Studies on the Utilization of Hydrocarbons by Microorganisms

Part XII. Screening of Alicyclic Hydrocarbon-Assimilating Microorganisms and *trans*-4-Ethylcyclohexanol Formation from Ethylcyclohexane

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In the course of investigation of alicyclic hydrocarbon-utilizing microorganisms, five strains of ethylcyclohexane-utilizing bacteria were isolated from soil samples.

Among those bacteria, the strain S6B1 that was identified as *Alcaligenes faecalis*, showed the best growth in shaking culture.

The strain S6B1 was found to produce 4-ethylcyclohexanol from ethylcyclohexane.

This substance separated from culture broth was purified and identified to be *trans*-4-ethylcyclohexanol by the use of NMR.

According to the advance of petroleum microbiology, many reports have been published as to the intermediates or end products accumulated during the microbial dissimilation of hydrocarbons. However, most of them were concerned with aliphatic or aromatic hydrocarbons and as to alicyclic hydrocarbons there are only a few reports, *i.e.*, the formation of valeric acid,¹⁾ formic acid, cyclohexanol and adipic acid from cyclohexane; the formation of adipic acid from decalin;²⁾ the co-oxidation of *n*-butylcyclohexane to cyclohexane-acetic-acid³⁾ and the co-oxidation of several cycloparaffines to alicyclic monoketones.⁴⁾

This report deals with the isolation of alicyclic hydrocarbon-utilizing microorganisms,

the taxonomical studies of isolated bacteria and microbial formation of *trans*-4-ethylcyclohexanol from ethylcyclohexane.

MATERIALS AND METHODS

Microorganisms. Five strains of bacteria were isolated from soil samples, using alicyclic hydrocarbons as the main source of carbon.

With regard to the microorganism that showed the best growth in shaking culture, diagnostic tests were carried out according to the "Manual of Microbiological methods," 1957, Society of American Bacteriologists and "Bergey's Manual of Determinative Bacteriology," 7th Ed., 1957.

Medium. The medium contained, per liter of distilled water, 1.0 g NH₄NO₃, 1.5 g KH₂PO₄, 1.5 g Na₂HPO₄, 0.2 g MgSO₄·7H₂O, 0.01 g CaCl₂·2H₂O, 0.005 g FeSO₄·7H₂O, 0.002 g MnSO₄·4H₂O, 0.05 g yeast extract and pH was adjusted to 7.0. A test tube (200 mm, 18 mm inner diameter) containing 5 ml of the medium was autoclaved at 115°C for 10 min and used for the screening of microorganisms. Before cultivation 0.1 ml of separately sterilized hydrocarbon was added to 5 ml of the medium. A

1) B. Imelik, *Compt. Rend. Acad. Sci.*, **226**, 2082 (1948).

2) C. Colla and V. Treccani, *Ann. Microbiol. ed Enzimol.*, **10**, 77 (1960).

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

4) J. Ooyama and J. W. Foster, *Antonie van Leeuwenhoek*, **31**, 45 (1965).

5 liters-shaking-flask containing 1 liter of the medium was used for the detection of the products.

Cultivation. For the detection of products a loopful of cells grown on hydrocarbon-agar-slant for 3 days was inoculated into 40 ml of the liquid medium which was shaken for 3 to 4 days at 30°C. Then this seed culture was transferred into 5 liter-shaking-flask containing 1 liter of the medium.

Hydrocarbons Cyclohexane, cyclohexanone, cyclohexene, methycyclohexane, ethylcyclohexane and decalin were used as the carbon sources.

They were purchased from Tokyo Chemical Industry Co., Ltd. Tokyo.

4-Ethylcyclohexanol (mixture of *cis*- and *trans*-isomers) was purchased from K & K Laboratories, Inc. New York.

Analytical methods and apparatus

Thin layer chromatography. Thin layer chromatography using silicagel G was employed for the detection of the metabolic products.

Solvent systems used are listed in Table I. The spots were visualized by spraying H_2SO_4 (containing 0.5 g of KMnO_4 in 15 ml).

Silicagel G was obtained from E. Merck Co., Ltd. Darmstadt.

TABLE I. SOLVENT SYSTEMS FOR SILICAGEL G THIN LAYER CHROMATOGRAPHY

a) Benzene-ethanol	10 : 1
b) Benzene-acetic acid	10 : 1
c) Carbon tetrochloride-acetic acid-water	10 : 1 : 1
d) Methanol saturated heptane	

Column chromatography. Silicic acid column chromatography was employed for the purification of the product and the separation of *cis*- and *trans*-isomers of authentic 4-ethylcyclohexanol.

Solvent system was benzene-ethylacetate. Column was 300 mm in length and 20 mm inner diameter.

Silicic acid was obtained from Mallinkrodt Chemical Works, New York.

Gas-liquid chromatography. Gas-liquid chromatography was performed with a model K-53 Hitachi Seisakusho Ltd., using 4 mm $\times$ 2 m column packed

with 10% PEG 1500 on diasolid (60~80 mesh). The operating conditions were as follows;

column temperature: 175°C
 carrier gas helium
 flow rate : 40 ml/min
 detector : thermal conductivity detector

Infrared spectra. The infrared absorption spectra were obtained on film with a Model IR-S infrared Spectrophotometer, Japan Spectroscopic Co., Ltd.

Mass spectra. Mass spectra were obtained by a model RMU-6D mass spectrometer with direct inlet system at 70 eV of electron accelerating voltage.

Nuclear magnetic resonance spectra. NMR spectra were obtained with a JEOL-4H-100 spectrometer at 100 mc.

RESULTS

Isolation of microorganisms

Enrichment culture with 0.1 g of a soil sample and 5 ml of the medium in a test was carried out at 30°C for 3 to 4 days.

Microorganisms were isolated after two successive enrichment cultures by the method shown in Fig. 1.

FIG. 1. Isolation Method of Bacteria.

From 350 soil samples 5 bacterial strains that grew on ethylcyclohexane as the main source of carbon were isolated.

Strain S6B1 showed the best growth in liquid culture.

Taxonomical studies of strain S6B1

The results of taxonomical studies are shown in Table II. Based on the description in the 7th edition of Bergey's Manual, the strain S6B1 was identified as *Alcaligenes faecalis*.

TABLE II. DESCRIPTIVE CHART OF STRAIN S6B1

Rods, 0.4 to 0.6 by 1.2 to 1.8 microns. Motile with peritrichous flagella. Gram-negative.

Nutrient agar colonies: Circular, smooth, entire, raised, opaque, glistening, pale yellowish brown, bitryous.

Nutrient agar slant: Growth moderate, filiform, glistening, yellowish gray, medium unchanged.

Nutrient broth: Strongly turbid.

Nutrient gelatin stab: No liquefaction.

Milk: Unchanged.

B.C.P. milk: Slightly alkaline, not peptonized.

Nitrite produced from nitrate.

Nitrate respiration: Positive.

Indole not produced.

MR test: Negative.

V-P test: Negative.

Starch not hydrolyzed.

Hydrogen sulfide not produced.

No acid and gas from glycerol, xylose, glucose, sucrose, lactose and starch in both aerobic and anaerobic conditions.

Citrate and succinate are utilized as a sole source of carbon with ammoniacal nitrogen, but glucose, gluconate, *p*-hydroxybenzoate and protocatechuate are not utilized.

Grows well at 20° to 30° No growth at 42°

Separation of metabolic product A

The ether extract of 72 hr culture broth of the strain S6B1 was applied to silicagel G thin layer chromatography.

Four spots were detected on the thin layer plate by using benzene-ethanol (10:1) as solvent system and H₂SO₄ (containing 0.5 g of KMnSO₄ in 15 ml) for spraying reagent.

FIG. 2. Isolation Procedure for Product A.

The ether extract was treated according to the procedure presented in Fig. 2 and the main product A was purified.

Analysis of product A

Purified product A was colorless, transparent, neutral liquid, soluble in ether, benzene, ethylacetate, ethanol, and insoluble in water. It gave a single spot on the thin layer chromatogram by using solvent systems shown in Table I.

Mass spectrum of this substance revealed a parent ion peak of *m/e* 178 with composition C₈H₁₆O. The presence of the functional groups illustrated in Table III was expected on the basis of infrared spectra and nuclear magnetic resonance spectra. Considering that ethylcyclohexane was used as the main source of carbon, these data suggest that this substance is ethylcyclohexanol. Among four isomers of ethylcyclohexanol, the product A will be 2 ethyl-, 3 ethyl- or 4 ethylcyclohexanol.

TABLE III. ASSUMED FUNCTIONAL GROUPS
ON THE BASIS OF INFRARED SPECTRA
AND NUCLEAR MAGNETIC
RESONANCE SPECTRA

	IR, $\nu_{\text{max}}^{\text{(Film)}}$ cm ⁻¹	NMR, $\tau_{\text{Me}_4\text{Si}}^{\text{(CDCl}_3\text{)}}$
-OH (alcoholic)	3330, 1055	
-CH ₂ CH ₃		9.12 (3H, t)
>CHOH		6.51 (1H, m)
-CH ₃ , -CH ₂ -	2920, 2840 1450, 1370	
>C=C<	none

FIG. 3. Predicted Fission Modes of Mass Spectra of Methylcyclohexanol and Ethylcyclohexanol.

According to the Herbert Budzikiewicz's

TABLE IV. RELATIVE ABUNDANCE OF m/e 57, AND m/e 71 IONS OF METHYL-
CYCLOHEXANOL, AND m/e 57 AND m/e 84 IONS OF PRODUCT A

	Relative abundance of m/e 57 and 71 ions of methylcyclohexanol			Relative abundance of m/e 57 and 85 ions of product A	
	m/e 57	m/e 71		m/e 57	m/e 85
2-Methylcyclohexanol	69	31			
3-Methylcyclohexanol	36	64			
4-Methylcyclohexanol	90	10	Product A	85	15

FIG. 5. Nuclear Magnetic Resonance Spectra of Substance A and B.

information concerning mass spectra of methylcyclohexanol,⁵⁾ 2-methyl and 3-methylcyclohexanols have two types of fission modes and 4-methylcyclohexanol has only one type as shown in Fig. 3 as demonstrated in Table IV. Ethylcyclohexanol is expected to have similar fission mode to methylcyclohexanol because of structural similarity. Therefore, it is suggested that the product A must be 4-ethylcyclohexanol considering the relative abundance of m/e 57 and m/e 84 ions in the mass spectra.

Separation of *cis*-, and *trans*-isomers of 4-

FIG. 4. Separation of *cis*-, and *trans*-Isomers of 4-Ethylcyclohexanol.

5) "Interpretation of Mass Spectra of Organic Compounds," ed. by H. Budzikiewicz, C. Djerassi

and D. H. Williams, Holden-Day Inc., San Francisco, 1964, P. 39.

FIG. 6. Infrared Spectra of Product A.

A: Product A

B: Authentic *trans*-4-ethylcyclohexanol

ethylcyclohexanol.

Cis- and *trans*-isomers of 4-ethylcyclohexanol were separated according to the procedure presented in Fig. 4.

Substance A and B was determined to be *trans*- and *cis*-isomers respectively with nuclear magnetic resonance spectra shown in Fig. 5.

Multiplex signals in the τ 6 to τ 7 region seem to show the spin-spin coupling of 1-hydrogen with the four hydrogens on the neighboring carbons. Comparing the half-width 50 cps and 20 cps of the two signals, substance A seemed to be *trans*-isomer and substance B *cis*-isomer.⁶⁾

Identification of *trans*-4-ethylcyclohexanol

The purified sample showed the identical *R_f* value in thin layer chromatography with solvent systems shown in Table I, retention time of 2.4 min under the condition previously listed, infrared spectrum shown in Fig. 6, and mass spectrum with those of *trans*-4-ethylcyclohexanol separated according to the procedure shown in Fig. 4.

SUMMARY

1. Five strains of bacteria were isolated from soil samples with ethylcyclohexane as the main source of carbon.

2. The strain S6B1 which showed the best growth in the liquid medium was identified

6) R. U. Lemieux, R. K. Kullning, H. J. Bernstein and W. G. Schneider, *J. Am. Chem. Soc.*, **80**, 6093 (1958).

as *Alcaligenes faecalis*.

3. The main product was separated from culture broth and identified as *trans*-4-ethylcyclohexanol.

4. The mixture of *trans*- and *cis*-isomers of 4-ethylcyclohexanol was separated and

submitted to the identification.

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