Studies on the Utilization of Hydrocarbons by Microorganisms

Part VI. Screening of Aromatic Hydrocarbon-Assimilating Microorganisms and Cumic Acid Formation from *p*-Cymene

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During the course of an investigation of the microbial assimilation of aromatic hydrocarbons, several strains were found to produce a large amount of cumic acid from *p*-cymene.

Five strains, S449B1, B2, B3, B4 and B6, were isolated from soil with the aromatic hydrocarbon substrates. They all assimilated both *p*-cymene and cumene. The strain S449B3 grew also on *p*-xylene, and S449B6 on *p*-xylene, toluene, and ethylbenzene.

They were all shown to be capable of producing an ultraviolet-absorbing substance from *p*-cymene. This substance was isolated in crystalline form and identified as cumic acid by infrared absorption spectrum and other observations.

The superior strain, S449B6, produced the acid as much as 1000 mg/l in shaking culture at 30°C after 24 hours. The yields were increased up to approximately 1700 mg/l after further investigations. Addition of calcium carbonate and considerable agitation were favorable conditions for the acid production.

The taxonomical studies of these strains were carried out, and they were all identified as closely resembling *Pseudomonas desmolytica*.

INTRODUCTION

In view of the industrial importance, increasing interest is being shown in the utilization of various hydrocarbons by microorganisms. Especially the fermentative production of amino acid and cells from *n*-alkane has been reported by several groups of workers.^{1~4)}

In this point of view, previous investigations of aromatic hydrocarbons have dealt almost exclusively with the formation of salicylic acid from naphthalene.⁵⁾ While, in recent years, a good deal of attention has been

paid for the microbial transformation of organic compounds.

Therefore, this work was initiated as a part of an extensive program on the microbial assimilation and transformation of aromatic hydrocarbons. In this paper, identification of newly isolated microorganisms, microbial formation of cumic acid from *p*-cymene and some aspects of their cultural conditions are presented.

MATERIALS AND METHODS

Microorganisms. Five microorganisms, S449B1, B2, B3, B4 and B6, were isolated from soil by a screening procedure with aromatic hydrocarbon substrates.

Diagnostic tests for morphological studies were carried out according to the "Manual of Microbiological Methods," 1957, Society of American Bacteriologists, and referred to the "Bergey's Manual

1) K. Yamada, J. Takahashi, K. Kobayashi and Y. Imada, *This Journal*, **27**, 390 (1963).

2) K. Yamada, J. Takahashi and K. Kobayashi, *This Journal*, **27**, 773 (1963).

3) A. Champagnat, Ch. Vernet, B. Laine and J. Filose, *the Sixth W.P.C.* (1963).

4) J. Takahashi, K. Kobayashi, Y. Imada and K. Yamada, *This Journal*, **27**, 836 (1963).

5) P. Hsler, *Biotech. Bioeng.*, **5**, 243 (1963).

of Determinative Bacteriology," 7th Ed., 1957.

Medium. The medium contained, per liter, 4.0 g NH_4NO_3 , 1.5 g KH_2PO_4 , 1.5 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002 g $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.01 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g yeast extract. A five hundred milli-liter-flask, containing 50 ml of the medium, was autoclaved at 110°C for 10 minutes. Before cultivation, 0.5 ml of sterile hydrocarbon was added to the medium in the flask.

Cultivation. When cultural conditions were tested, 1.5 ml of cell suspension, grown on *p*-cymene-agar-slant for 2 days, was inoculated into 50 ml of the medium. The medium was incubated at 30°C on reciprocal shaker for 1 to 7 days.

Hydrocarbons. All of the hydrocarbons (toluene, *p*-xylene, ethylbenzene, *m* & *p*-diethylbenzene, cumene and *p*-cymene) were obtained from Tokyo Chemical Industry Co., Ltd. and were used without further purification.

In isolation procedures, hydrocarbon mixture, containing equal volume of the all hydrocarbons listed above, was used as a sole carbon source.

For hydrocarbon assimilation tests, *n*-alkane mixture, containing equal volume of *n*-dodecane and *n*-hexadecane, was employed.

TABLE I. SOLVENT SYSTEMS AND DEVELOPING REAGENTS FOR THE DETECTION OF METABOLIC PRODUCTS

Solvent	Reagent	Metabolic Product
1. $(\text{NH}_4)_2\text{CO}_3\text{-NH}_4\text{OH}$ - <i>n</i> -Butanol*	M. R.** Diazotized Benzidine*** U.V.-Ray Irradiation	Organic Acid Phenolic compound U.V.-Ray-absorbing compound
2. <i>n</i> -Butanol :Acetic acid :Water (4:1:2)	Ninhydrin	Amino acid

* *n* Butanol was saturated with ammonia/ammonium carbonate buffer (1.5N with respect to each).⁶⁾

** A mixture of 5 parts of 0.1% alcoholic methyl red solution with 10 parts of M/15 phosphate buffer solution pH 7.0.

*** A: Five grams of benzidine was dissolved in 14 ml of concentrated hydrochloric acid and the solution was filled up to 11 with water.
B: Ten per cent sodium nitrite solution in water. The solutions A and B were mixed at equal volume before spraying.

Paper Chromatography. Paper chromatography was employed for detection of metabolic products. Solvent systems used are listed in Table I. The spots were visualized by ultraviolet-ray irradiation and/or the spraying of the developing reagents (Table I). Tôyô filter paper No. 50 was used.

Acid. Cuminic acid, produced in cultural broth, was extracted by equal volume of ether. The ether solution was titrated by 1/20 N-NaOH, then amount of the acid produced was calculated from the titration value.

RESULTS AND DISCUSSION

Isolation of Microorganisms from Soil.

Accumulation cultures with 1g of a soil sample and 50 ml of the medium were carried out at 30°C for 4 to 7 days. Organisms, isolated after second accumulation culture, were tested for their abilities to utilize the individual hydrocarbons. Results are shown in Table II.

TABLE II. ASSIMILATION OF HYDROCARBONS BY ISOLATED BACTERIA

Strain No.						<i>n</i> -alkane*
S449B1	-	-	-	+	+	-
B2	-	-	-	+	+	-
B3	-	+	-	+	+	-
B4	-	-	-	+	+	-
B6	+	+	+	+	+	-

+: growth
-: no growth
* A mixture of equal volume of *n*-dodecane and *n*-hexadecane.

Sixty-eight enriched cultures were screened but only five microorganisms (S449B1, B2, B3, B4 and B6) were isolated. It seems to be very important reason for poor isolation that toluene was rather toxic for growth of some microorganisms.

Taxonomical Studies of Isolated Microorganisms.

The results of taxonomical studies are shown in Fig. 1 and Table III, together with the com-

6) M. E. Fewster and D. A. Hall, *Nature Lond.*, **168**, 78 (1951).

FIG. 1. Electron Microscopic Photograph of Strain S449B3.

parable descriptions in the Bergey's Manual, 7th Ed. The morphological characteristics of the all strains were exactly the same to each other.

From these results, it was concluded that all the strains were very closely resembling *Pseudomonas desmolytica*.

It was reported by Tabak, Chambers and Kabler⁷⁾ that pseudomonads readily adapted to aromatic derivatives. However, it could not be determined whether the newly isolated strains were adaptive or not.

Determination of Metabolic Products.

Each strain was cultivated on the individual hydrocarbons. After 24, 63 and 193 hours, the cultural broth was submitted to the paper chromatography.

From *p*-cymene substrates, methyl-red positive and ultraviolet-absorbing spot was detected from the broth of all 5 strains (Solvent 1).

This substance was isolated in crystalline form from the cultural broth of the strain S449B6 by extraction with ether and was recrystallized from water. The chemical

TABLE III. DESCRIPTIVE CHART OF THE ISOLATED MICROORGANISMS

	<i>Pseudomonas desmolytica</i>	S449B1, B2, B3, B4 and B6
Morphological characteristics	Rods, 0.7 to 0.8 by 2.0 to 3.0 μ , occurring singly and in pairs. Motile, with 1 to 5 polar flagella. Gram-negative.	Rods, 0.6 to 0.9 by 1.5 to 2.0 μ , occurring singly and in pairs. 1 to 6 polar flagella. Gram-negative.
Gelatine colonies	Circular, gray to buff. Smooth, glistening, entire. Raised or umbonate.	Irregular, gray to buff. Rough, dull, undulate. Umbonate.
Gelatin stab	No liquefaction.	No liquefaction.
Agar colonies	Circular or amoeboid, whitish, flat or convex, smooth, translucent to opaque, entire.	Circular, convex, smooth, translucent or opaque, glistening, pale yellowish green with luminescence.
Agar slant	Filiform, pale buff, raised, smooth, undulate.	Filiform, pale yellowish green, raised, smooth, undulate.
Broth	Turbid.	Turbid.
Action on nitrate	Nitrites often produced.	Nitrites produced.
Hydrolysis of starch	Negative.	Negative.
Cleavage of sugar	Acid usually produced from glucose.	Acid produced only from glucose.
Free oxygen	Aerobic, facultative.	Aerobic.
Temperature	Grows at 35°C.	Grows at 35° and 37°C.
Source	Isolated from soil.	Isolated from soil.

characteristics of the crystal were compared with those of the authentic cumic acid.

As shown in Figs. 2 and 3, the samples had identical infrared absorption spectra, and m.p. 118 to 118.5°C.

Production of Cumic Acid by the Organisms.

The amounts of the acid formed are shown in Table IV. Maximum amount of the acid formed was observed with strain S449B6, which was employed for the following experiments as a test organism.

Some Aspects of Cumic Acid Formation by Strain S449B6.

The course of the cultivation of the strain

7) H. H. Tabak, C. W. Chambers and P. W. Kabler, *J. Bacteriol.*, **87**, 910 (1964).

FIG. 2. Infrared Absorption Spectra of the Product.

A; Product B; Authentic cumic acid

FIG. 3. Ultraviolet Absorption Spectra of the Product.

1.2 mg of the sample was dissolved in 100 ml of 0.01 N-NaOH.

TABLE IV. PRODUCTION OF CUMIC ACID BY THE ORGANISMS (mg/l)

Strain No.	Time (hr.)		
	22	73	116
S449B1	887	888	798
B2	917	934	832
B3	927	874	824
B4	284	251	257
B6	974	965	905

S449B6 was followed. The results are shown in Fig. 4. It is clear that maximum productions of both the acid and the cells were obtained at 15 hours after inoculation, when pH value of the cultural broth was minimal 4.6.

FIG. 4. Time Course of the Acid Production.

So it seems to be very likely that the control of the pH value results in higher yield of the acid as shown in Fig. 5.

Then the effect of *p*-cymene concentration

on the acid production was tested (Fig. 6). Although the highest yield of the acid was obtained from 10% *p*-cymene, the most effective conversion of *p*-cymene to the acid was observed at the concentration of 0.5%.

FIG. 5. Effect of CaCO_3 on the Acid Production.

—●— CaCO_3 addition
 ↓ 50 mg of CaCO_3 per 50 ml of medium was added at each time.
 ---○--- no addition

FIG. 6. Effect of *p*-Cymene Concentration on the Acid Production.

Because the acid formation is oxidative process, aeration will be indispensable factor. So the relationship between the volume of the medium and the amount of the acid formed was investigated in the range of the volume from 25 ml to 200 ml per 500 ml-flask.

The results were obscure as shown in Table V. But as shown in Table VI the agitation seemed to be more strictly effective. It was concluded that the acid formed at 160 reciprocation per minute (Re. P.M.) was some 30% higher than at 132 Re. P.M.

Fig. 7 shows that the acid formation was not significantly effected at either 26.5°, 30° or 37°C.

TABLE V. EFFECT OF VOLUME OF MEDIUM ON THE ACID PRODUCTION (mg/l)

Medium (ml/500 ml flask)	Time (hr.)	
	25	117
25	970	1060
50	939	1070
100	970	977
200	844	892

TABLE VI. EFFECT OF AGITATION ON THE ACID PRODUCTION

Agitation (Recip. P.M.*)	Exp. No.	Acid found (mg/l)
132	1	1250
	2	1130
160	1	1680
	2	1650

* Reciprocation per minute
 The cultivation was carried out at 37°C

FIG. 7. Effect of Temperature on the Acid Production.

Consequently, it is suggested that the control of the pH value and the agitation would be very important factors to improve the production of the acid from *p*-cymene.

The microbial transformation of *p*-cymene to cumic acid was reported by Davis and Raymond.⁸⁾ The work showed that cumic acid was produced during the growth of a *Nocardia* on *n*-alkane. It was not described whether the *Nocardia* grew on *p*-cymene or not.

Further investigations on the cumic acid formation from *p*-cymene, including reaction mechanisms, and on other aromatic hydrocarbon-utilizing microorganisms are in pro-

gress. The results will be shown elsewhere.

SUMMARY

1. Five strains were isolated from soil with the aromatic hydrocarbon substrates as a sole carbon source.

2. They were identified as closely resembling *Pseudomonas desmolytica*.

They produced cumic acid during the growth on *p*-cymene.

3. Addition of calcium carbonate and considerable agitation were favorable cultural conditions for the acid production.

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8) J. B. Davis and R. L. Raymond, *Appl. Microbiol.*, **9**, 383 (1961).