Studies on the Utilization of Hydrocarbons by Microorganisms

Part III. Production of Bacterial Cell from Hydrocarbons*

By Joji TAKAHASHI, Kaetu KOBAYASHI, Yasuro KAWABATA
and Koichi YAMADA

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo

Received June 26, 1963

The production of microbial cell substances from hydrocarbons has become the object of commercial attention. We have tested hydrocarbon-utilizing bacterial strains for cell production, and found out one strain of high cell yield, which is very similar to *Pseudomonas aeruginosa*. The effect of medium composition on cell yield and the utilization of individual hydrocarbons by this strain were investigated. Sixty percent of the added kerosene was converted into cell materials in the following medium of optimum composition: kerosene 2.5%, urea 0.13%, dipotassium phosphate 0.25%, magnesium sulfate 7 aq. 0.1% and tap water. Aliphatic series lower than C₁₀, aromatic and naphthenic hydrocarbons were not or very slightly assimilated. However, aliphatic members higher than C₁₂ were utilized with increasing ease. Especially, *n*-docosane and octadecene-1 were utilized very effectively, and 70% of them were converted into cell substances.

The ability of microorganisms to utilize hydrocarbons as a sole carbon source has been recognized for many years. The formation of several kinds of organic and fatty acids, alcohols, aldehydes, and others from hydrocarbons has also been reported by various workers¹⁻³⁾.

We have been studying on the microbial dissimilation products of hydrocarbons, and reported the amino acid formation from kerosene by fermentation process in the previous paper⁴⁾.

Recently, the production of microbial cell substances from hydrocarbons has become

the object of not only scientific but also commercial attention, and it is said that the process converting hydrocarbons into feed supplements has been piloted by a few firms^{5,6)}.

We have also tested hydrocarbon-utilizing bacterial strains isolated by our laboratory for cell production, and found out several strains of high cell yields. Successive investigations are carried out in order to increase cell yields of those strains.

In this paper, the effect of medium composition on cell yield of S7B1 strain which is very similar to *Pseudomonas aeruginosa*, and results of experiments on the utilization of individual pure hydrocarbons by this strain are reported.

* This paper was read at the Annual Meeting of Agricultural Chemical Society of Japan held on 4, April, 1963.

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

2) E. Beerstecher, Jr., "Petroleum Microbiology", Elsevier Press Inc., Huston, New York (1954).

3) V. Treccani, *Progress in Industrial Microbiology*, **4**, 3 (1963).

4) K. Yamada, J. Takahashi, K. Kobayashi and Y. Imada, *This Journal*, **27**, 390 (1963).

5) "New Aims for Biosynthesis", *Chemical Week*, Feb. 2, 49 (1963).

6) "Paraffin Feedstock for BP Bugs", *European Chemical News*, Dec. 28, 26 (1962).

EXPERIMENTAL AND RESULTS

Screening Test of Bacterial Strains for Cell Production.

Forty seven bacterial strains isolated by the procedure described previously⁴⁾ were tested for cell production. Fifty ml of two kinds of media shown in Table I, in 500 ml shaker flasks, were inoculated with testing strains respectively, and incubated at 26.5°C on the reciprocal shaker. During the cultivation, pH of fermenting broths was adjusted to 7.0 as occasion demands.

After four days cultivation, cell concentration in the fermented broth was determined by packed volume of cells which were collected centrifugally (for fifteen min. at 5,200 G) at the bottom of the measuring tube shown in Fig. 1.

Results were presented in Table II, showing seven strains, S7B1, S24B1, S44B1, S44B2, S46B2, S64B1 and S65B1 which were able to produce remarkably larger amounts of cells

TABLE I. COMPOSITIONS OF MEDIA EMPLOYED IN SCREENING TEST

	N medium	U medium
Kerosene	35.0 g	35.0 g
Liquid paraffin	35.0 "	35.0 "
NH ₄ NO ₃	5.0 "	—
Urea	—	3.5 "
K ₂ HPO ₄	2.5 "	2.5 "
MgSO ₄ ·7H ₂ O	1.0 "	1.0 "
Tween-20	0.5 "	0.5 "
Tap water	1.0 l	1.0 l
pH	7.0	7.0

FIG. 1. Graduated Tube for the Determination of Packed Volume of Cells.

from kerosene than the others. Six strains out of those 7 strains were isolated from oil-soaked soils around oil wells and refineries, as shown in Table II, and the other was isolated from non-oil soaked field soil.

Following investigations were begun with the taxonomical studies of S7B1 strain.

Taxonomical Studies of S7B1 Strain.

Diagnostic tests of S7B1 strain were carried out, and the results showed that this strain was very similar to *Pseudomonas aeruginosa* described in "Bergey's Manual of Determinative Bacteriology", 7th Ed. (1957). The descriptions of this strain are as follows:

Descriptions:

Rods, 0.6 by 1.2 to 1.6 microns. Motile with polar flagella. Gram negative.

Nutrient agar colonies: Circular, smooth, entire to slightly erose, flat, transparent, brownish green, butyrous.

Nutrient agar slant: Moderate growth, filiform to spreading, glistening, transparent, brownish green, butyrous, medium becomes dark green.

Nutrient broth: Fragile pellicle, turbid.

Gelatin stab: Liquefied.

Milk: Peptonized, medium becomes yellow.

B.C.P. milk: Alkaline, peptonized, soft coagulum formed.

Nitrate reduced to nitrite in nitrate broth and succinate-nitrate.

Indole not produced.

Hydrogen sulfide produced.

Starch not hydrolyzed.

No acid and no gas from glycerol, xylose, glucose, lactose, sucrose and starch in peptone, and synthetic media. (Acid produced from glucose aerobically by Hugh & Leifson's method.)

Anaerobically no acid and no gas from glucose and lactose.

Glucose, gluconate, 2-ketogluconate, citrate, succinate, benzoate, protocatechuate, gen-

TABLE II. RESULTS OF THE SCREENING TEST FOR CELL PRODUCTION

Strain No.	Kind of soil from which strains were isolated	Kind of medium	Cell yields (ml of packed volume per 10ml of broth)	Strain No.	Kind of soil from which strains were isolated	Kind of medium	Cell yields (ml of packed volume per 10ml of broth)
S2B2	oil soaked soil	N	0.02	S44B1	"	N	0.27
"	"	U	0.04	"	"	U	0.03
S5B2	road side soil (non-oil soaked)	N	0.03	S44B2	"	N	0.22
"	"	U	0.03	"	"	U	0.03
S6B2	field soil (")	N	0.01	S45B1	"	N	0.11
"	"	U	0.02	"	"	U	0.06
S7B1	" (")	N	0.29	S45B2	"	N	0.10
"	"	U	0.29	"	"	U	0.19
S10B1	road side soil (")	N	0.01	S46B1	"	N	0.08
"	"	U	0.02	"	"	U	0.06
S10B2	" (")	N	0.07	S46B2	"	N	0.20
"	"	U	0.03	"	"	U	0.38
S11B1	" (")	N	0.01	S47B2	"	N	0.04
"	"	U	0.04	"	"	U	0.04
S11B2	" (")	N	0.01	S48B1	"	N	0.01
"	"	U	0.01	"	"	U	0.05
S12B2	" (")	N	0.01	S48B3	"	N	0.07
"	"	U	0.01	"	"	U	0.05
S14B1	field soil (")	N	0.01	S50B1	"	N	0.07
"	"	U	0.05	"	"	U	0.05
S17B1	" (")	N	0.06	S51B1	road side soil (non-oil soaked)	N	0.02
"	"	U	0.05	"	"	U	0.02
S18B1	road side soil (")	N	0.01	S52B1	oil soaked soil	N	0.07
"	"	U	0.06	"	"	U	0.05
S18B2	" (")	N	0.02	S58B1	"	N	0.15
"	"	U	0.04	"	"	U	0.10
S19B1	field soil (")	N	0.02	S59B1	"	N	0.07
"	"	U	0.04	"	"	U	0.09
S19B2	" (")	N	0.03	S61B1	"	N	0.05
"	"	U	0.05	"	"	U	0.05
S20B1	" (")	N	0.05	S62B1	"	N	0.11
"	"	U	0.04	"	"	U	0.07
S24B1	oil soaked soil	N	0.32	S64B1	"	N	0.11
"	"	U	0.35	"	"	U	0.16
S31B1	"	N	0.05	S64B2	"	N	0.14
"	"	U	0.05	"	"	U	0.40
S32B2	"	N	0.06	S65B1	"	N	0.05
"	"	U	0.08	"	"	U	0.30
S33B1	"	N	0.02	S67B1	"	N	0.11
"	"	U	0.05	"	"	U	0.18
S39B1	"	N	0.04	S72B1	"	N	0.09
"	"	U	0.04	"	"	U	0.07
S39B2	"	N	0.05	S74B1	"	N	0.18
"	"	U	0.05	"	"	U	0.09
S43B1	"	N	0.10	S77B1	marsh soil (non-oil soaked)	N	0.01
"	"	U	0.05	"	"	U	0.05
S43B2	"	N	0.03				
"	"	U	0.03				

tisate, and anthranilate assimilated.

Salicylate, *m*-hydroxybenzoate and *p*-hydroxybenzoate not assimilated.

Catalase: Positive.

Optimum temperature: 25 to 30°C. No or scant growth at 42°C.

Aerobe.

Source: Field soil.

Effects of Kerosene and Urea Concentrations on Cell Yields.

Various amounts of kerosene (ranging from 0.8 to 8%) and urea (0.1~1.5%) were added to 50 ml of mineral salts solutions containing 0.25% of dipotassium phosphate and 0.1% of magnesium sulfate 7 aq., in 500 ml shaker flasks. After five min. heat sterilization at 110°C, each flask was inoculated with 1 ml of seed culture of S7B1 strain grown at 26.5°C for ninety six hrs. in 50 ml of the following medium: kerosene 2.5%, urea 0.1%, dipotassium phosphate 0.25%, magnesium sulfate 7 aq. 0.1%, Tween-20 0.05% and tap

water. Flasks were shaken on the reciprocal shaker at 26.5°C.

Some of the typical growth curves are illustrated in Fig. 2. These curves show that the growth rate decreases according as the initial kerosene concentration decreases, and cell concentrations in every broth reach their maximum in about four days irrespective of initial kerosene and urea contents of the broths.

Cell yields after ninety six hrs. cultivation which were, as known above, regarded as the maximum in each culture system containing various amounts of kerosene and urea were shown in Table III. In this table, it is reasonable that the cell yield per unit volume of

FIG. 2. Typical Growth Curves of S7B1 Strain in Media Containing Various Amounts of Kerosene and Urea.

	Kerosene (g/100 ml)	Urea (g/100 ml)	Kerosene/urea
○ ——— ○	0.8	0.1	8.0
△ ——— △	2.5	0.9	2.8
× ——— ×	5.0	0.1	50.0
□ ——— □	8.0	0.4	20.0

TABLE III. EFFECTS OF KEROSENE AND UREA CONCENTRATIONS ON CELL YIELDS

Concentration of kerosene (K) (g/100 ml)	Concentration of urea (U) (g/100 ml)	K/U	Cell yields	
			ml of packed volume* per 10 ml of broth	added kerosene per (ml/g)packed volume
8.0	1.5	5.3	0.41	0.51
	0.4	20.0	0.42	0.51
	0.25	32.0	0.32	0.39
	0.1	80.0	0.23	0.29
5.0	0.9	5.6	0.29	0.58
	0.4	12.5	0.22	0.44
	0.25	20.0	0.31	0.62
	0.13	38.5	0.27	0.54
	0.1	50.0	0.22	0.44
2.5	0.9	2.8	0.11	0.44
	0.4	6.3	0.15	0.60
	0.25	10.0	0.17	0.68
	0.13	19.3	0.19	0.76
	0.1	25.0	0.18	0.72
1.25	0.4	3.1	0.06	0.48
	0.25	5.0	0.08	0.64
	0.1	12.5	0.06	0.48
0.8	0.4	2.0	0.025	0.31
	0.25	3.2	0.02	0.25
	0.1	8.0	0.015	0.19

* 1 ml of packed volume was equal to 0.165 g of dried cell.

broth increases in accordance with the increase of kerosene content of the medium. The optimum kerosene concentration, however, at which kerosene is converted most effectively into cell materials is about 2.5%. On the other hand, the ratio of urea concentration to kerosene has little influence on cell yield within the limits of this experiment.

Effect of Various Nitrogen Sources on Cell Yields.

Effect of five kinds of nitrogen sources on cell yields was tested by the same cultural procedures as described above. Kerosene contents of every medium employed were 2.5%, and fermenting broths were neutralized with alkali or acid as occasion demands during the cultivation.

Results presented in Table IV show that urea is the most suitable nitrogen source for cell production, and ammonium is less suitable than nitrate, though every type of nitrogen sources is assimilable. But it is not always concluded that ammonium is the least assimilable, because bacterial utilization of the ammonium ion lowers the pH strongly

TABLE IV. EFFECT OF VARIOUS NITROGEN SOURCES ON CELL YIELDS

Nitrogen source		Cell yields
Kind	Ratio of nitrogen to kerosene (%)	(ml of packed volume per 10 ml or broth)
NH ₄ Cl	{ 10	0.06
	{ 2.5	0.06
	{ 0.6	0.03
(NH ₄) ₂ SO ₄	{ 10	0.04
	{ 2.5	0.05
	{ 0.6	0.03
NaNO ₃	{ 10	0.08
	{ 2.5	0.11
	{ 0.6	0.07
KNO ₃	{ 10	0.08
	{ 2.5	0.10
	{ 0.6	0.06
CO(NH ₂) ₂	{ 10	0.11
	{ 2.5	0.19
	{ 0.6	0.17

and necessitates a most frequent neutralization with alkali.

Tests of Individual Pure Hydrocarbons for the Utilization by S7B1 Strain.

Fifty ml of sterile mineral solutions, in 500 ml shaker flasks, containing 0.13% of urea and 2.5% of pure hydrocarbons to be tested, were inoculated with 1 ml of seed culture

described above, and shaken for six days at 26.5°C. Cell concentrations in fermenting broths were determined at two days intervals.

When the cell concentration exceeded 0.3 ml of packed volume per 10 ml of broth, 0.05 g of additional urea was fed to the culture

TABLE V. ASSIMILATION OF INDIVIDUAL HYDROCARBONS BY *Pseudomonas aeruginosa* S7BI STRAIN

Hydrocarbon	Cell yield (ml of packed volume per 10 ml of broth)	Hydrocarbon	Cell yield (ml of packed volume per 10 ml of broth)
Paraffinic series (straight chain)		Olefinic series (with side chains)	
<i>n</i> -Pentane	0.03	4-Methylpentene-2	0.03
<i>n</i> -Hexane	0.02	2-Ethylhexene-1	0.03
<i>n</i> -Heptane	0.02	2,2,4-Trimethylpentene-1	0.03
<i>n</i> -Octane	0.02		
<i>n</i> -Nonane	0.06	Aromatic series	
<i>n</i> -Decane	0.08	Benzene	0.02
<i>n</i> -Undecane	0.11	Toluene	0.03
<i>n</i> -Dodecane	0.21	Ethylbenzene	0.03
<i>n</i> -Tridecane	0.25	<i>p</i> -Xylene	0.03
<i>n</i> -Tetradecane	0.13	<i>m</i> -Xylene	0.02
<i>n</i> -Hexadecane	0.23	<i>o</i> -Xylene	0.01
<i>n</i> -Octadecane	0.44	<i>n</i> -Propylbenzene	0.02
<i>n</i> -Eicosane	0.42	iso-Propylbenzene	0.02
<i>n</i> -Docosane	0.85	1,2,3-Trimethylbenzene	0.02
		1,2,4-Trimethylbenzene	0.02
Paraffinic series (with side chains)		1,3,5-Trimethylbenzene	0.01
iso-Pentane	0.03	1,2,4,5-Tetramethylbenzene	0.01
2-Methylpentane	0.02	Naphthalene	0.03
3-Methylpentane	0.02	1-Methylnaphthalene	0.02
2,2-Dimethylbutane	0.02	2-Methylnaphthalene	0.01
2,3-Dimethylbutane	0.04		
3-Methylheptane	0.03	Cycloparaffinic series	
2,2,4-Trimethylpentane	0.03	Cyclopentane	0.02
2,2,5-Trimethylpentane	0.03	Cyclohexane	0.02
		Cycloheptane	0.02
Olefinic series (straight chain)		Cyclooctane	0.03
Pentene-1	0.02	Methylcyclopentane	0.02
Pentene-2	0.03	Methylcyclohexane	0.02
Hexene-1	0.03	Ethylcyclohexane	0.02
Hexene-2	0.02		
Heptene-1	0.02	Cycloolefinic series	
Heptene-2	0.01	Cyclopentene	0.03
Heptene-3	0.01	Cyclohexene	0.02
Octene-1	0.02	4-Methylcyclohexene	0.03
Octene-2	0.01		
Decene-1	0.02	Hydrocarbon mixtures	
Dodecene-1	0.04	Kerosene	0.23
Tetradecene-1	0.25	Light oil	0.04
Hexadecene-1	0.19	Liquid paraffin	0.01
Octadecene-1	0.96		
Tricosene-11	0.28		

system every day in order to avoid growth limitation due to the depletion of nitrogen source. Maximum packed volume of cell yielded from 10 ml of each fermented broth was shown in Table V. When cell yields are less than 0.03 ml per 10 ml of broth, it is uncertain whether hydrocarbons in those media of poor growth are assimilated and converted into cell materials or not. In other words, the method used does not permit the conclusion that those hydrocarbons are not assimilated.

From the results presented in this table, it may be concluded:

1. Liquid paraffinic hydrocarbons with straight or branched chain having fewer than eight carbon atoms are not or very slightly assimilated, whereas higher members than nonane are utilized with increasing ease.

2. Olefinic hydrocarbons lower than decene are not or very slightly assimilated, but higher members than dodecene are utilized. Especially, octadecene-1 is utilized more readi-

ly than any other compounds tested.

3. Aromatic and naphthenic hydrocarbons, and their derivatives with methyl or ethyl groups are not or very slightly attacked by S7B1 strain.

4. Light oil and liquid paraffin are not or very slightly assimilated. This is an unaccountable result, because those petroleum products should contain larger amounts of aliphatic hydrocarbons ranging from C_{16} to C_{22} which have been known as readily assimilable to S7B1 strain, than kerosene. To explain this fact, further investigations will be necessary.

Determination of RNA Content.

Ribonucleic acid content of vacuum dried cell of this strain was 5.0% according to the determination by Schmidt-Thannhauser method.

Acknowledgement. We are grateful to the staffs of the Central Research Laboratory of Ajinomoto Co. Inc. for the taxonomical studies of S7B1 strain.