Studies on the Utilization of Hydrocarbons by Microorganisms

Part X. Screening of Aromatic Hydrocarbon-Assimilating Microorganisms and *p*-Toluic Acid Formation from *p*-Xylene

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Received June 16, 1967

In the course of investigation of aromatic hydrocarbon-utilizing bacteria, a certain strain S668B2 was found to produce a large amount of *p*-toluic acid from *p*-xylene.

Strain S668B2 was shown to be capable of producing an ultraviolet-absorbing substance from *p*-xylene. This substance was isolated in the form crystal and identified to *p*-toluic acid by several observations.

From the results of taxonomic studies on S668B2, it was identified to be *Pseudomonas* species.

The optimal medium composition and culture condition were studied in order to increase *p*-toluic acid production. The addition of CaCO₃ was favorable for the *p*-toluic acid production. The amount of *p*-toluic acid formation increased from 0.4 g/l to 3.4 g/l after the investigation of optimal culture condition.

INTRODUCTION

The utilization of various hydrocarbons by microorganisms has recently received a good deal of attention. Especially the studies on fermentative production of amino acids and cells from *n*-alkane were carried out by several groups of workers.¹⁻³⁾ In spite of these many reports, there are few concerning the industrial application of aromatic hydrocarbons. Only the formation of salicylic acid from naphthalene⁴⁾ and of cumic acid from *p*-cymene⁵⁾ have been almost exclusively investigated. While, in recent years, increasing

interest has been shown in the microbial conversion of organic compounds, yet there is no systematic study on the transformation and assimilation of aromatic hydrocarbons, such as methylbenzenes, by microorganisms. So this work was initiated to form a part of above program. This report deals with the identification of newly isolated microorganisms, microbial formation of *p*-toluic acid from *p*-xylene and some observations on the *p*-toluic acid production.

MATERIALS AND METHODS

Microorganisms. A bacterial strain S668B2 was isolated from soil of rice field by a screening procedure using *p*-xylene as the sole source of carbon. Diagnostic tests for the morphological studies were carried out mainly according to the "Manual of Microbiological Methods," 1957, Society of American Bacteriologists, and referred to the "Bergey's Manual of Determinative Bacteriology", 7th Ed., 1957.

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

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5) K. Yamada, S. Horiguchi and J. Takahashi, This Journal, **29**, 943 (1965).

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

Solvent	Reagent	Metabolic Product
1. $(\text{NH}_4)_2\text{CO}_3\text{-NH}_4\text{OH-}n\text{-Butanol}^*$	B.C.G.	Organic Acid
2. $n\text{-Butanol; Acetic acid; Water}$ (3:2:95)	Diazotized Benzidine** UV Ray Irradiation	Phenolic compound UV-Ray-absorbing compound
3. $n\text{-Butanol; Acetic acid; Water}$ (4:1:2)	Ninhydrin	Amino acid

* $n\text{-Butanol}$ was saturated with ammonia/ammonium carbonate.

** 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.

Media. The composition of screening medium was as follows $p\text{-Xylene}$ 20 ml/l, NH_4NO_3 4.0 g, KH_2PO_4 1.5 g, Na_2HPO_4 1.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.005 g, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.002 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 g, Yeast ext. 0.5 g and distilled water to make 1 l.

After culture conditions were investigated, the optimal composition of the medium was established as follows: $p\text{-Xylene}$ 20 ml/l, $(\text{NH}_4)_2\text{CO}_3$ 4.0 g, KH_2PO_4 1.5 g, NaH_2PO_4 1.5 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.001 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.01 g, Yeast ext. 0.5 g, CaCO_3 40 g, distilled water to make 1 l and pH was adjusted to 7. A five hundred milliliter-flask containing 50 ml of the medium was autoclaved at 115°C for 10 minutes. Before cultivation 1.0 ml of sterile hydrocarbon was added to the medium in the flask.

Cultivation. A loop of cells grown on $p\text{-xylene-agar-slant}$ for 2 days was inoculated and incubated at 30°C on reciprocal shaker for 24 hours during the investigation of the culture conditions.

Hydrocarbons. All of the hydrocarbons (toluene, $p\text{-xylene}$, $m\text{-xylene}$, $o\text{-xylene}$, ethylbenzene, $m\text{-}$ and $p\text{-diethylbenzene}$) were obtained from Tokyo Chemical Industry Co., Ltd., and used without further purification.

Paper Chromatography. Paper chromatography of the ascending technique was employed for the detection of metabolic products. Solvents systems used⁶⁻⁷⁾ are listed in Table I. The spots were visualized by ultraviolet irradiation and/or by spraying the developing reagent (Table I). Toyo filter paper No. 50 was used.

Determination of Growth Cell and Acid produced. Cell growth was determined by weighing dried cells.

FIG. 1. Procedure for the Determination of Cell Growth and Acid Produced.

Usually main part of cells did not precipitate to the bottom of culture broth through the centrifugation so long as $p\text{-xylene}$ substrate remained. Therefore the amount of cells was determined according to the procedure presented in Fig. 1. Also $p\text{-toluic acid}$ produced in culture broth was extracted by equal volume of ether as shown in Fig. 1.

The ether solution was titrated by NaOH (N/100, N/50, N/20) and then the amount of acid produced was calculated from the titration value.

RESULTS AND DISCUSSION

Isolation of Microorganisms from Soil

Accumulation culture was carried out by adding 0.1 g of soil sewage sample into 5 ml of medium in a test tube. After second accumulation culture microorganisms were isolated by the general method.

Seven microorganisms were obtained from seventy enriched cultures. But only one of them showed good growth in liquid medium.

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

7) M. S. Bardinskaya and T. A. Shubert, *Biokhimiya*, **27**, 58 (1962).

TABLE II. ASSIMILATION OF HYDROCARBONS BY ISOLATED BACTERIA

Substrate	Growth
Toluene	—
<i>p</i> -Xylene	+
<i>m</i> -Xylene	+
<i>o</i> -Xylene	—
Ethylbenzene	—
Diethylbenzene	—
1, 2, 3-Trimethylbenzene	—
1, 2, 4-Trimethylbenzene	+
1, 3, 5-Trimethylbenzene	—
+: growth —: no growth	

TABLE III. DESCRIPTIVE CHART OF THE ISOLATED MICROORGANISMS (S668B2)

Rods, 0.6 by 1.2 to 1.6 microns. Motile with a polar flagellum. Spore not formed. Gram-negative.

Nutrient agar colonies: Circular, smooth, entire, convex, glistening, pale yellowish brown, butyrous.

Nutrient agar slant: Growth moderate, filiform, flat, glistening, pale yellowish brown.

Glutamate agar slant: Growth moderate, filiform, glistening, yellowish gray.

Nutrient broth: Strongly turbid.

Nutrient gelatin stab: Liquefaction.

B.C.P. milk: Alkaline, not peptonized.

Nitrite is not found after 1, 3 and 5 days' incubation in nitrate broth.

Nitrate respiration: Positive.

Indole not produced.

Starch not hydrolyzed.

Acid but no gas is aerobically produced from glycerol, xylose, glucose and starch according to Hugh and Leifson's method. No acid and gas are anaerobically produced from the carbohydrates mentioned above.

Glucose, gluconate, citrate, succinate and *p*-hydroxybenzoate are utilized as a sole source of carbon with ammoniacal nitrogen.

Reducing substance is not produced from gluconate.

Soluble pigment is not produced on King's A and B media.

Grows well at 37° and 42°C.

Cytochrome oxidase: Positive.

Source: Soil.

And the strain (S668B2) was tested for its abilities to utilize the individual hydrocarbons. As shown in Table II, the strain (S668B2)

utilized *p*-xylene, *m*-xylene and 1, 2, 4-trimethylbenzene as a sole source of carbon.

Taxonomical Studies of Microorganisms Isolated

From the results of taxonomic studies, shown in Table III, the strain S668B2 obviously belonged to *Pseudomonas* species. Through the results of physiological tests it seemed to be achromogenic variety of *Ps. aeruginosa*. But the strain differed from typical *Pseudomonas aeruginosa* in the following points; 1) 2-Ketogluconate was not formed from gluconate. 2) Gelatin was liquefied but milk was not peptonized. 3) Nitrate respiration was positive.

Determination of Metabolic Products. After 20 and 40 hours cultivation on *p*-xylene, the ether extract of the culture broth was submitted to paper chromatography. Bromocresol green-positive and ultraviolet-absorbing spot was detected. Usually other spot could not be found. The substance was isolated in crystalline form from the ether extraction of culture broth and was recrystallized from water. The paper chromatography was done on two kinds of solvent system shown in Table I. The R_F values (solvent system 1, 0.4; solvent system 2, 0.65) were essentially the same with those of authentic *p*-toluic acid. The physical properties of the crystal which was recrystallized from water two times were compared with those of authentic *p*-toluic acid. As shown in Fig. 2 and 3, the product and authentic sample had identical infrared and ultraviolet ray absorption spectra and m.p. of 178°C.

Also elementary analysis of the product was

FIG. 2. Infrared Absorption Spectra of the Product

A: Authentic *p*-toluic acid
B: Product

FIG. 3. Ultraviolet Absorption Spectra of the Product.

The sample was dissolved in 0.01 N-NaOH.

FIG. 4. Time Course of the Acid Production.

in good agreement with the calculated value of the *p*-toluic acid (Found: C, 70.38; H, 6.07 Calcd. for: C, 70.58; H, 5.92; O, 23.50%).

Some Observations Concerning *p*-Toluic Acid Production

The time course of the *p*-toluic acid production was pursued with the composition of screening medium. The growth curve, *p*-toluic acid production and change of pH are given in Fig. 4. From the results, a maximum amount of acid production was given at 15 hours after inoculation. Then the pH value of the culture broth moved downward with the acid production and showed 5.0 at the lowest. The cell growth had similar tendency with the curve of acid production.

Effect of Aeration and Agitation on *p*-Toluic acid Production

As the formation of *p*-toluic acid should be due to oxidative process, aeration and agitation seem to be essential factors. The aeration effect was investigated with varying the volume of medium within the range from 50 ml to 300 ml per 500-ml flask. The results given in Table IV showed that aeration was effective for the higher acid formation. And also agitation had obvious effect on acid formation, which was concluded by comparing 170 and 120 reciprocations per minute. The results are listed in Table V

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

Medium (ml/500-ml flask)	Acid (mg/l)
50	410
100	450
200	380
300	300

TABLE V. EFFECT OF AGITATION ON THE ACID PRODUCTION

Agitation (R.P.M.)*	Acid found (mg/l)
120	370
170	530

* Reciprocations per minute

Effect of Nitrogen Source on *p*-Toluic Acid Production

In order to find out the suitable nitrogen source for *p*-toluic acid production, various kinds of nitrogen source were examined by replacing ammonium nitrate with the same

TABLE VI. EFFECT ON NITROGEN SOURCES ON THE ACID PRODUCTION

N-Source (4 g/l)	pH	Acid found (mg/l)
NH ₄ NO ₃	5.4	490
NH ₄ Cl	5.4	260
(NH ₄) ₂ SO ₄	5.6	370
(NH ₄) ₂ HPO ₄	5.6	880
NH ₄ H ₂ PO ₄	5.6	920
NaNO ₃	7.0	370
(NH ₂) ₂ CO	5.8	1080
Peptone	5.4	1240

TABLE VII. EFFECT OF NITROGEN CONCENTRATION ON THE ACID PRODUCTION

N. Source	g/l	Acid (20 hr.) (mg/l)	Acid (40 hr.) (mg/l)
(NH ₂) ₂ CO	1	1040	1100
	4	810	1100
	8	820	1110

TABLE VIII. EFFECT OF MINERAL SALTS ON THE ACID PRODUCTION

Omission	Growth	Acid (mg/l)
-(Mg+Mn+Ca+Fe)	—	—
-(Mn+Ca+Fe)	+	240
-(Mg+Mn+Ca)	+	80
-(Mg+Mn+Fe)	—	—
-(Mg+Ca+Fe)	—	—
-(Fe+Ca)	+	140
-(Fe+Mn)	+	260
-(Mg+Mn)	+	130
-(Mg+Ca)	+	10
-(Mg+Fe)	+	20
-(Mn+Ca)	+	250
-Fe	+	110
-Mg	+	90
-Mn	+	370
-Ca	+	340
-Yeast ext.*	+	390

Mg: MgSO₄·7H₂O Mn: MnSO₄·7H₂OFe: FeSO₄·7H₂O Ca: CaCl₂·2H₂O

* Yeast ext. was not added throughout this omission test.

weight per cent of individual nitrogen source. As shown in Table VI, urea, ammonium phosphate and peptone were chosen as favorable nitrogen sources. Above all, urea was superior in reappearance to the others. The concentration of urea was also studied within the extent from 1 g/l to 8 g/l as presented in Table VII. But there was no difference in acid production regarding to the level of nitrogen concentration.

Effect of Mineral Salts

Omission test was carried out. The result is shown in Table VIII. MnSO₄·4H₂O showed inhibitory effect, while FeSO₄·7H₂O and MgSO₄·7H₂O stimulatory. CaCl₂·2H₂O had little effect. The investigation was made to find out the optimal concentration of MgSO₄·7H₂O and FeSO₄·7H₂O. From these studies it was concluded that MgSO₄·7H₂O 200 mg/l and FeSO₄·7H₂O 1 mg/l were optimal concentration.

Effect of pH and CaCO₃ on *p*-Toluic Acid Production

For the purpose of investigating the effect of pH on the formation of *p*-toluic acid, the initial pH of the culture medium was adjusted

TABLE IX. EFFECT OF INITIAL pH ON THE ACID PRODUCTION

Initial pH	pH*	Acid (mg/l)
9.0	9.0	—
8.0	5.4	720
7.0	5.2	350
6.0	5.2	250
5.0	5.0	—

* pH after 20 hr. incubation

Ammonium nitrate was used as nitrogen source.

TABLE X. EFFECT OF INITIAL pH ON THE ACID PRODUCTION

Initial pH	pH*	Acid (mg/l)
9.0	9.0	—
8.0	7.4	540
7.0	6.9	900
6.0	5.7	430
5.0	5.0	—

* pH: After 20 hr incubation.

Urea was used as nitrogen source.

to 5, 6, 7, 8 and 9. The results are given in Table IX. When ammonium nitrate was used as nitrogen source, the initial pH 8 was suitable for the higher yield of acid. But during the early period of cultivation the cell growth was not so well and after 24 hours cultivation the pH value gradually fell down to 5.4. But when urea was used as nitrogen source, the initial pH 7 was the most favorable for the higher production of the acid. The result is shown in Table X.

It was concluded that the initial pH 7 was effective for the acid production. Also it was necessary to maintain pH value during cultivation to some degree higher than 6. From the results of Fig. 4 and Tables IX and X, it appeared that the control of the pH value was necessary for the higher yield of the acid. So the CaCO_3 was added for the maintenance of pH. As shown in Fig. 5, the amount of the production of *p*-toluic acid increased by the addition of CaCO_3 . However there was no great difference among the concentrations of CaCO_3 from 0.2% to 8% concerning the yield of the acid.

Consequently, it is concluded that the aeration and agitation are effective for the production of the acid and also that the control of pH value is important factor to improve the production of *p*-toluic acid from *p*-xylene. Further studies on the *p*-toluic acid formation from *p*-xylene including not only reaction mechanisms but also the relation between

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

●—● + CaCO_3 ×—× - CaCO_3
Urea was used as nitrogen source.

substrates and organisms are in progress. The results will be shown elsewhere.

Acknowledgement. Grateful acknowledgement is made to Dr. K. Komagata for the taxonomic studies.

This paper was read at the Annual Meeting of the Agric. Chem. Soc. Japan which was held at Tokyo on April 4th, 1967.