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Formation of 3-Ethylsalicylic Acid from 3-Ethyltoluene by *Pseudomonas ovalis*

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To elucidate the microbial oxidation mechanism of aromatic hydrocarbons, the works were carried out by comparing the microbial oxidation patterns of following two different groups of aromatic compounds. One is aromatic hydrocarbons substituted with *n*-alkyl or *n*-alkenyl groups, such as xylene, ethyltoluene and various *n*-alkyl and *n*-alkenylbenzenes. The other is aromatic hydrocarbons substituted with *iso*-alkyl or *iso*-alkenyl groups which have a branched carbon chain, such as isopropylbenzene, isobutylbenzene and α-methylstyrene *etc.* As a part of above program, this report deals with the result of screening of methyl or ethyl substituted aromatic hydrocarbons assimilating microorganisms and the identification of oxidation product from 3-ethyltoluene.

Isolation of microorganisms from soil was carried out as follows. 0.1 gram of a soil sample and 5 ml of the medium in a test tube was shaken at 30°C for 3 or 4 days. The composition of medium containing 1% (v/v) aromatic hydrocarbon was same as reported already.[1] After second enrichment culture, microorganisms were isolated by the general method. Three bacterial strains grown on 3-ethyltoluene as a source of carbon and energy were isolated. Among them a strain SlBl showed the best growth in liquid medium and the strain SlBl was used for the experiment. Growth of the strain SlBl on various methyl or ethyl substituted aromatic hydrocarbons was as follows; utilized 3-ethyltoluene, *m*-xylene and *p*-xylene as a source of carbon, but not 2- and 4-ethyltoluene, *o*-xylene, ethylbenzene and diethylbenzene.

From the result of taxonomic studies, the strain SlBl obviously belonged to *Pseudomonas* species. Through the results of physiological tests it seemed to be achromogenic variety of *Ps. ovalis*. But the strain differed from typical *Pseudomonas ovalis* in the following points: 1) Nitrate was reduced weakly to nitrite in nitrate broth. 2) Galactose, mannose and arabinose were assimilated but 2-ketogluconate and ethanol were not.

In order to examine the oxidation products, the strain SlBl was grown at 30°C in 5 liter erlenmeyer flask with 1 liter of medium containing 10 ml of 3-ethyltoluene. The flask was shaken on a rotary shaker for 2 days. The culture broth (pH 4.8) was acidified with 5% HCl solution and extracted with ether. The ether extract was subjected to steam distillation and a crystalline product was formed from the distillate. The product was recrystalized from methanol and water. A purified product was colorless crystal, soluble in ether, benzene and ethanol. It gave a single spot on thin-layer chromatogram with following two different solvents: benzene-dioxane-acetic acid (90: 25: 4, v/v), *Rf* 0.72; chloroform-acetic acid (9: 1, v/v), *Rf* 0.81.

The IR spectrum of the product was shown in Fig. 1. A rather weak absorption at 3160 cm^{-1} due to OH group indicated OH group's chelation with *ortho* functional group, perhaps with COOH group. Its absorption at 1660 cm^{-1} showed carbonyl group due to carboxylic acid and the absorption at 1610 cm^{-1}, 770 cm^{-1} and 700 cm^{-1} due to benzene nucleus were sharp and characteristic. The other properties of the product were as follows. The product melted at 113°C was positive to the reagent of bromocresol green, diazotized benzidine and negative to that of 2,4-dinitrophenyl hydrazine. UV λ_{max}^{EtOH} nm: 305, 240, 206.

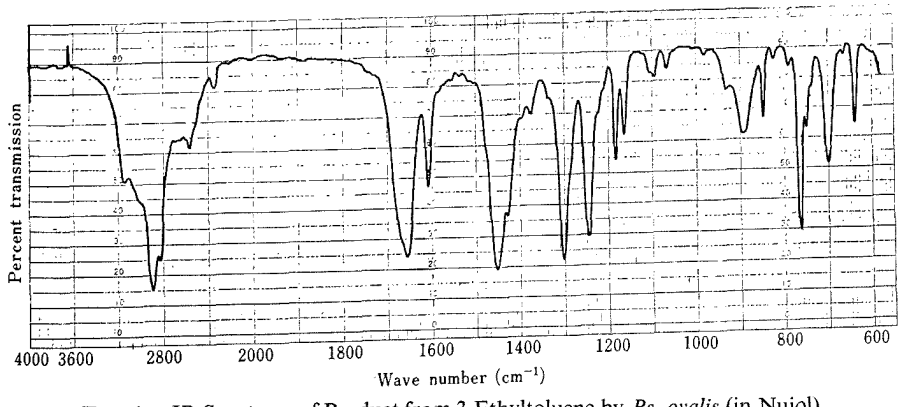

FIG. 1. IR Spectrum of Product from 3-Ethyltoluene by *Ps. ovalis* (in Nujol).

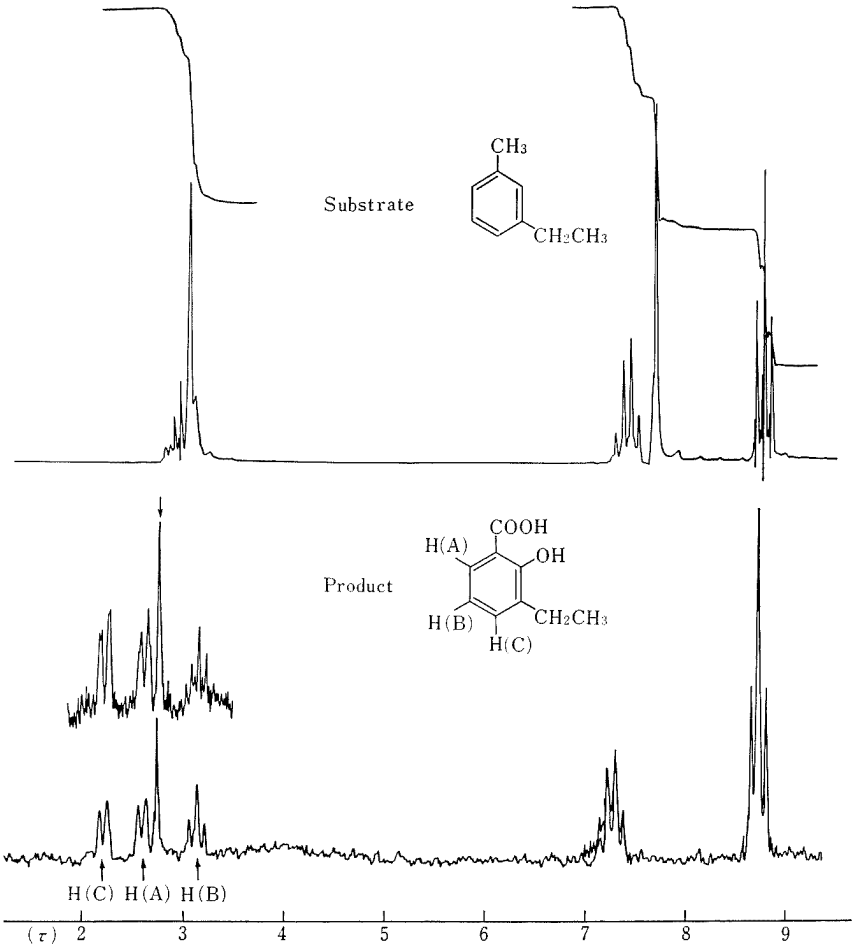

FIG. 2. NMR Spectra of Product and Substrate (in $CDCl_3$).

MS *m/e*: 166 (M^+), 149 (M^+-OH), 133 (M^+-H_2O-CH_3), 121 (M^+-COOH), 105, 102, 77, 65, 51. Found: C, 65.38; H, 6.10. Calcd. for $C_9H_{10}O_3$: C, 65.06; H, 6.02%.

Furthermore, to determine a position of OH group and COOH group on benzene nucleus, NMR spectral data in $CDCl_3$ were analysed. As shown in Fig. 2, the triplet at 8.80 τ of methyl proton connected to methylene proton and the quartet at 7.40 τ of methylene proton connected to methyl proton were not changed between substrate and product. But, the singlet at 7.70 τ of methyl proton in the spectrum of substrate was not observed in that of product. These data indicated methyl group of substrate was attacked by the microbial oxidation of 3-ethyltoluene and ethyl group of substrate was not attacked. In addition, concerning the protons of benzene ring, a spectrum of product showed bands at 2.20 τ, H(C) (double doublet J_{CB}=8.0 cps, J_{CA}=1.0 cps); 2.60 τ, H(A) (double doublet J_{BA}=8.0 cps, J_{AC}=1.0 cps); 3.15 τ, H(B) (double doublet J_{BA}=8.0 cps, J_{BC}=8.0 cps). The observed coupling constants (8.0 cps) show that H(B) proton has two protons in its *ortho* position, namely H(A) and H(C). From the analytical data mentioned above, the structure of product from 3-ethyltoluene by *Ps. ovalis* was determined to be 3-ethylsalicylic acid, as shown in Fig. 2.

The strain SlBl could also assimilate *m*- and *p*-xylene, and produced 3-methylsalicylic acid from *m*-xylene and *p*-toluic acid from *p*-xylene, which was the same results already reproted.[1,2] Among isomers of ethyltoluene, only 3-ethyltoluene was oxidized and 2- and 4-ethyltoluene was not used by the strain SlBl which could assimilate *p*-xylene, although the reason was not clear. In addition, the strain SlBl did not oxidize *p*-diethylbenzene which was reported to be oxidized to *p*-ethylphenylacetic acid by a *Pseudomonas* strain.[3]

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