Agar Plate Method for Detecting Microorganisms Which Produce Equilin and Other Estrogens from Various Steroids

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The intense red color produced by a reagent specific for Δ1-estrogens is used to directly detect microorganisms which produce these estrogens from various steroids.

In the microbiological transformation of steroids, it is desirable to detect rapidly and to recognize directly microorganisms which transform a particular substrate into a desired product. Since, in most cases, the product does not markedly differ from the substrate, no known reagent is specific and sensitive enough to attain this ideal. The aromatization of various 19-substituted steroids to equilin, equilenin, dihydroequilin, and dihydroequilenin represents an exception. These products respond specifically to para-nitrobenzene diazonium fluoroborate (1% solution in 50% acetic acid) to yield an intense red color. Steroids with saturated ring B or non-aromatic ring A, or both, give a yellow-to-brown color and, therefore, are difficult to discern from the background of the solidified medium. The yellow background does not interfere with the red color which develops at the edge of the colonies when the specific estrogens are present.

Agar plates were prepared as follows. A 20-ml amount of a liquefied culture medium was poured into a Petri dish 100-mm in diameter. When the medium had solidified, 4 to 5 ml of the same medium (or of a 1.5% agar suspension) containing 5 to 25 mg of a steroid substrate in very fine suspension was poured evenly over the bottom layer. When the top layer had solidified, the plate was partially dried at 37 C. Microorganisms to be tested were inoculated by point inoculation according to a pattern, and the inoculated plate was incubated. At the end of the incubation period, the plate was sprayed with the above reagent. When equilin, equilenin, and dihydroequilin (or dihydroequilenin) were formed, a red ring developed at the edge of the colony 15 to 30 min after the reagent had been sprayed. The identity of the products was confirmed by thin-layer chromatography by use of the following procedure. A piece of medium containing a duplicate colony which had not been sprayed was extracted with methylene dichloride; the solvent was evaporated to dryness, and the residue was dissolved in a methanol-chloroform mixture (1:1) to a final concentration of 10 mg/ml. The solution was applied (5 to 10 μl) on a Silica Gel G thin-layer chromatography plate, and the plate was developed for 2 to 3 hr with 10% collidine in CCl4; it was dried thoroughly in air and sprayed with the reagent. Identification was made by comparing RF values and colors of the unknown to the RF values and colors of an authentic mixture of estrogens and substrate. Equilin and dihydroequilin (one double bond in ring B) and equilenin and dihydroequilenin (two double bonds in ring B) yield red and purple colors, respectively; estrone and estradiol (saturated ring B) and the substrates (one double bond in ring B, but nonaromatic ring A) form a yellow-to-brown color with the reagent.

On the agar plate, the reagent does not reveal the presence of estrone and estradiol conclusively, since the resulting yellow color fades out on the yellow background produced by the reaction of the unconverted substrate with the reagent. However, the characteristic red color stands out nicely on the yellow background; the red shades, however, are not subtle enough to identify equilin, equilenin, and their dihydro derivatives directly on the plate. Therefore, when a microorganism yields positive result (red color), it is necessary to extract a duplicate colony and identify the individual products by thin-layer chromatography. In spite of this limitation, the method proves very useful for eliminating inactive organisms and for detecting the few active ones without running
the tedious and time-consuming transformation in shake flasks. The agar plate method was applied successfully to a variety of Δ7-19-substituted substrates, such as 19-hydroxy- and 19-acetoxy-androsta-4,7-diene-3,17-dione.

Several hundreds of bacteria were screened at the rate of 25 to 75 colonies per plate, and a few species yielded positive results. A modification of the standard procedure is illustrated in Fig. 1. After incubation, the plate was sampled by means of a cork borer; the discs were transferred to a microscopic slide and sprayed with the reagent. From the intensity of the color developed, it was possible to observe semi-quantitative differences between various treatments (media, temperature, incubation periods). For this purpose, replica plating was used as inoculum. When very dark media had to be used, the top layer consisted of a 1.5% agar suspension containing the substrate and 5% CaCO₃ or, preferably, 3% TiO₂ (which is completely inert) to insure a lighter background.

For screening fungi, large Petri plates (150 mm) are desirable; fungi sometimes produced aromatic metabolites which yielded false positive results (red color with the reagent), and the thin-layer chromatography technique cleared these uncertainties. The persistent red color produced by equilin, equilenin, and their dihydro derivatives could also be easily distinguished from the color produced by fungal aromatic metabolites which faded out rapidly on standing.

Since the agar plate method is specific for Δ7-estrogens, it can be extended to the search for microorganisms that yield these compounds from various substrates through diverse reactions.

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