Continuous Microbiological Transformation of Steroids

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ABSTRACT

Reusser, F. (The Upjohn Company, Kalamazoo, Mich.), H. J. Koepsell, and G. M. Savage. Continuous microbiological transformation of steroids. Appl. Microbiol. 9:346–348. 1961.—Continuous fermentation trials on the bioconversions of pregnadiene to pregnatriene by Septomyxa affinis and progesterone to 11α-hydroxyprogesterone by Rhizopus nigricans were conducted successfully in an eight-stage pilot plant reactor. The first stage was used as the mycelial growth stage while the steroid solutions were added continuously to stage 2, thus using the remaining stages as conversion vessels. Recoveries of 50 to 60% oxidized steroid (based on total steroid supplied) were obtained in both cases upon a contact time of 5 hr between mycelium and steroid. Longer contact times resulted in a gradual net loss of steroid. It was concluded that two-stage reactors (one growth stage and one conversion stage) were adequate for efficient continuous operation of such processes. The reaction volumes of both stages have to be kept in proper balance to insure optimal holdup times for both the cell growth and conversion steps.

Numerous reviews on the microbiological transformations of steroids and their application to synthesis of hormones (Fried et al., 1955; Wettstein, 1955; Shull, 1956; Eppstein et al., 1956; Vischer and Wettstein, 1958; Peterson, 1959; Owen, 1960) have appeared. Most of these fermentations are based on conventional batch methods consisting of (i) generation of microbial cells and (ii) bioconversion of the steroid substrate. With the exception of the work of Mateles and Fulld (1959) who investigated the continuous transformation of progesterone to 11α-hydroxyprogesterone by Aspergillus ochraceus, no results on the continuous processing of such reactions have been published to date. The present paper deals with the continuous bioconversion of pregnadiene [11β,21-dihydroxy-4,17(20)-pregnadien-3-one] to pregnatriene [11β,21-dihydroxy-1,4,17(20)-pregnatrien-3-one] (Murray and Sebek, 1959) and of progesterone (4-pregnen-3,20-dione) to 11α-hydroxyprogesterone [11α-hydroxy-4-pregnen-3,20-dione] (Peterson and Murray, 1952; Peterson et al., 1952). For convenience the trivial steroid names will be used in this paper.

MATERIALS AND METHODS

Continuous Culture Apparatus

The design of the continuous culture apparatus will be described in another paper and only details pertinent to this work will be given here. The apparatus used was a multistage reactor of small pilot plant style and was fabricated of stainless steel. It consisted of eight individual stages connected together. The first stage had an operating volume of 20 liters, and each subsequent stage had a volume of 10 liters. Each stage was provided with the accessories of conventional tanks such as agitator, supply of sterile air, sample valves, and temperature controls. The level in each stage was kept constant by horizontal overflow to the next stage and finally to the drain. Air was sterilized by filtration through glass wool filters and its flow was measured by rotameters and controlled by needle valves. Continuous feed of sterile medium was provided into stage 1 by an injection device which delivered a constant amount of medium at short, variable time intervals. Steroid dissolved in suitable water-soluble solvents was added into stage 2 at a controlled rate by the use of a peristaltic-action metering pump. Temperatures were controlled by circulating thermostatically controlled cooling water (28°C) through the jackets of each individual stage.

Operation

The entire fermentation system was steam sterilized empty and then charged with sterile medium, and stage 1 was inoculated with 10 liters of a 1-day-old batch culture in the same medium. Continuous feed was started 2 to 3 hr after inoculation. The feed rate was adjusted to give a holdup time of 10 hr for the first stage and 5 hr for subsequent vessels, thus giving an over-all holdup time of approximately 45 hr. The air supply was 10 liters/min to the first stage and 5 liters/min to subsequent stages. No antifoam was used. Continuous steroid feed was started into stage 2 at 24 hr after inoculation.

Steroid Feed Solutions

Pregnatriene fermentation. In the case of the pregnatriene fermentation, the steroid feed rate was adjusted to deliver 0.5 g of steroid/liter of fermentation medium.
The steroid solution contained 50 g/liter of pregnadiene dissolved in propylene glycol.

11α-Hydroxyprogesterone fermentation. The steroid feed rate was adjusted to deliver 1 g of progesterone/liter of fermentation medium. The steroid solution contained 100 g of progesterone/liter dissolved in acetone.

Organisms
The organism used for the conversion of pregnadiene to pregnatriene was Septomyxa affinis. Rhizopus nigricans Ehrb. (ATCC 6227b) was used for the conversion of progesterone to 11α-hydroxyprogesterone. Stocks of both cultures were maintained on agar slants.

Culture Medium
Medium of the following composition was used in all experiments: glucose monohydrate, 10 g; corn steep liquor, 20 g; novobiocin, 50 mg; neomycin, 50 mg; tap water to 1 liter; pH was adjusted to 4.8 to 5.0 with concentrated NaOH before sterilization. The antibiotics were added to minimize possible bacterial contamination during runs. Batch results indicated that the addition of these antibiotics had no effect on growth rate, steroid conversion rate, or extent of conversion. Medium was sterilized for 30 min at 19 psi.

Sampling
Samples from each stage, as well as from the medium storage tanks, were withdrawn daily and tested for steroid levels.

Steroid Assays
One-hundred-milliliter samples of bioconversion medium were extracted with an equal volume of chloroform under reflux. The extracts of pregnadiene conversion media were subjected to paper chromatography on the benzene-formamide system (Zaffaroni and Burton, 1951), eluted, and the ultraviolet absorption determined at 243 m\(\mu\). For progesterone bioconversions the paper chromatogram system methylcyclohexane-carbitol (Murray and Peterson, 1952) was used for the separation of progesterone and the system benzene-cyclohexane-propylene glycol (Kochakian and Stidworthy, 1952) was used for the separation of 11α-hydroxyprogesterone and elution extracts were read at 232 m\(\mu\) according to the method of Reineke (1956).

Results
The addition of steroid to culture medium containing proliferating cells was found to inhibit cell growth, and
it was necessary to separate the cell proliferation and steroid bioconversion phases of the process. This was done by using a 20-liter, 10-hr holdup first stage without steroid feed for cell growth. Steroid was then introduced in the second stage, and further cell growth was negligible.

**Pregnatriene Fermentation**

Steroid assays for stages 2 and 8 are shown in Fig. 1 and 2. Stage 2 assays averaged 40 to 60% pregnatriene and 0 to 20% residual substrate, on the basis of substrate supplied. Assays of stage 3 were essentially the same, and for stage 8 showed about 10% loss of steroid. Thus, under the conditions used a contact time of 5 to 10 hr between steroid and mycelium was adequate for maximal reaction.

**11α-Hydroxyprogesterone Fermentation**

Assays of stage 2 showed 60% 11α-hydroxyprogesterone and 0 to 10% residual substrate. There was progressive steroid loss in further stages, and stage 8 assays gave a steroid recovery of 40 to 60%. These results are shown in Fig. 3 and 4. Thus, a contact time of 5 hr was sufficient for maximal product formation.

**Discussion**

The results presented indicate that continuous steroid bioconversion is feasible. The short contact time observed should make reductions in processing costs possible. The growth process should be limited to the first stage, while the steroid to be converted is added to the second stage where the actual bioconversion takes place. The volumes of the two stages must be properly balanced in order to insure optimal holdup times for both growth and conversion processes.

The experiments reported were conducted to explore the feasibility of continuous steroid bioconversions and operational conditions were chosen arbitrarily. The results do not necessarily represent maximal achievable recoveries or efficiencies and are therefore of relative rather than absolute value. It should be expected that the efficiency of steroid conversion processes could be increased considerably by proper adjustment of all operational variables, particularly by minimizing the portion of steroid loss due to degradation by the organism (usually 30% in these experiments) and by adding higher steroid levels continuously to the conversion stage.

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**Literature Cited**


