This may lead to the occurrence of unwanted side reactions, affect reaction rates, or exercise inhibitory or toxic effects in biological processes. Under such conditions, batch data will be of very limited use for predicting the performance of continuous reactors.

However, the method described may still prove very helpful for the initial design and operation of pilot units and the preparation of preliminary cost estimates. Experimental verification of the theoretical results will ultimately be required.

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LITERATURE CITED


Continuous Fermentation of Novobiocin

F. Reusser

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan

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ABSTRACT

Reusser, F. (The Upjohn Company, Kalamazoo, Mich.). Continuous fermentation of novobiocin. Appl. Microbiol. 9:366-370. 1961.—Continuous fermentation trials with Streptomyces niveus in a nine-stage fermentation system (7-liter reaction volume per stage) indicated that the cultures used gradually lost their ability to produce novobiocin when cultured over periods from 10 to 25 days. It was found that mycelial degeneration could be circumvented by operational means during continuous culture using the following technique: Two interchangeable 24-liter stages were installed at the front end of the nine-stage system and connected in parallel with the latter. Alternatingly one of these two tanks was then used as first stage of the continuous fermentation system. The holdup time in the first vessel was adjusted to limit cell growth chiefly to this stage so that most of the antibiotic production took place in subsequent stages. The first stages were switched at approximately weekly intervals. Each of the new tanks was prepared as a batch, inoculated with a high-producing cell population, and allowed to grow for 3 days before it was connected to the remaining system for continuous operation. Using this technique no evidence of culture degeneration was encountered in subsequent novobiocin production stages over a period of 33 days. In conventional runs without periodic replacement of the first stage, culture degeneration with the novobiocin fermentation occurred within a period of 10 to 25 days of continuous operation. This observation indicates that the described technique offers a solution to the problem of maintaining high steady-state titers in continuous novobiocin fermentations. Extension of this technique to other continuous fermentations where culture degeneration is a problem is indicated.

An excellent review of the current pertinent literature and status of continuous antibiotic fermentation processes, by Gerhardt and Bartlett (1959), indicates that, although several laboratories are known to have investigated the feasibility of continuous antibiotic fermentations, few data have been published to date.

The main problem in the development of successful continuous antibiotic processes involves culture degeneration. Most industrially important antibiotics are produced by polynucleate microorganisms (Streptomyces, Penicillium) where the maintenance of a high-producing genetical composition within a given population upon repeated transfer has proved very difficult.
Ample evidence for progressive loss of ability to produce a certain antibiotic by *Streptomycetes* upon repeated transfer has been presented in the literature (Williams and McCoy, 1953; Perlman, Greenfield, and O’Brien, 1954; Reusser, Koepsell, and Savage, 1961). Quite extensive continuous fermentation studies have been carried out with *Streptomycetes* strains producing three different antibiotics which were propagated in submerged culture over periods from 30 to 60 days of continuous operation. All strains without exception showed a progressive loss of their ability to produce antibiotics. In some cases this ability was completely lost. Based on literature reports and our own experience, it is concluded that progressive loss of the ability to produce antibiotics is a general phenomenon among *Streptomycetes*.

The present paper describes a method which circumvents loss of biosynthetic potential despite unstable cultures in continuous fermentations. The novobiocin fermentation was used as a model process.

**Materials and Methods**

*Continuous Fermentation System*

Mechanical aspects of the apparatus used will be described elsewhere and only details pertinent to the present work are therefore mentioned here. The apparatus was of small pilot plant style and fabricated wholly of stainless steel. In principle, it consisted of a multistage system of nine stages having a reaction volume of approximately 7 liters each. In later experiments, as outlined in the text below, two 24-liter stainless steel stages were connected in parallel ahead of the nine-stage system and used alternatingly as first stages thus increasing the total number of stages to ten. All stages were equipped with the usual accessories of conventional tanks such as air spargers, baffles, agitators, sample valves, temperature controls, and antifoam systems.

The level of culture in each stage was kept constant by horizontal overflow to the neighboring vessel and finally to the drain. No separate air exhaust lines were used. Air was sterilized through filters packed with glass wool. Air flow rates were controlled through rotameters adjusted manually by needle valves. Sterile medium was metered into stage 1 by a device delivering constant amounts of medium at adjustable short time intervals controlled by an electric timer. Sterile medium was prepared and stored in two 400-liter standard fermentors (operating volume, 200 liters) which were used alternatingly to provide continuous feed to the fermentation system. Temperatures were kept constant at 30°C by circulating thermostatically controlled water through the jacket of each stage. Foam levels were controlled by continuous additions of sterile lard oil.

*Operation.* The complete fermentation system was steam sterilized empty for 2 hrs at 24 psi pressure. The apparatus was cooled and charged with sterile medium. The first stage was then inoculated with 30 liters of a 3-day-old batch culture and continuous feed was started 2 or 3 hr after inoculation. The reaction volume of each stage was approximately 7 liters.

*Sampling.* Samples of all stages and feed tanks were withdrawn daily and examined microscopically for contamination, and tested for pH value, wet solid matter, total carbohydrate, and novobiocin.

*Organism.* The organism used throughout these experiments was *Streptomycyes niveus* strain BC-342. To ensure initial culture homogeneity it was isolated as a single conidium from a high-producing population.

*Medium.* The medium used had the following composition per liter: glucose monohydrate, 40 g; distiller's solubles,² 40 g; lard oil, 1 ml; tap water to 1 liter; pH was adjusted to 8.0 with concentrated NaOH before sterilization.

**Analytical Methods**

Total carbohydrates were determined by the anthrone method as described by Neish (1952). Spin solids were used as a relative measure of mycelial growth. Since the medium per se contained appreciable amounts of solids, no true mycelial weight could be obtained. The following method was used: A 10-ml sample aliquot was withdrawn directly from each stage into a graduated centrifuge tube. The tubes were spun for 20 min at 2,000 rev/min and read immediately after the centrifuge had come to a stop. Both the total volume remaining after centrifugation and the volume of solids were read and recorded. The value of per cent solids in the medium was calculated according to the formula:

\[
\text{Per cent solids} = \frac{\text{volume of solids, ml}}{\text{total volume after spin, ml}} \times 100.
\]

Novobiocin was determined spectrophotometrically as described by Smith et al. (1958) and the values were uncorrected for isonovobiocin.

**Results**

Several test runs with the novobiocin fermentation were conducted in the nine-stage reactor. The feed rate was adjusted to give a holdup time of 15 hr per stage thus accounting for an over-all holdup time of approximately 135 hr. Air flow rates were adjusted to 10 liters/min/stage.

Results of a typical run are shown in Fig. 1 through 3. Steady-state conditions at the outlet were attained

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1. The trade-mark of The Upjohn Company for novobiocin is Albamycin.

² Brown Forman Company, Louisville, Kentucky.
on about the seventh operational day at an output titer of approximately 400 μg of novobiocin per ml of culture medium. Steady state conditions were maintained until the 25th day, whereupon loss of activity occurred progressively and the output titer on the 33rd day had dropped to 172 μg of novobiocin per ml. Other runs operated under the same conditions corroborated the results of this run. As outlined in another paper (Reusser, 1961), antibiotic processes consist of at least two successive phases: (i) the generation of cells and (ii) the elaboration of antibiotic by these cells. Although the amount of antibiotic formed is proportional to the amount of cells present in the reactor, antibiotic formation lags behind cell formation in respect to time. Therefore, it seems feasible to separate cell growth and antibiotic formation to a large extent in continuous multistage reactors by proper adjustment of holdup times in each stage. The first stage(s) where cell growth occurs and only minor amounts of antibiotic are produced can be replaced with high-producing culture at a time interval shorter than that required for culture degeneration to occur. The antibiotic formation phase of the process taking place in subsequent stages should then be maintained at a steady high output titer over an indefinite period of time despite culture degeneration. In other words, the whole process is divided spatially by operational means into the two phases mentioned above where the first phase (cell growth) is operated continuously over a much shorter time cycle than the second phase (antibiotic production). This proposal was tested with the novobiocin fermentation.

Two 24-liter tanks were installed at the head end of the nine-stage reactor and connected in parallel with the latter. Alternatingly, one of the two 24-liter tanks was used as the first stage of the continuous fermentation system, where sterile medium was supplied continuously. The feed rate (1 liter/min), corresponding to a holdup time of 24 hr for the first stage, was selected to essentially limit cell growth to this first tank. A comparison between the observed values of the spin

![Fig. 1. Continuous novobiocin fermentation without periodic replacement of first stage. Novobiocin, total carbohydrate and per cent spin solids in stage 1.](image)

![Fig. 2. Continuous novobiocin fermentation without periodic replacement of first stage. Novobiocin, total carbohydrate and per cent spin solids in stage 2.](image)

![Fig. 3. Continuous novobiocin fermentation without periodic replacement of first stage. Novobiocin, total carbohydrate and per cent spin solids in stage 3.](image)

![Fig. 4. Continuous novobiocin fermentation with periodic replacement of first stage. Novobiocin, total carbohydrate and per cent spin solids in stage 1.](image)
solids in stages 1 and 2 (Fig. 4 and 5), indicates that growth was indeed limited chiefly to stage 1. Since one of the 24-liter tanks was connected at all times through a horizontal overflow line with the remaining nine stages, these later stages served strictly as antibiotic production vessels. Each of the stages had a holdup time of 7 hr thus accounting for an over-all holdup time of 87 hr for the whole ten-stage system.

It was our intention to switch the two growth stages (24-liter tanks) at weekly intervals. Each of these new tanks was prestarted as a batch, inoculated with a high-producing cell population, and allowed to grow for 3 days before it was connected to the remaining system for continuous operation, thus supplying constantly fresh high-producing mycelium to the remaining stages (2 to 10). Due to occasional mechanical or contamination difficulties, the exchange of the first stages was not always done at regular intervals and the actual times of change are indicated in Fig. 4 to 6. Antibiotic titers in stage 2 (first antibiotic production stage) and stage 10 (output titer) are assembled in Fig. 5 and 6. The run was operated for 33 days and was discontinued due to contamination. An average output titer of 500 μg of novobiocin per ml was maintained over the entire period. No evidence of culture degeneration was observed. In earlier fermentations without periodic replacement of the cell growth stage, progressive loss of activity in the output generally occurred within 10 to 25 days of continuous operation.

**DISCUSSION**

The results obtained with this run indicate that the replacement of the cell production stage(s) at short intervals with a freshly prepared growth stage definitely offers a solution to the problem of maintaining high steady-state outputs in continuous novobiocin fermentations. Extension of this technique to other continuous antibiotic fermentations appears entirely feasible.

Operational difficulties were encountered very seldom with this rather complex fermentation system over long periods of time. Most runs lasted from 2 to 3 months without any detectable contamination with foreign organisms and were generally discontinued voluntarily due to low titers in the output.

The main emphasis in these experiments was on the establishment of prolonged steady states in continuous novobiocin fermentation processes. No special attention was given to the establishment of optimal operational variables such as different aeration levels, temperature or pH variations, addition of precursors to different stages, etc. Actual output titers obtained in our trials do therefore not necessarily represent maximal obtainable outputs to be expected with such operations. On the contrary, it is suspected that upon proper adjustment of all operational variables, productivities, and absolute product concentration in the outlet of such reactors could be increased considerably. Also, Malek (1958) has amply emphasized the possibility that physiological states of continuous vs. batch cultures may differ entirely, and quantitative and qualitative differences between the two culturing methods may result.

The time interval of 7 days for the replacement of the growth stage was chosen arbitrarily. An experimental determination concerning the maximal possible time interval to be used before culture degeneration occurs was not done.

The possibility of withdrawing aseptically the whole content of stage 1 at periodic intervals without re-sterilization was also explored. The tank was flushed several times with sterile water and charged with 24 liters of a 3-day-old fresh, high-producing batch culture. This operation proved unsuccessful and titer loss oc-
curred in spite of the replacement of content of stage 1 due to interaction of remaining mycelium on the agitator shaft and tank walls with the freshly supplied mycelium.

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Cacao Fermentation
V. Yeasts Isolated from Cacao Beans during the Curing Process

H. L. Martelli and H. F. K. Dittmar

Escola N. de Química, Universidade do Brasil, Rio de Janeiro, Brazil

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Abstract
Martelli, H. L. (University of Brazil, Rio de Janeiro), and H. F. K. Dittmar. Cacao fermentation. V. Yeasts isolated from cacao beans during the curing process. Appl. Microbiol. 9:370-371. 1961.—Cacao beans must be subjected to fermentation before they are used in making chocolate, and their commercial value is related to a proper procedure. Saccharomyces rosei, Hansenula anomala, Pichia fermentans, Pichia membranaefaciens, and Trichosporon cutaneum were found in fermenting cacao beans. All species isolated during the investigation grew on cacao pulp, but only S. rosei, H. anomala, and P. fermentans exhibited fermenting capacity on the sugars of cacao pulp. Species of the genus Saccharomyces were identified as the agents responsible for the alcoholic phase of the cacao fermentation.

Before being used in making chocolate, cacao beans are subjected to a curing process known as cacao fermentation. The pulp and the beans from ripe fruits are accumulated in large wooden tanks and an alcoholic fermentation starts, followed later by an acetic phase (Martelli, 1955a). Through this process the temperature of the mass rises to 50°C, the embryo of the bean dies, and autolysis of the cotyledons is induced; then, the polyphenolic substances of the beans are liberated and transformed (Martelli, 1955b, c). The beans become brown and sweet, and upon roasting develop the typical smell of cocoa. Although a proper fermentation is essential to obtain a product of value (Martelli, 1960), existing knowledge about the yeasts participating in the alcoholic phase is very incomplete, and former descriptions of some species isolated from cacao fermentations do not fit into the system of yeast classification in current use. A case in point is the species isolated by Peyer (1913) and described as Saccharomyces theobroma, later reclassified by Knapp (1937) as Torulopsis theobroma, but no longer recognized by Lodder and Van Rij (1952). A similar situation exists for the species referred to by Ciferri (1931). Martelli (1955d) isolated a yeast from the husk and from the beans in fermentation which undoubtedly participated in the alcoholic process. That organism was reported at the time to represent T. theobroma, but was later reclassified as Saccharomyces carlbergensis. In the study reported here, strains of yeast were isolated from several