Note

Isolation and Identification of Styrene Assimilating Bacteria

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Styrene monomer is widely used, important material for synthetic resins, such as polystyrene and styrene-butadiene rubber.

Styrene can be found in nature also. Finkle *et al.*^{1,2)} have reported that *p*-hydroxycinnamic, ferulic and caffeic acids are converted to styrene by enzymatic decarboxylation in the decay of vegetative material. Styrene is also known to be formed by the decarboxylation of cinnamic acid with microorganisms, such as *Penicillium*,³⁾ *Aspergillus*⁴⁾ and *Saccharomyces*.⁵⁾ Styrene is biologically degraded in mammalian tissues.⁶⁾

Microbial degradation of styrene, however, is not well understood, as discussed by Omori *et al.*^{7,8)} They reported that isolated bacteria utilizing aromatic hydrocarbons did not grow on styrene when it used as the sole carbon source. Recently, Sielicki *et al.*⁹⁾ obtained a mixed population of bacteria which could utilize styrene from landfill soil. The metabolites were traced and identified, and they proposed two degradative pathways for styrene: one was *via* β -phenethyl alcohol (β -PA) and phenylacetic acid and the other was *via* low-molecular-weight oligomer.

The present authors tried to obtain a pure culture of bacterial strain which assimilated styrene independently of Sielicki's work. This paper deals with the isolation methods, identification of the isolated bacteria and some comparisons of styrene metabolism by these isolates.

The medium for screening and cultivation contained NH_4NO_3 (2 g), $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (1.5 g), KH_2PO_4 (1.5 g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2 g), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (10 mg), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mg), yeast extract (0.1 g), corn steep liquor (0.1 g) and tap water to make 1 liter. Initial pH was adjusted to 7.0 by 0.5 N NaOH. Styrene was added to the pre-sterilized medium as the sole carbon source by procedure described below. Soil samples were collected mainly from rice fields, vegetable gardens and forests in the southern part of Chiba Prefecture.

No microbial growth was observed on styrene, although common methods including the method for benzene assimilating bacteria described by O. Connor

*et al.*¹⁰⁾ were tested for 200 samples of soil. However, medium became turbid, and pellicle was formed on the surface of the medium, when soil-percolation method¹¹⁾ which is an usual technique in soil microbiology was used. Three hundred milliliter of medium was placed in a 500 ml percolator and sterilized at 120°C for 20 min. Then soil was packed in a column 50~70 mm in length. After addition of 3 ml of styrene, percolation was started. Incubation was carried out at 30°C for 1 week. Styrene was dissolved in the medium at very low concentration, and the excess floated on the medium to keep the concentration low and constant. When styrene was consumed by microbial utilization and vaporization, additional styrene was fed. A significant growth of microorganisms was observed in 10 of 29 soil samples. These 10 samples were diluted with sterilized water and spread on bouillon plates. A colony was then transferred to a bouillon slant or agar slant of the medium containing 2% styrene. Thirty-one strains which assimilated styrene were isolated. Most of them were short rod, motile bacteria.

One loopful of bacteria pre-cultured on bouillon slant at 30°C for 12 hr was inoculated to a test tube (20 × 200 mm) containing 5 ml of the medium and 0.1 ml of styrene and incubated at 30°C for 3 days. Most of 31 strains grew on styrene and lowered pH of the culture less than 5.0 as shown in Table I. Since some cultures developed a rose-like fragrance, which indicated production of β -PA, the microbial product from styrene was analyzed by gas liquid chromatography. Hitachi Gas Chromatograph 063 equipped with a glass column (3 mm × 3 m), packed with 10% PEG-20M on Diasolid (60~80 mesh), was used. The operational conditions were as follows: column temp, 180°C; carrier gas, helium; flow speed, 50 ml/min; detector, flame ionization detector. As the result, strain 305-STR-1-4 and 311-STWR-1-2 were found to produce a substance of identical retention time with authentic β -PA. The

TABLE I. STYRENE ASSIMILABILITY
OF ISOLATED STRAINS

Strain	Final pH	Growth ^a	β -PA($\mu\text{g/ml}$) ^b
213-STR-2-4	4.5	1.25	0
267-STR-2-2	4.6	1.30	0
268-STR-2-3	5.1	1.50	0
303-STR-2-2	4.7	1.50	0
305-STR-1-4	4.8	1.40	30
305-STR-1-1	4.7	1.48	0
311-STWR-1-2	4.7	1.50	40
311-STWR-2-1	4.8	1.46	0
312-STR-1-1	4.4	1.50	0
313-STR-1-1	6.2	0.32	0

^a OD at 610 nm.

^b β -PA, β -phenethyl alcohol detected by gas liquid chromatography.

TABLE II. TAXONOMICAL DATA OF TYPICAL STRAINS

305-STR-1-4	311-STWR-1-2
Rods, 0.7 by 1.0 to 1.5 nm. Nonmotile. Gram negative.	Rods, 0.7 to 1.0 by 1.0 to 1.5 nm. Motile with 1 to 3 polar flagella. Gram negative.
Nutrient agar colonies: Growth moderate, circular, smooth, entire, convex, homogeneous, dark yellow and glistening.	
Nutrient agar slant: Growth moderate and filiform.	
Nutrient broth: Turbid moderate, ring and sediment moderate.	Nutrient broth: Turbid strong, membranous and sediment moderate.
Gelatin stab: Growth slight and no liquefaction.	Gelatin stab: Growth moderate and no liquefaction.
Litmus milk: Alkaline, decoloring and unchanged. Nitrate reduction and denitrofication: Positive.	Litmus milk: Alkaline, decoloring and peptonization.
Indole not formed. Acetylmethylcarbinol not formed. Starch hydrolysed.	Hydrogen sulfide formed.
Catalase, urease and oxidase: Positive.	
Citrate utilized. Acid from glucose, fructose, xylose and maltose. No acid from galactose, sucrose, lactose, trehalose and glycerol. Anaerobically no acid from glucose.	
Hugh and Leifson test: Oxidative. Aerobic.	
Water soluble, greenish yellow and fluorescent pigment formed in King's B medium.	No pigment.
Good growth at 30°C to 35°C. Good growth at pH 6.5 to pH 8.5.	

amount was calculated at 30 µg/ml and 40 µg/ml in the culture broth, respectively, by peak area comparison with the standard β -PA peak as control.

From these results, styrene is presumed to be metabolized via β -PA by these two strains, in the way similar to that Sielicki *et al.* reported in a mixed population of bacteria. Although the decrease in pH may suggest accumulation of phenylacetic acid, it has not yet been detected. In addition, Sielicki *et al.* proposed another pathway via styrene oligomer. The existence of this pathway in the strains authors isolated has not yet been examined. A detailed discussion on styrene metabolism by isolated strains must be left for future investigation.

Then strains listed in Table I were chosen at random and tested as described in "Manual of Microbiological Methods," 1975, Society of American Bacteriologists. All of them were identified as *Pseudomonas* species on the basis of "Bergey's Manual of Determinative Bacteriology," 7th Ed., 1957 and 8th Ed., 1974. The taxonomical data of typical strains (305-STR-1-4 and 311-STWR-1-2) are shown in Table II.

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REFERENCES

- 1) B. J. Finkle, *Nature*, **207**, 604 (1965).
- 2) B. J. Finkle, J. C. Lewis, J. W. Corse and R. E. Lundin, *J. Biol. Chem.*, **237**, 2926 (1962).
- 3) F. Jaminet, *J. Pharm. Belg.*, **5**, 191 (1950).
- 4) D. R. Clifford, J. K. Faulkner, J. R. L. Walker and D. Woodcock, *Phytochemistry*, **8**, 549 (1969).
- 5) S. L. Chen and H. J. Peppler, *J. Biol. Chem.*, **221**, 101 (1956).
- 6) K. C. Leibman, *Environ. Health Perspect.*, **11**, 115 (1975).
- 7) T. Omori, Y. Jigami and Y. Minoda, *Agric. Biol. Chem.*, **38**, 409 (1974).
- 8) T. Omori, Y. Jigami and Y. Minoda, *ibid.*, **39**, 1775 (1975).
- 9) M. Sielicki, D. D. Focht and J. P. Martin, *Appl. Environ. Microbiol.*, **35**, 124 (1978).
- 10) R. J. O'Connor, B. W. Weinrich and W. A. Darlington, Report from Monsanto Company (1963).
- 11) F. M. Collins and C. M. Smis, *Nature*, **178**, 1073 (1956).