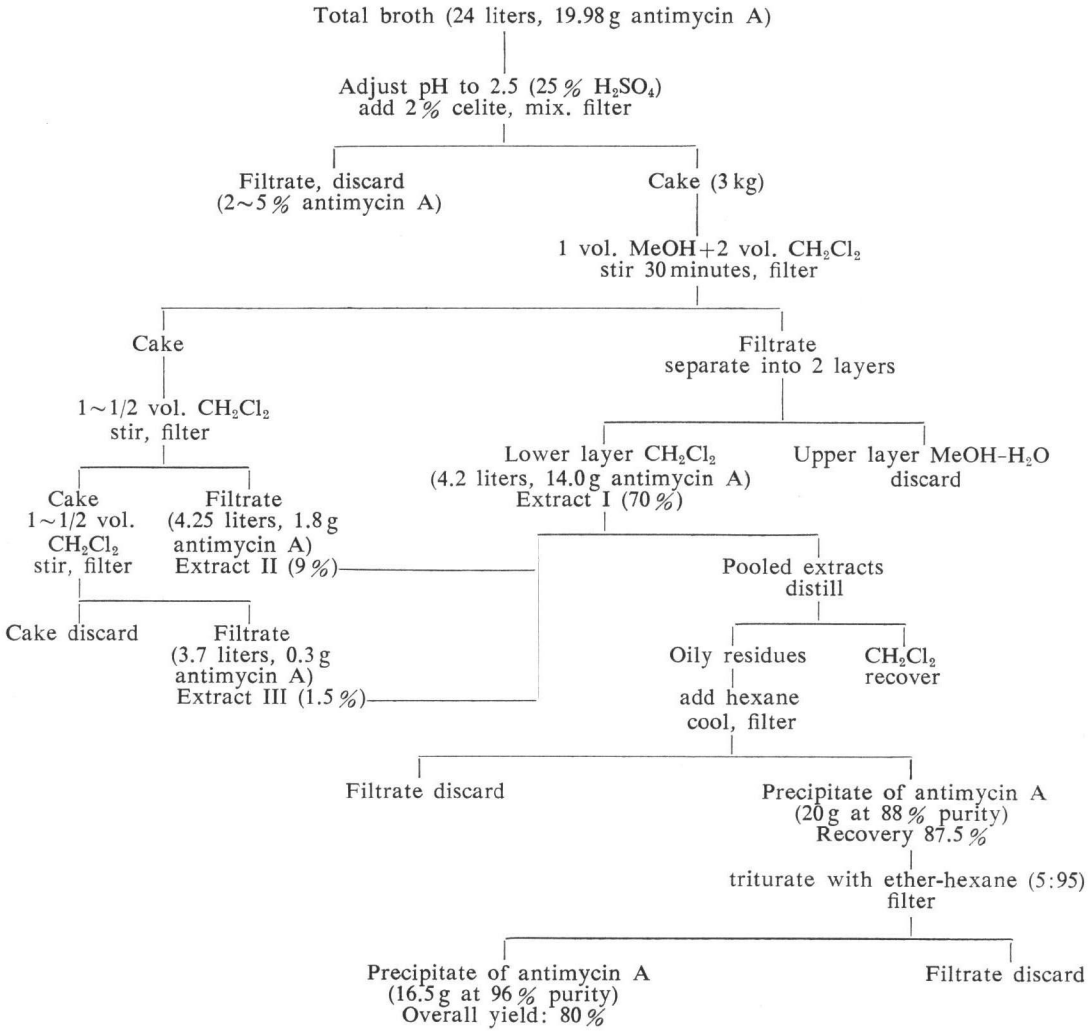


higher but occurred one day later. The pH shoulder observed between 36 and 42 hours probably reflects a lag in oil utilization. The role of $NH_4OH$ is not only to neutralize the acidity produced; it also serves as a readily available nitrogen source, as observed in subsequent experiments: $NH_4OH$ could not be replaced by NaOH, KOH or phosphate buffers the effect of which was disastrous on antimycin A yields.

## Extraction from Fermentation Broths

Antimycin A is a lipophilic substance. It is produced intracellularly, and not more than 2~5% is excreted into the medium during fermentation. Therefore, it is practical to separate the mycelium from the liquid medium at the end of fermentation, extract the antibiotic from the mycelium, and discard the filtrate. A flow diagram of antimycin A extraction is shown in Fig. 6. Total broth was acidified to pH 2.5 with sulfuric acid, 2% Celite added, and the mixture filtered on a vacuum rotary drum filter coated with Celite. The mycelium-celite cake was mixed with 1 volume of methanol and 2 volumes of methylene dichloride, and the mixture stirred

Fig. 6. Flow diagram of antimycin A extraction from microbial broth.



for 30 minutes. This mixed solvent extraction procedure has been used successfully for lipid extraction from tissue cells. The extract separated into two layers: the upper layer of methanol-water contained no antimycin A and was discarded; the lower layer of methylene dichloride contained about 70% of the antibiotic estimated in the mycelium. The extract was filtered to recover the organic solvent containing antimycin A, and the cake was extracted two more times with 1.5 volume of methylene dichloride. The methylene dichloride extracts were pooled, dehydrated over sodium sulfate, and evaporated to recover the solvent and obtain an oily residue. Antimycin A was precipitated from the oil with cold hexane and recovered by filtration on a Buchner funnel. At this stage 85~90% of the antimycin A originally present in the mycelium were recovered as a precipitate. Further purification was achieved by washing the precipitate with a mixture of ether-hexane (5:95). Overall recovery was 80% and purity 96%.

## Discussion

It was recognized early in this work that successful fermentation runs were always associated with rapid utilization of glucose and rapid pH drop in the first two days of incubation. This observation, first made in shake flasks, is depicted in Fig. 1 for aerated-agitated fermenters. Addition of soybean oil and a slowly utilized sugar, such as lactose, was beneficial in terms of productivity, but always led to excessive fermentation time. Soybean oil and lactose cannot be replaced by increasing initial glucose concentration over 2%, the maximum concentration tolerated by the microorganism. They could be replaced, however, by continuous addition of glucose, as in penicillin fermentation[4,7,18]. The effect of increasing the carbon source concentration was not apparent during the first fermentation phase. The effect was apparent during the second phase, when pH remained at 4, and growth and antimycin A production were arrested. The length of this intermediate lag phase was about 4 days in medium B (Fig. 2); it lasted 6 more days when soybean oil and lactose were added to the medium; when glucose was fed continuously to medium B the second phase lasted as long as glucose was added. This intermediate lag phase came to an end when the carbon source was exhausted; this phase was not detrimental to the organism which could always manage to restore suitable conditions (third phase) for resuming growth and antibiotic production, probably by utilizing acidic compounds produced previously, and certainly by excreting ammonia into the medium. This third phase generally lasted 2 days and came to an end when pH reached 8.2~8.4 and antimycin A attained a maximum (it was destroyed as rapidly as it was produced). Addition of phosphate buffer caused the sudden arrest of antibiotic production, hence could not be made to maintain pH at 7. During this last phase the organism sporulated abundantly; and antibiotic production was growth dependent. The effect of increasing the carbon source concentration was very apparent during the third phase: overall antibiotic production was increased.

Corrections of fermentation characteristics were brought about by pH control: the optimum value was 6. These corrections prolonged the initial growth and production phase, and eliminated the second and third phases. Antibiotic production paralleled the growth curve and extended to the stationary growth phase. Continuous addition of soybean oil was superior to that of glucose, probably because of the higher energy value of the former. In spite of these corrections it was not possible to prolong growth and/or production beyond 6 days.

Acknowledgement

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