Studies on the Utilization of Hydrocarbons by Microorganisms

Part I. Isolation of Amino Acids Producing Bacteria from Soil*

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It is well known that a good many microorganisms can utilize hydrocarbons as a sole carbon source. We have as yet little information, however, as to what kinds of intermediate or end products are produced by the microbial dissimilation of hydrocarbons. Above all, the formation of amino acids from hydrocarbons has not been reported. We have isolated many strains of microorganisms from soil samples by selective culture techniques in a medium containing kerosene and mineral salts, in order to examine if the products of economical value such as amino acids, organic acids, fatty acids, steroids, nucleic acids and their related compounds, can be produced by those microorganisms. Shaking cultures of those microorganisms were carried out in the medium containing 3.5% kerosene, 3.5% liquid paraffin and mineral salts (K_2HPO_4 0.25%; $MgSO_4 \cdot 7H_2O$ 0.1%; NH_4NO_3 0.5% or urea 0.3%); we found that 55 strains out of 127 strains tested produced amino acids in their broths, after 4 days cultivation at 26.5°C. The kinds and maximum yields of amino acids were as follows: alanine 50 mg/l, aspartic acid 23 mg/l, glutamic acid 485 mg/l, glycine 8.5 mg/l, isoleucine 15 mg/l, lysine 23.9 mg/l, and valine 38 mg/l.

The ability of microorganisms to utilize hydrocarbons as a sole carbon source has been recognized for many years. According to Zobell's reviews^{1,2)}, more than one hundred species representing about forty genera of bacteria, yeasts, and molds have been shown by various workers to be able to utilize one or more kinds of hydrocarbons, in which gaseous, liquid, and solid hydrocarbons of aliphatic, olefinic, aromatic, and naphthenic series are included. As to the intermediate or end products produced by the microbial dissimilation of hydrocarbons, formic, acetic, valeric, lactic, salicylic, phthalic and long chain fatty acids, formaldehyde, acetaldehyde,

glycol, glycerol, etc. have been reported by various investigators. The amounts of those products, however, are very small, and the useful fermentation products such as amino acids, nucleic acids and their related compounds, and antibiotics have not been detected.

We have isolated many strains of hydrocarbon utilizing microorganisms, in order to examine if the reasonable amounts of fermentation products of economic value can be produced by those microorganisms from such mixtures of hydrocarbons as kerosene, ligroin, and liquid paraffin which are considered to be more economical carbon sources than carbohydrates.

In this paper, the isolation method of hydrocarbon utilizing microorganisms from soil

1) C.E. Zobell, *Bact. Revs.*, **10**, 1 (1946).

2) C.E. Zobell, *Advances in Enzymology*, **10**, 443 (1950).

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samples and the results of the screening of amino acids producing bacteria are reported.

EXPERIMENTAL

Isolation Media.

As shown in Table I, isolation media were consisted of mineral salts solutions, kerosene and liquid paraffin which contained many kinds of $C_{10}\sim C_{20}$ hydrocarbons. The mineral salts solutions used were slight modifications of the medium employed by Bushnell and Hass³⁾. Tap water was used instead of distilled water, because we had little information on the mineral requirements of hydrocarbon utilizers. Hydrocarbons were effectively dispersed by Tween-20 (polyoxyethylene sorbitan monolaurate) so that they could be attacked easily by microorganisms. Heat sterilization was carried out at 105°C for 5 min. Solid media for stock and plate cultures were prepared fairly homogeneous by adding 2% agar into the isolation media, autoclaving for 5 min. at 115°C, and passing through a homogenizer under aseptic conditions.

Method of Isolation.

It was very difficult to isolate hydrocarbon utilizing

microorganisms by using only the ordinary plate culture technique. Therefore, accumulation culture and plate culture were alternately repeated for the isolation of those microorganisms as shown in Fig. 1.

Fifty ml of M-1 and M-2 media shown in Table I, in 500 ml shaker flasks, were inoculated with about 0.1 g of soil samples, and the flasks were shaken on reciprocal shaker for 3 days at 26.5°C (first accumulation culture). Plate cultures were carried out by

TABLE I. THE COMPOSITIONS OF ISOLATION MEDIA

	For yeasts and molds (M-1)	For bacteria (M-2)
Kerosene	35.0 g	35.0 g
Liquid paraffin	35.0	35.0
NH_4NO_3	5.0	5.0
KH_2PO_4	2.5	—
K_2HPO_4	—	2.5
$MgSO_4 \cdot 7H_2O$	1.0	1.0
Tween-20	0.5	0.5
Tap water	1.0 l	1.0 l
pH	5.0	7.0

FIG. 1. Method of Isolation

3) L.D. Bushnell and H.F. Hass, *J. Bact.*, **41**, 653 (1941).

the ordinary culture technique using solid media mentioned above, and incubated for 4~6 days at 26.5°C. Each different colony developed on the plate cultures was transferred into 5 ml of isolation liquid medium in 30 ml test tube, and the test tube was shaken for 3 days at 26.5°C (second and third accumulation culture). Purity test of the first stock culture was carried out by the ordinary plate method using nutrient-agar or Koji-agar. The second stock cultures which were regarded as pure strains, were used for further investigations.

Occurrence of Hydrocarbon Utilizing Microorganisms.

By the procedure above mentioned, 127 strains of

bacteria, 16 strains of yeasts, and 24 strains of molds, as shown in Table II, were isolated from 89 kinds of samples in which large varieties of soil and water from different origins were included. Those results show that hydrocarbon utilizing microorganisms are not always abundant in oil soaked soil around oil wells and refineries, but they also occur in road side, garden, farm, rice field, pond and river water. That is, hydrocarbon utilizers appear to be quite commonly present in soil and water.

Screening of Amino Acids Producing Bacteria.

Five ml of M-2 medium in 30 ml test tubes, was inoculated with hydrocarbon utilizing bacteria isolated

TABLE III. STRAINS OF AMINO ACIDS PRODUCERS AND KINDS OF AMINO ACIDS PRODUCED BY THEM

Strain No.	Amino acids detected by paper chromatography							Strain No.	Amino acids detected by paper chromatography						
	Ala-nine	Aspar-tic acid	Glu-tamic acid	Glycine	Lysine	Iso-leucine	Val-nine		Ala-nine	Aspar-tic acid	Glu-tamic acid	Glycine	Lysine	Iso-leucine	Val-nine
S 2B 2	—	+	+	+	—	—	—	S 46B 2	+	+	+	+	+	—	—
S 5B 2	+	+	+	+	+	—	—	S 47 B 2	+	+	+	+	+	—	—
S 6B 1	+	+	+	+	+	+	+	S 48B 1	+	—	—	—	+	—	—
S 7B 1	++	++	++	—	—	++	++	S 48 B 3	+	+	+	+	+	—	—
S 10B 1	—	+	++	+	—	—	—	S 50B 1	+	+	+	+	+	+	+
S 10B 2	—	—	++	—	—	—	—	S 51 B 1	+	+	+	+	+	—	—
A 1B 2	—	—	+	—	—	—	—	S 52B 1	+	+	+	+	+	—	—
S 11B 1	+	+	++	+	—	—	—	S 58B 1	—	—	+	—	+	—	—
S 11B 2	—	+	++	+	+	—	—	S 59B 1	+	+	—	—	—	—	—
S 12B 2	—	++	++	+	+	—	—	S 61B 1	+	+	+	+	+	—	—
S 14B 1	—	+	+	+	+	—	—	S 62B 1	+	+	+	+	+	—	—
S 17B 1	—	+	++	+	—	—	—	S 64B 1	+	+	++	+	—	—	—
S 18B 1	—	—	+	—	—	—	—	S 64B 2	+	+	+	+	+	—	—
S 18B 2	—	—	+	—	+	—	—	S 65B 1	+	+	+	+	+	—	—
S 19B 1	—	—	—	—	+	—	—	S 67 B 1	—	+	+	+	—	—	—
S 19B 2	—	—	+	+	—	—	—	S 72B 1	—	+	+	+	—	—	—
S 20B 1	+	+	++	+	+	—	—	S 77B 1	+	+	+	+	—	—	—
S 24B 1	+	+	++	+	+	+	++	S 82B 2	—	+	+	—	—	—	—
S 31B 1	—	+	++	+	+	—	—	S 82B 3	+	+	+	+	+	—	—
S 33B 1	—	+	++	—	—	—	—	S 84B 1	—	+	—	—	—	—	—
S 39B 1	+	+	++	+	—	—	—	S 85B 3	—	+	+	+	+	—	—
S 43B 1	+	+	+	+	—	—	—	S 86B 1	+	+	+	—	—	—	—
S 43B 2	—	—	+	+	—	—	—	S 86B 2	+	+	+	—	—	—	—
S 44B 1	—	—	+	—	—	—	—	S 87B 2	+	—	—	—	—	—	—
S 44B 2	+	+	+	+	+	—	—	S 74B 1	+	+	—	—	—	—	—
S 45B 1	—	—	+	—	—	—	—	S 88B 1	+	+	+	—	—	—	—
S 45B 2	—	—	+	—	—	—	—	S 89B 2	+	+	+	+	+	—	—
S 46B 1	+	+	+	+	+	—	—								

—: spot of amino acid was not detected.

+: spot of amino acid was detected.

++: strong spot of amino acid was detected.

above. Test tubes were shaken on the reciprocal shaker for 6 days at 26.5°C, and pH of culture broths was controlled to about 7.0 by adding aqueous ammonia at suitable intervals. After 6 days cultivation, culture broths were centrifuged for 15 min. at 5,400× G, and the existence of amino acids in the supernatants were tested by paper chromatography (solvent: butanol 4, acetic acid 1, water 2; detector: ninhydrin).

As the results of this screening procedure, 55 strains among 127 strains of bacteria, as shown in Table III, were found to be able to accumulate amino acids in their culture broths. Kinds of amino acids produced were alanine, aspartic and glutamic acid, glycine, lysine, isoleucine, and valine. Among those amino acids, glutamic and aspartic acid were produced by almost all amino acids producers, but the strains which could produce isoleucine or valine were very few.

Occurrence of Amino Acids Producing Bacteria.

The amino acids producing bacteria obtained above, are equally abundant in either oil soaked soil around oil wells and refineries or gardens and farms, as shown in Table II, but they rarely occur in rice fields, ponds

and rivers.

Test of the Suitability of Urea as the Nitrogen Source.

In the above procedures, to add frequently aqueous ammonia into the culture broths was very troublesome. It was expected that culture broths would be easily kept neutral if urea could be used instead of ammonium nitrate as the nitrogen source. Using 11 strains of bacteria which produced relatively large amounts of amino acids, tests were carried out as to whether the growth and amino acids production of these bacteria were supported by urea. Fifty ml of M-2 and U media (0.3% urea was added to M-2 medium instead of 0.5% ammonium nitrate), in 500 ml shaker flasks, were inoculated with those 11 strains respectively. Flasks were shaken on the reciprocal shaker for 4 days at 26.5°C, and pH of M-2 broths were controlled by the method mentioned previously. After 4 days cultivation, packed volumes of cell per unit volume of broths were determined by centrifuging 10 ml culture broths for 15 min. at 5,400× G, and the existence of amino acids in the supernatants were detected by paper chromatography.

The results are presented in Table IV, which show that those amino acids producers grow almost equally

TABLE II. THE OCCURRENCE OF HYDROCARBON UTILIZING MICROORGANISMS AND AMINO ACIDS PRODUCING BACTERIA

Kinds of samples for isolation	Station	Number of samples	Number of isolated strains			Number of strains of amino acids producing bacteria
			Molds	Yeasts	Bacteria	
Crude oil soaked clayey soil	{ Yabase Oil Field, Akita	2	0	0	5	2
	{ Toyokawa Oil Field, Akita	5	1	1	6	5
	{ Funakawa Oil Refinery, Akita	1	0	0	1	0
Crude oil soaked sandy soil	{ Gasoline stand, Tokyo	4	2	2	4	1
	{ Oil refinery, Kanagawa	7	5	3	8	3
	{ Funakawa Oil Refinery, Akita	2	0	0	0	0
	{ Kurokawa Oil Field, Akita	3	0	0	1	1
Crude oil soaked wet soil	{ Turumi River, Kanagawa	1	0	0	1	0
	{ Yabase Oil Field, Akita	10	2	0	23	12
	{ Funakawa Oil Refinery, Akita	1	0	0	1	1
	{ Toyokawa Oil Field, Akita	1	0	0	2	0
	{ Kurokawa Oil Field, Akita	3	0	0	1	1
	{ Omono River, Akita	3	0	0	2	2
Garden and road side soil	{ Tokyo	3	0	1	6	4
	{ Saitama	3	3	0	6	5
	{ Akita	1	0	0	1	1
Farm soil	{ Hiroshima	2	0	0	4	2
	{ Tokyo	6	2	1	10	5
	{ Chiba	14	4	6	22	9
Rice field soil	Toyama	2	3	0	5	0
Pond, marsh and river water	{ Tokyo and Kanagawa	6	1	2	9	0
	{ Akita	9	1	0	9	1
Total		89	24	16	127	55

well on either urea or ammonium nitrate as nitrogen source, and accumulate a little more amino acids in U-medium than in M-2 medium, except for S12B2 and S31B1 strain. But it is not clear whether these results are attributed to the difference of suitability

as nitrogen source between urea and ammonium nitrate or the range of pH changes during the cultivation. Anyway, to simplify experimental procedures, it is advisable to use urea as nitrogen source.

TABLE IV. SUITABILITY OF UREA AS NITROGEN SOURCE

Strain No.	Medium used	Yield of cell (ml. of packed cell / 10 ml. of broth)	Amino acids detected by paper chromatography						
			Alanine	Aspartic acid	Glutamic acid	Glycine	Lysine	Isoleucine	Valine
S 7 B 1	{U	0.530	++	++	++	+	—	+	+
	{M-2*	0.410	+	+	+	—	—	+	+
S 10 B 1	{U	0.021	+	+	++	++	+	—	—
	{M-2*	0.017	+	+	++	—	—	—	—
S 10 B 2	{U	0.038	++	++	++	++	—	—	—
	{M-2*	0.098	—	+	+	—	—	—	—
S 11 B 1	{U	0.017	—	++	++	++	—	—	—
	{M-2*	0.038	—	++	+	+	—	—	—
S 12 B 2	{U	0.043	—	+	—	+	—	—	—
	{M-2*	0.013	+	++	++	—	+	—	—
S 17 B 1	{U	0.068	—	++	++	++	+	—	—
	{M-2*	0.089	—	+	++	—	+	—	—
S 20 B 1	{U	0.051	—	+	++	+	—	—	—
	{M-2*	0.077	+	+	+	+	+	—	—
S 31 B 1	{U	0.064	—	+	+	+	—	—	—
	{M-2*	0.069	+	++	++	++	+	—	—
S 39 B 1	{U	0.051	+	++	++	+	—	—	—
	{M-2*	0.055	—	+	+	+	—	—	—
S 48 B 3	{U	0.069	++	+	++	+	+	—	—
	{M-2*	0.094	+	+	+	+	+	—	—
S 64 B 1	{U	0.443	+	+	++	+	—	—	—
	{M-2*	0.315	+	+	+	+	—	—	—

* pH of broth was controlled to 7.0 by adding aqueous ammonia during the cultivation.

U medium: 0.3% urea was added to M-2 medium instead of 0.5% NH_4NO_3 .

TABLE V. RESULTS OF THE DETERMINATION OF AMINO ACIDS BY MICROBIOASSAY*1

Strain No.	Medium used	Amounts of amino acids produced (mg/L)						
		Alanine	Aspartic acid	Glutamic acid	Glycine	Isoleucine	Lysine	Valine
S 7 B 1	U	17.0	23.0	23.0	—	15.0	—	38.0
S 10 B 1	U	18.5	0	485.0	2.0	—	7.5	—
S 10 B 2	U	25.0	0	200.0	8.5	—	—	—
S 11 B 1	U	—	0	98.0	0.5	—	—	—
S 12 B 2	M-2*2	2.5	10.0	42.0	8.0	—	2.4	—
S 17 B 1	U	—	0	54.0	4.0	—	23.9	—
S 20 B 1	U	8.0	0*3	0*3	8.0	—	3.2	—
S 31 B 1	M-2*2	50.0	11.0	74.0	7.0	—	2.4	—
S 39 B 1	U	1.5	0*3	0*3	0*3	—	—	—
S 48 B 3	U	38.0	10.5	94.5	8.5	—	17.0	—
S 64 B 1	U	42.0	10.5	24.5	1.5	—	—	—

*1 *Lactobacillus arabinosus* and *Leuconostoc mesenteroides* were used.

*2 pH of broth was controlled to 7.0 by adding aqueous ammonia during the cultivation.

*3 Microbioassay was hindered by some unknown factor in the culture broth.

Determination of Amino Acids Produced.

The amounts of amino acids in 11 kinds of culture broths obtained above were determined by microbioassay method using *Lecuconostoc mesenteroides* and *Lactobacillus arabinosus*. As shown in Table V, maximum yield of each amino acid was as follows: alanine 50 mg/l, aspartic acid 23 mg/l, glutamic acid

485 mg/l, glycine 8.5 mg/l, isoleucine 15 mg/l, lysine 23.9 mg/l and valine 38 mg/l. On the other hand, it seemed that culture broths of S20B1 and S39B1 strain contained some unknown growth inhibiting factor of test organisms, by which microbioassay was hindered.