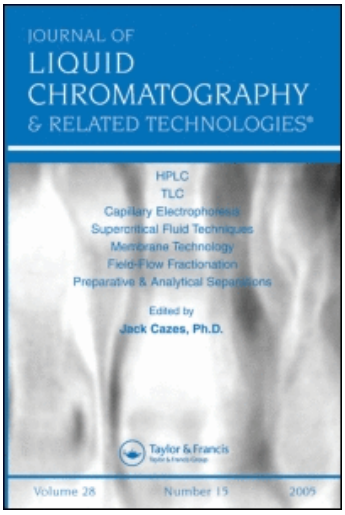

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Quantitative Analysis of Phenyl-Acetic Acid in *Proteus Mirabilis* Cultures by High Performance Liquid Chromatography

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QUANTITATIVE ANALYSIS OF PHENYLACETIC ACID IN *PROTEUS MIRABILIS* CULTURES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method was developed for the analysis of phenylacetic acid produced by Proteus mirabilis during a 10-day incubation period. A daily sample was acidified and extracted with petroleum ether for 24 hours in a continuous extraction apparatus. The extract of each day was analyzed by reverse-phase liquid chromatography using UV detection at 254 nm. A 10-µl aliquot of the concentrated extract was injected onto an octadecylsilane column. The mobile phase consisted of 20% methanol and 80% sodium acetate buffer, 0.01 M, pH 4.2. This method may be used for screening other microbial fermentations for the production of similar lipophilic acids.

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INTRODUCTION

The inhibition of bacterial growth in food and beverage items has become an important part of the food industry (1). This form of control requires the use of agents which possess inhibitory action on a wide variety of microorganisms. The compounds currently used are either acidic, neutral, or slightly basic and are substantially lipophilic in nature (2). The site of action of these agents is on the cell membrane, and they appear to affect the transport of amino acids into the cell (2,3). Mammalian cells, when exposed to the same concentrations required to inhibit microbial growth, also show a change in cell morphology (4, 5). The bactericidal effect appears to be both pH and concentration dependent (5). The dilution of these agents in the body following ingestion apparently precludes the possibility of any harmful effect in man.

There is a continual search for other compounds which may be more effective as antimicrobial food preservatives. One source for the production of lipophilic acids are microorganisms (6). However, methods for determining the elaboration of these compounds must be simple and quantitative in order to be useful. The extraction of complex growth media must be capable of removing the majority of undesirable metabolites. This paper describes a method to quantitate the production of phenylacetic acid by Proteus mirabilis during a 10-day fermentation using reverse-phase high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Instrumentation

A Hewlett-Packard Model 1081 liquid chromatograph with a Schoeffel Model SF 770 UV variable wavelength detector was equipped with an ODS (IBM Instruments) column, 150 mm long and 4.5 mm i.d. The degassed mobile phase was pumped through the column at 1.2 ml/min using isocratic conditions. The column compartment was maintained at 28°C, and the detector was set at a wavelength of 254 nm. The mobile phase was prepared by mixing 20% methanol and 80% sodium acetate, 0.01 M, pH 4.2.

Chemicals and Reagents

All constituents used in the preparation of the growth medium were reagent grade. The petroleum ether, b.p. 20-40°C, (Matheson Coleman and Bell Manufacturing Chemists) was reagent grade. The methanol was HPLC grade (Fisher Scientific Co.). The phenylacetic acid and 3-indoleacetic acid were obtained from Sigma Chemical Co.

Drug Solutions

Two phenylacetic acid concentrations were prepared in methanol; 4 mg/ml and 0.4 mg/ml. 3-indoleacetic acid was prepared in methanol at a concentration of 1 mg/ml.

Bacterial Strains

One strain of *P. mirabilis*, ATCC #19692 (isolated from a gypsy moth), was used in this study.

Bacterial Medium and Culture Preparation

The fermentation medium consisted of the following components per liter of distilled water: 0.5 g ammonium chloride, 0.04 g magnesium sulfate, 0.0024 g nicotinic acid, 7.0 g casaminoacids (Difco Laboratories), and 6.8 g sodium lactate. The pH was adjusted to 7.2 with sodium hydroxide and 150-ml volumes were dispensed into 250-ml erhlenmeyer flasks. The flasks were topped with cotton plugs and autoclaved at 121°C and 15 psi for 15 min. A solution of ferrous ammonium sulfate (2.18 mg/ml) was prepared in 0.1 M hydrochloric acid. The solution was filtered through a 0.2 μm Millipore membrane filter and an appropriate amount was added to each flask of the sterilized medium to a final concentration of 0.030 mg/ml.

An 18-24 hour Brain Heart Infusion (Difco Laboratories) culture of P. mirabilis was centrifuged at 1800 x g for 5 min. The cells were collected, washed with saline, centrifuged and resuspended in 2.5 ml of saline. One-milliliter of the suspension was transferred to each of 10 flasks containing 150 ml of the growth medium.

Sample Preparation

Immediately following incubation, and on each successive day of the 10-day incubation period, the contents of one flask was autoclaved for 15 min (121°C, 15 psi) and then centrifuged at 10,000 x g for 10 min to remove the cells. The supernatant

was collected and acidified to pH 3 with concentrated hydrochloric acid. The volume of the medium was measured and 3 x 25 ml aliquots were extracted individually with 100 ml of petroleum ether for 24 hours in a continuous extraction apparatus. The petroleum ether was evaporated by rotary distillation. The residue was washed 3 times with methanol, transferred to a test tube and evaporated to dryness under nitrogen. All samples were stored at $4^{\circ}C$ until analyzed.

Quantitation

A standard curve for phenylacetic acid was constructed utilizing 3 replicates simulating concentrations from 100 to 1400 µg/ml. The chromatograms were recorded at a chart speed of 5 mm/min. The peak heights were measured and the ratios (drug/internal standard) were calculated and plotted versus concentration expressed as micrograms per milliliter of medium.

Recovery

Extraction efficiency was determined by preparing 3 x 25 ml volumes of uninoculated growth medium with a 1 mg/ml concentration of phenylacetic acid. The spiked solutions were extracted and compared with 3 samples prepared in water at the same concentration but not extracted. All the values obtained from the culture analysis were corrected for per cent recovery.

RESULTS AND DISCUSSION

Although extraction of the culture medium was performed with a very nonpolar solvent, the presence of other metabolites was still evident and increased as the incubation time increased. It was therefore necessary to extend the analysis time to 20 min, which offered adequate separation of all the extracted components from the internal standard and phenylacetic acid peaks. A chromatogram of the standard representing 1 mg/ml of phenylacetic acid and one showing a sample taken on day 3 are shown in Fig. 1. The retention time of phenylacetic acid was 10.7 min, while the internal standard was retained for 16.4 min.

The ratios of the peak heights of phenylacetic acid to the peak heights of the internal standard were calculated. Statistical analysis of the data by linear regression indicated excellent linearity and reproducibility with a correlation coefficient of 0.9980, a slope of 0.0005, and an intercept of 0.00168 in the range of 100 to 1400 μg of phenylacetic acid/ml of growth medium.

A graphic representation of the amount of phenylacetic acid present in the medium on each day of the 10-day period is shown in Fig. 2. The points representing phenylacetic acid concentration are calculated averages for each day (n=3), and have been corrected for the 80% efficiency in extraction. Table 1 contains a listing of the data.

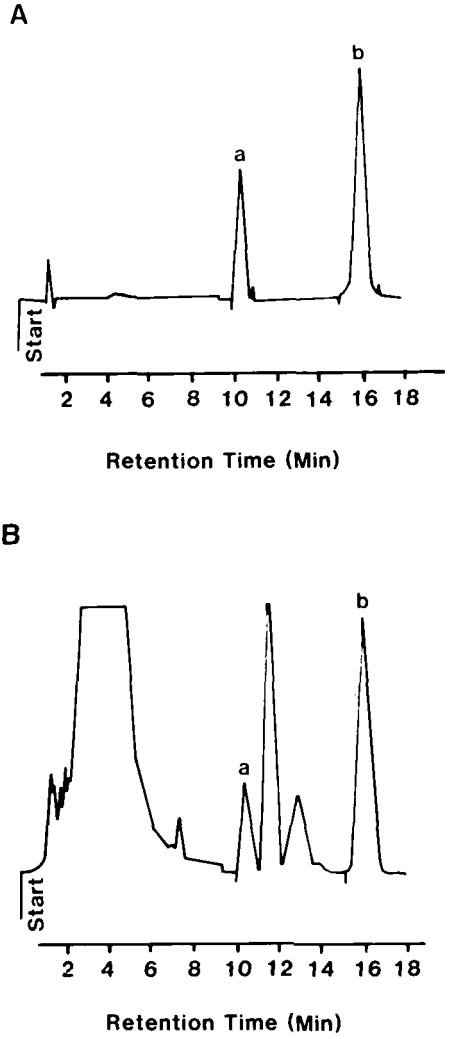

FIGURE 1. HPLC of: Phenylacetic Acid Standard (A), Three-day Culture Extract (B).
Key: a) Phenylacetic Acid
b) Internal standard

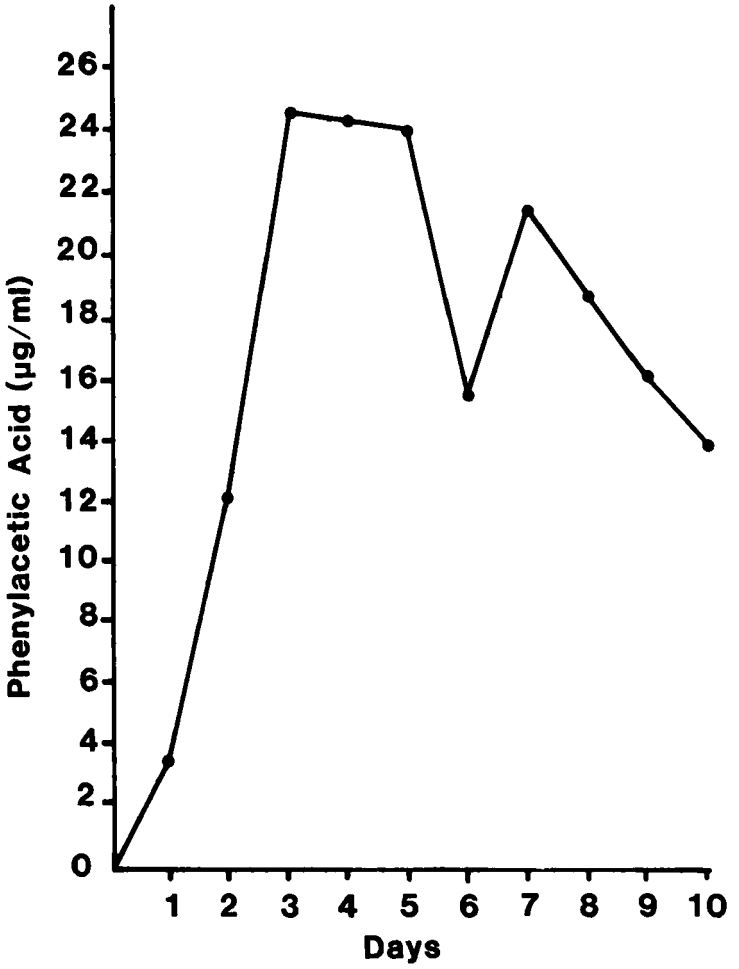

FIGURE 2. Concentration of Phenylacetic Acid During a 10-Day Fermentation.

TABLE I

Phenylacetic Acid Production

During

A Ten Day Incubation Period

Day	n	Average Conc of Phenylacetic Acid (ug/ml)	S.D.
0	-	---	---
1	3	4.0	± 0.6
2	3	14.7	± 0.8
3	3	29.3	± 2.8
4	3	29.0	± 2.2
5	3	28.9	± 5.8
6	3	18.6	± 2.8
7	3	25.9	± 3.5
8	3	22.4	± 1.6
9	3	19.3	± 1.5
10	3	14	± 1.4

Production of phenylacetic acid began during the first day of incubation and reached a peak concentration by day 3 (29.3 µg/ml). This concentration remained relatively constant until day 5. However, on the sixth day a dramatic drop in phenylacetic acid concentration was noted (18 µg/ml). The decrease was fol-

lowed by another steep increase in production (days 6 to 7) to 25.9 µg/ml. The remaining 3 days showed a steady decrease from 25.9 µg/ml on day 7 to 14 µg/ml on day 10.

The evaluation of microbial fermentations requires quick and simple means in order to determine the efficiency at which the products are being formed. The fermentations employed for the production of several of the antibiotics may also result in the elaboration of many chemically related constituents (7,8). These products are usually undesirable and may suggest the dominance of an unfavorable biochemical pathway (8).

The method described in this paper suggests a relatively easy procedure for following the production of a lipophilic acid, phenylacetic acid, during a 10-day fermentation. Extraction of the cultures with petroleum ether involved one simple step and results in a solution which could be injected directly onto the column. This method may also be expanded for the screening of other bacterial strains for the production of similar lipophilic acids.

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