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Determination of *p*-Hydroxyphenoxyacetic Acid in Phenoxymethyl Penicillin Fermentations

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Abstract □ A method based on TLC separation and subsequent colorimetric determination was developed for the determination of *p*-hydroxyphenoxyacetic acid in phenoxymethyl penicillin (penicillin V) fermentation samples. The *p*-hydroxyphenoxyacetic acid is extracted from penicillin culture fluid into amyl acetate. A portion of the extract is dried and then dissolved in acetone. An aliquot is chromatographed on a silica gel thin-layer plate by ethyl acetate–chloroform–acetic acid (9:1:0.15 v/v). The zones are located by spraying with an aqueous mixture of equal volumes of 1% ferric chloride and 1% potassium ferricyanide. The zones are transferred into glass tubes, treated by sodium nitrite in dilute sulfuric acid, and neutralized by ammonia. The absorbance of the orange supernate, which is proportional to the concentration of *p*-hydroxyphenoxyacetic acid, is measured spectrophotometrically at 478 nm.

Keyphrases □ *p*-Hydroxyphenoxyacetic acid—from phenoxymethyl penicillin fermentations, TLC separation, colorimetric determination □ Phenoxymethyl penicillin fermentations—determination of *p*-hydroxyphenoxyacetic acid, TLC separation, colorimetric determination □ TLC—separation of *p*-hydroxyphenoxyacetic acid from phenoxymethyl penicillin fermentations □ Colorimetry—determination of *p*-hydroxyphenoxyacetic acid separated from phenoxymethyl penicillin fermentations

The presence of *p*-hydroxyphenoxymethyl penicillin was first detected in phenoxymethyl penicillin fermentation samples by de Flines *et al.* (1), and the authors assumed that this compound is formed either by: (*a*) direct hydroxylation of penicillin by the mold or (*b*) by oxidation of the precursor which is then used to form *p*-hydroxyphenoxymethyl penicillin. A method for quantitative determination of this compound in fermentation samples was developed (2). A colorimetric method for the determination of *O*-hydroxyphenylacetic acid in benzylpenicillin (penicillin G) was developed by Pan in 1955 (3). When this method was applied to phenoxymethyl penicillin fermentation samples, it gave positive results, indicating that phenolic compounds were present in the broth. An attempt to apply this method for the determination of *p*-hydroxyphenoxyacetic acid was unsuccessful due to interference by other phenolic or similar substances present in fermentation media.

When *p*-hydroxyphenoxyacetic acid is treated with a small amount of sodium nitrite in dilute sulfuric acid, heated for 15 min., and then neutralized by ammonia, an orange compound is produced with maximum absorbance at 478 nm. When a dried amyl acetate extract from a penicillin fermentation sample is similarly treated, the formed compound has a yellow color and higher absorbance at 420 nm. than at 478 nm. It is thus evident that for the determination of *p*-hydroxyphenoxyacetic acid in extracts, this compound first must be separated from interfering substances. This is achieved by extracting the acidified culture fluid, saturated with sodium chloride, with amyl acetate. A portion of the amyl acetate extract is dried by an air current, and the dry residue is dissolved in acetone. An aliquot is chromatographed on silica gel by ethyl acetate–chloroform–acetic acid (9:1:0.15 v/v). After spraying with a freshly prepared mixture of ferric chloride and potassium ferricyanide, the zones appear as blue spots of the same R_f value as *p*-hydroxyphenoxyacetic acid controls. The zones are transferred into glass tubes, treated with sodium nitrite in dilute sulfuric acid, and then neutralized by ammonia. After separation from silica by centrifuging, the orange phenolic nitroso compound is measured spectrophotometrically.

EXPERIMENTAL

Reagents, Solutions—The following were used: sodium chloride A.R.; amyl acetate A.R.; sulfuric acid, 12 *N*, A.R.; sulfuric acid, 1 *N;* sodium nitrite, 5% w/v; ammonia (0.880 specific gravity)–water (1:2); silica gel according to Stahl (mean particle size 10–40 μ,

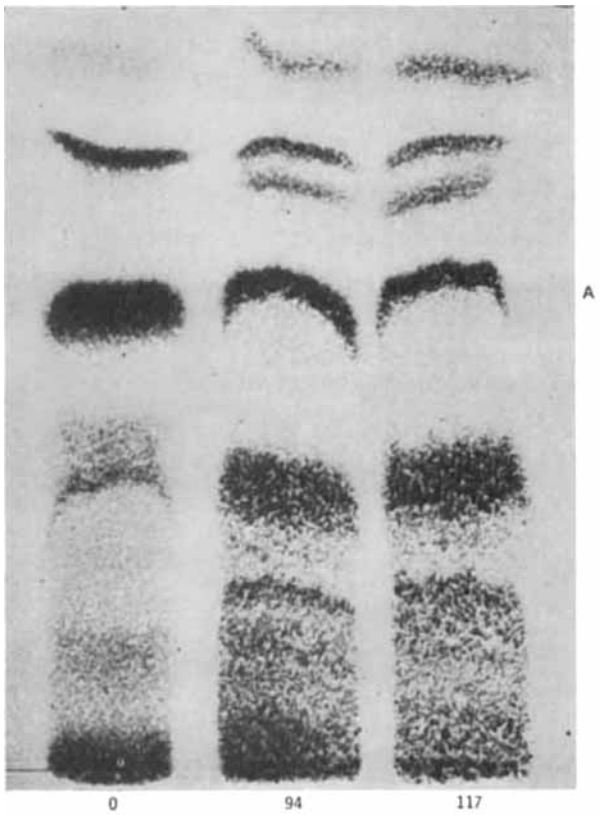

Figure 1—*Chromatogram of extracts from 0.25 ml. of fermentation samples and standard solution of* p-*hydroxyphenoxyacetic acid (A) in unfermented broth.*

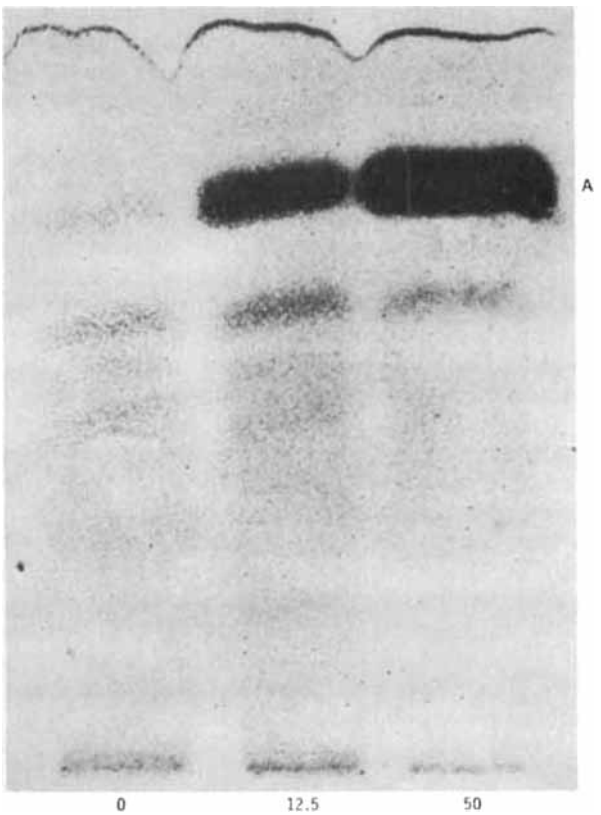

Figure 2—*Sprayed chromatogram of extracts from 0.25 ml. of standard solutions of* p-*hydroxyphenoxyacetic acid (A) in unfermented broth.*

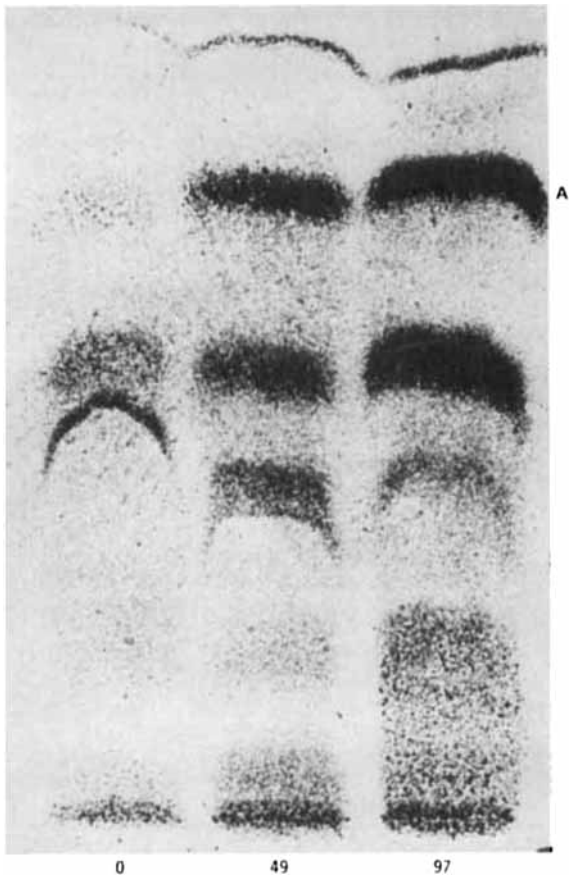

Figure 3—*Sprayed chromatogram of extracts from 0.25 ml. of samples of* p-*hydroxyphenoxyacetic acid (A) taken during a fermentation.*

containing approximately 13% gypsum) for TLC; ferric chloride, 1% w/v in water; potassium ferricyanide, 1% w/v in water; ethyl acetate A.R.; chloroform BP; glacial acetic acid A.R.; acetone A.R.; and *p*-hydroxyphenoxyacetic acid[1] used without further purification.

Procedure—*Step A. Preparation of Thin-Layer Plates*—Using a hand-operated apparatus (4)[2], coat glass plates, 20 × 10 cm., with silica gel slurry to produce a thickness of 250–300 μ after drying. Dry the plates in an oven at 100° and finally keep them at room temperature exposed to the atmosphere.

Step B. Preparation of Sample—Pipet 2 ml. of filtered culture fluid into a suitable centrifuge tube, add about 1 g. of sodium chloride, and shake to dissolve. Add 0.15 ml. of 12 *N* sulfuric acid and then 5 ml. of amyl acetate. Stopper and shake the tube for 45 sec.; then centrifuge to separate the layers. Transfer 2.5 ml. of amyl acetate extract into a 10–15-ml. test tube fitted with a ground-glass stopper. Remove the amyl acetate by warming the test tube in a 50–70° water bath while blowing a gentle air current into the tube. After cooling, add 0.2 ml. of acetone to the dry residue, stopper immediately, and rotate gently to dissolve the residue.

Step C. Chromatographic Separation and Determination of p-*Hydroxyphenoxyacetic Acid*—Using a microsyringe, apply 50 μl. of the acetone solution to the plate, using three application sites for one plate. Insert the plate into a covered jar containing the solvent ethyl acetate–chloroform–acetic acid (9:1:0.15 v/v) and develop until the solvent front advances to 14–16 cm., which takes about 1 hr. After drying, spray the plate with a freshly prepared solution of equal volumes of 1% ferric chloride and 1% potassium ferricyanide. The blue spots appearing on the plate at R_f values between 0.68 and 0.72 indicate the position of *p*-hydroxyphenoxyacetic acid. These spots also appear first, with those resulting from other compounds

[1] Eastman Organic Chemicals.
[2] Camag.

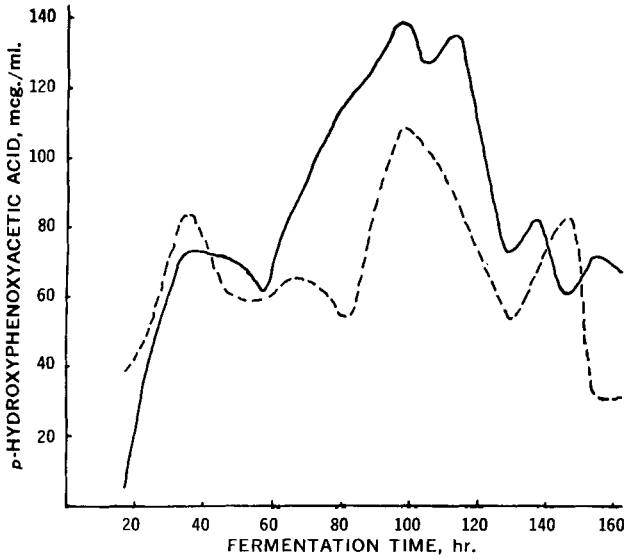

Figure 4—p-*Hydroxyphenoxyacetic acid content of samples during production tank fermentations. Key:* ——, *production tank U795; and* - - -, *production tank R801.*

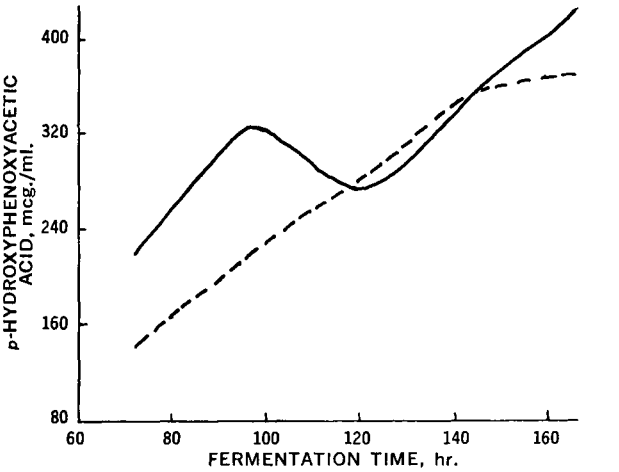

Figure 5—p-*Hydroxyphenoxyacetic acid content of samples of shaken flask fermentations. Key:* - - -, *experimental run A; and* ——, *experimental run B.*

appearing later. Figure 1 shows the sprayed chromatogram of samples taken during fermentation at 0, 94, and 117 hr. To the 0-hr. sample, *p*-hydroxyphenoxyacetic acid was added to furnish a concentration of 50 mcg./ml. to serve as a location control.

Scrape each of the blue zones containing *p*-hydroxyphenoxyacetic acid onto a square of glossy paper and quantitatively transfer into 11.5 × 1.5-cm. glass centrifuge tubes. Add 2 ml. of 1 *N* sulfuric acid and 0.1 ml. of freshly prepared 5% sodium nitrite solution to each tube and stir. Immerse the unstoppered tubes in boiling water for 15 min. After cooling in running water, add 5 ml. of diluted ammonia [ammonia (0.880 specific gravity)–water (1:2)] to each tube from a buret, cool, and mix the contents of the tubes by swirling to disperse the silica. Centrifuge the tubes for 10–15 min. at 2000 r.p.m. Remove and measure absorbance of the orange supernate spectrophotometrically at 478 nm. using 10-mm. cells after setting the instrument on a clear blank obtained by boiling 2 ml. of 1 *N* sulfuric acid and 0.1 ml. of 5% sodium nitrite solution neutralized by 5 ml. of diluted ammonia. From the undeveloped part of the glass plate, scrape off, in duplicate, areas of surface equal to the areas extracted for *p*-hydroxyphenoxyacetic acid and put them through the same procedure as the samples to serve as a blank. Any value obtained from the blanks is subtracted from the values for samples and standards.

The data from the samples may then be converted to *p*-hydroxyphenoxyacetic acid concentrations using a standard curve.

Preparation of Standard Curve—To a filtered 0-hr. broth sample, add solid *p*-hydroxyphenoxyacetic acid to furnish a 400-mcg./ml. solution. Dilute aliquots of this solution, using broth, to produce solutions containing 200, 100, and 50 mcg./ml. Put these standard solutions in duplicate through the procedure already described (Steps A–C) and use the data to plot a standard curve. The quantities of *p*-hydroxyphenoxyacetic acid used for the standard curve range from 12.5 to 100 mcg. in 7.1-ml. volume.

It has been shown that the formation of color follows Beer's law from the concentration of 5 to 200 mcg. in 7.1-ml. volume. The color is stable for at least 24 hr. A photograph of a developed and sprayed chromatogram of standards is shown in Fig. 2.

By using the method already described, several production tanks of phenoxymethyl penicillin were examined during all stages of fermentation. The medium used for fermentation in production was corn-steep based and contained phenoxyacetic acid as precursor.

A photograph of a developed and sprayed chromatogram from samples taken during fermentation at 0, 49, and 97 hr. is shown in Fig. 3. Values for two production tanks, U795 and R801, are shown in Fig. 4. The amount of *p*-hydroxyphenoxymethyl penicillin relative to the total penicillin content was determined (2) at harvest time (161 hr.) for both tanks, being 5% for U795 and 4.5% for R801.

To obtain special samples with high *p*-hydroxyphenoxyacetic acid content, several samples were fermented in 500-ml. conical flasks on a shaking machine, the volume being 120 ml. of broth. They were shaken at 270 r.p.m. at an amplitude of 2.5 cm. The medium consisted of: corn steep (50% solids), 20 g.; soya oil, 10 g.; calcium carbonate, 5 g.; ammonium sulfate, 2 g.; phenoxyacetic acid, 3 g.; lactose, 50 g.; and water to 1 l.; pH 5.5.

The flask contents were fermented for 168 hr. and samples were taken every 24 hr., commencing at 72 hr. The values from two separate experiments, A and B, are shown in Fig. 5, each value being the mean from four flasks.

DISCUSSION

The extraction efficiency of *p*-hydroxyphenoxyacetic acid under the described conditions lies between 89 and 92%; but since the standard curve is prepared by the same extraction procedure from a common broth base, the error due to extraction inefficiency is eliminated in the method. The ferric chloride–potassium ferricyanide spray reagent was selected because it does not interfere with further color formation and quantitative determination. The TLC separation leads to determination of *p*-hydroxyphenoxyacetic acid and excludes interference by other phenolic compounds. The spray reagent also locates penicillin and other components or metabolites of the fermentation medium which appear at R_f positions different from that of *p*-hydroxyphenoxyacetic acid. The method allows estimation of the concentration of the free acid at any given time during a fermentation cycle. To obtain a value for total *p*-hydroxyphenoxyacetic acid formed at any stage of a fermentation cycle, it is necessary to determine the amount of *p*-hydroxyphenoxymethyl penicillin, to calculate the equivalent amount of *p*-hydroxyphenoxyacetic acid, and to add this to the amount of free *p*-hydroxyphenoxyacetic acid present in the same sample.

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