Single Cell Protein Production from Liquefied Petroleum Gas*

by Shinichi Kawakami**, Hideo Shoji**, Norimasa Nonaka***,
Mikitake Nakayama** and Takuya Hatano**

Summary: A propane and butane-utilizing bacterial strain, capable of growth at a comparatively high temperature with a specific growth rate of 0.12 hr^{-1} , has been isolated from soil samples by enrichment culture. The optimum temperature and pH range for the growth of the isolate, designated SB-1701, were $35\sim 40^\circ\text{C}$ and $5.7\sim 6.7$, respectively.

Cultivation was carried out with a mixed gas of 20% n-butane and 80% air, using only inorganic salts as the medium. The optimum medium composition which contained trace metallic salts was determined to obtain high productivity. Continuous culture was maintained at a steady state for more than 400 hours in a 20-liter jar fermenter during which the productivity was $1.4 \text{ g cell/l}\cdot\text{hr}$. The yield based on propane was $0.95 \text{ g cell/g propane}$ and the yield based on oxygen was $0.41 \text{ g cell/g oxygen}$. All experiments were carried out aseptically.

SB-1701 has been identified as a new strain of the genus *Flavobacterium*.

The crude protein content and the amino acid profile of SB-1701 were determined.

1 Introduction

Processes for producing SCP (Single Cell Protein) from n-paraffin and methanol have already been established. Although gaseous hydrocarbon fermentation has certain problems related to bio-engineering¹⁾, there are advantages of using gaseous hydrocarbons as raw materials, such as

- (i) pure raw materials can be made available in abundance
- (ii) unused substrate can be removed easily from fermentation broth or biomass

A considerable number of papers on SCP production using methane as the substrate have been reported²⁾, but only a limited number of papers such as those of Sugimoto et al.³⁾, Akiba et al.⁴⁾ and McLee et al.⁵⁾ are available on SCP production from LPG (Liquefied Petroleum Gas).

Moreover, the cultivation temperatures used in these studies were around 30°C . We consider it advantageous to cultivate the microorganisms at higher temperatures for SCP production, because the difference in temperatures between cooling water and fermentation medium will become greater and then the heat of fermentation can be effectively removed.

This paper concerns with isolation and charac-

teristics of propane and butane utilizing bacteria which are capable of growth at temperatures above 35°C .

2 Materials and Methods

2.1 Medium and LPG

The composition of medium A used for isolation of organisms is shown in **Table 1**. The medium consists only of inorganic salts and it does not contain organic nutrients and vitamins. The initial fermentation pH was 6.8, and a solid medium containing 2% agar was used for plate and stock culture. Sterilization was carried out for 15 minutes at 120°C . The composition of medium B used to obtain high productivity, is shown in **Table 1**. The purity of the n-butane used was over 99.9% and that of the propane over 98%.

Table 1 Composition of Media

Component	Medium A (g/l)	Medium B (g/l)
$(\text{NH}_4)_2\text{SO}_4$	2.0	8.0
KH_2PO_4	0.5	4.0
Na_2HPO_4	0.5	0.5
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.2	1.6
$\text{FeSO}_4\cdot 7\text{H}_2\text{O}$	1.0×10^{-2}	4.0×10^{-2}
$\text{MnSO}_4\cdot 4\text{H}_2\text{O}$	2.0×10^{-3}	8.0×10^{-3}
$\text{CaCl}_2\cdot 2\text{H}_2\text{O}$	1.0×10^{-2}	4.0×10^{-2}
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	—	9.6×10^{-4}
$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	—	9.6×10^{-3}
$\text{CoCl}_2\cdot 6\text{H}_2\text{O}$	—	4.8×10^{-5}
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	—	1.3×10^{-3}
KI	—	8.2×10^{-4}
H_3BO_3	—	4.0×10^{-3}

* Received June 1, 1977.

** Bridgestone Liquefied Gas Co., Ltd. (1-2, Kyobashi, Chuo-ku, Tokyo 104)

*** Present address: DIC Kiko Co., Ltd. (3-7-20, Nihonbashi, Chuo-ku, Tokyo 104)

2.2 Isolation of Organisms and Screening

Bacteria were isolated by the enrichment culture method. 100 ml of medium A was placed in a 500 ml Sakaguchi flask, inoculated with about 0.5 g of a sample. Then air containing 20% *n*-butane was sealed in with a cotton filter tube, and cultivation was carried out at 37°C on a reciprocal shaker for 1~2 weeks. After incubation, the broth was diluted with sterilized water in the amount 5~10 times that of the broth, streaked onto the agar plates with a loop and incubated for 4~6 days at 37°C. Incubation was carried out in a desiccator in an atmosphere of air containing 20% *n*-butane. After incubation, large dense colonies on the plates were selected and transferred to the agar slants and incubated in a *n*-butane-air atmosphere and they were finally purified.

The growth rate of the isolated bacteria was obtained by batch culture. Cultivation of the isolated bacteria was carried out in a 500 ml Sakaguchi flask under the same conditions as those of the enrichment culture. Cell concentrations during culture were measured by following the optical densities to obtain the growth rate.

2.3 Cell Concentration

After centrifugation of the culture broth, the sedimented cells were washed twice with water and dried for 24 hours at 105°C to obtain dry cell weight (g cell/l). The optical cell density (O.D.) of the broth was obtained by measuring absorbancy at 610 nm using a spectrophotometer (Hitachi Model 124). 1 O.D. was equal to 0.2 g cell/l.

2.4 Gas Analysis

The composition of the gas at the inlet and outlet of the fermenter was analyzed by gas chromatography using a thermal conductivity detector (Shimadzu Model GC-3BT). Propane, butane and carbon dioxide levels were determined with DBM columns (3 mm dia. $\times$ 3 m), and oxygen and nitrogen levels were determined with molecular sieves 5A (3 mm dia. $\times$ 1.5 m). The column temperature was 40°C, and 40 ml/min helium was used as the carrier gas. The gas flow rate at the outlet of the fermenter was measured with a wet-type flow meter.

2.5 Cell Analysis

The broth cultured in a 20-liter fermenter using propane as the substrate (O.D. 80) was centrifuged, and the harvested cells were washed twice with a 0.85% physiological saline solution and

dried *in vacuo* for 30 hours at 40°C. The crude protein contents of the dry cells were calculated by multiplying the nitrogen content, determined by elementary analysis by 6.25. The amino acid distribution of the dry cells was analyzed by the Japan Scientific Feeds Association.

2.6 Identification of Strain

Mycologic characteristics of the isolated bacteria were examined by the following methods. Gram staining was observed by the modified Hucker method⁶⁾. Barsiekow medium and the synthetic medium of Ayers et al. were used for observing the acid formation from sugars. An NH_4NO_3 -inorganic synthetic medium was used for observing the utilization characteristics of sugars. The O-F test was performed in accordance with the Hugh & Leifson method. G.C. content in DNA was obtained by measuring $\text{Tm}^{7)}$ after extracting DNA by the Marmur method⁸⁾. Other tests were carried out by conventional methods^{9)~11)}.

2.7 Experiments with a 2-liter Fermenter

Batch culture using a 2-liter fermenter was carried out to determine (i) the optimum growth temperature and (ii) the optimum growth pH. One liter of medium A was charged into a standard, agitated-type 2-liter fermenter (Kanto Rikaki) and sterilized. The temperature and pH were maintained constant and agitation was carried out at 600 rpm. A feed gas mixture of 20% propane and 80% air was sparged at a rate of 1 vvm. The specific growth rate by O.D. measurement during culture was used as the reference for deciding the optimum condition.

2.8 Experiments with a 20-liter Fermenter

The optimum cultivation medium for obtaining high productivity was decided from batch culture using a 20-liter fermenter (Kanto Rikaki). Eight liters of medium A was charged into the fermenter as the basal medium. A batch culture was carried out aseptically at 37°C, pH 6.1 and agitation speed of 600 rpm. A mixed gas of 20% propane and 80% air was sparged continuously at a rate of 0.5 vvm. Inorganic salts and trace quantities of metallic salts (Cu^{2+} , Zn^{2+} , Co^{2+} , Mo^{6+} , I^- , B^{3+}) were injected separately into the broth when any growth depression was observed during cultivation. The combination of medium A and injected salts was taken as medium B.

2.9 Continuous Culture Experiment

Experiments on continuous culture were carried out using a 20-liter fermenter. A schematic dia-

1 20 l Jar fermenter, 2 Motor, 3 Bottle with stock medium, 4 Metering pump, 5 Harvesting pump, 6 Harvesting bottle, 7 Air compressor, 8 LPG cylinder, 9 Gas flow meter and controller, 10 PVA air filter, 11 Condenser, 12 Flow meter, 13 Bottle with 28% NH_4OH solution, 14 Dosing pump, 15 Temperature controller, 16 pH controller

Fig. 1 Schematic Diagram of Continuous Cultivation Apparatus

gram of the apparatus is shown in **Fig. 1**. Cultivation was carried out at 37°C , pH 6.1 using an agitation speed of 600 rpm. A mixed gas of 20% propane and 80% air was sparged continuously at a rate of 0.5 vvm. The exhaust gas from the fermenter was not recycled. The liquid volume in the fermenter was maintained constant at 8 liters by means of dosing and harvesting peristaltic pumps (Tokyo Rikakikai Model MP-1011). Medium B was used in this experiment. The dilution rate was 0.06 hr^{-1} .

Productivity was calculated by the following equation.

$$P = D \cdot X$$

D : dilution rate, hr^{-1}

X : cell concentration, g cell/l

And yields based on propane and oxygen were calculated by the following equations.

$$Y_c = \frac{P}{V_c}$$

$$Y_o = \frac{P}{V_o}$$

Y_c : yield based on propane, g cell/g propane

Y_o : yield based on oxygen, g cell/g oxygen

V_c : propane uptake rate, g propane/l·hr

V_o : oxygen uptake rate, g oxygen/l·hr

The uptake rates of propane and oxygen were obtained from the mass balance around the fermenter, which was obtained from gas analysis and exhaust gas volume measurements.

3 Results and Discussion

3.1 Isolation and Screening

Twenty three bacterial strains capable of growth on *n*-butane as its sole carbon-energy source at 37°C were isolated from 31 soil samples.

The soil samples were taken from an oil seepage area in Shizuoka Prefecture. As a result of screening, SB-1701 indicated the fastest growth with a doubling time of 5 hours. The doubling time of the other bacteria was over 6 hours. It was found that SB-1701 can also utilize propane besides *n*-butane with the same growth rate.

3.2 Identification

The results of the identification tests are shown in **Table 2**. SB-1701 is a nonmotile, Gram negative and yellow rod bacteria with positive catalase and oxidase. Therefore, it was concluded that this strain belongs to the genus *Flavobacterium*. A comparison was made between this strain and

Table 2 Taxonomic Properties of Strain SB-1701

1. Morphological Characteristics.
 - Rods, $2.5-5.0 \mu \times 0.7 \mu$, Gram-negative, Non motile, No endospores, Not acid-fast.
 2. Cultural Characteristics.
 - 1) Nutrient agar colonies.
 - $2.5-3.0 \times 3.0-4.0 \text{ mm}$, rabulate, verrucose, pulvinate, labate, amorphous, pale reddish yellow, opaque.
 - 2) Nutrient broth.
 - Pellicle, Strongly turbid, Viscid.
 - 3) Potato slant.
 - Abundant growth, Non soluble light reddish yellow pigments.
 - 4) Litmus milk : Alkaline.
 - 5) B.C.P. milk : Alkaline.
 3. Biochemical characteristics.
 - 1) Indol : Not produced.
 - 2) Nitrate reduced to nitrite : Positive.
 - 3) Hydrogen sulfide : Produced.
 - 4) Urease : Positive.
 - 5) Oxidase : Negative.
 - 6) Catalase : Positive.
 - 7) Starch : Not hydrolyzed.
 - 8) Gelatin stab : Not liquefied.
 - 9) No acid and no gas from carbohydrates.
 - 10) D-glucose, D-mannose, D-fructose, D-galactose, sucrose, D-sorbitol and D-mannitol are utilized as a sole source of carbon with ammoniacal nitrogen.
 4. Physiological Characteristics.
 - 1) Aerobic.
 2. Optimum temperature : $35-40^\circ \text{C}$.
- Moles%G+C : 43.2%

Table 3 Analysis of the Cell Components

Crude Protein	65.13%
C	44.80%W/W
H	7.33%
N	10.42%
O	28.14%
Ash	9.30%

Table 4 Amino Acid Distribution in Strain SB-1701 and Typical SCP

Amino Acid	Source of Protein			
	SB-1701 (Propane)	Bacteria ⁽¹²⁾ (Methane)	Bacteria ⁽¹³⁾ (Methanol)	Yeast ⁽¹⁴⁾ <i>n</i> -Paraffin
Isoleucine	2.89	3.45	4.30	5.1
Leucine	6.70	5.61	6.77	7.4
Phenylalanine	3.21	5.03	3.43	4.7
Tyrosine	2.33	—	3.07	3.6
Lysine	4.12	3.52	5.88	7.4
Cystine	0.54	—	0.61	1.1
Methionine	0.89	1.32	2.41	1.8
Threonine	5.08	3.83	4.59	4.9
Tryptophane	1.38	9.29	0.89	1.4
Valine	5.09	12.47	5.23	5.9
Glycine	4.55	5.14	5.04	—
Alanine	8.23	2.19	6.82	—
Histidine	1.63	2.23	1.53	2.1
Proline	3.58	2.87	3.01	—
Serine	2.13	4.30	3.40	—
Arginine	6.12	5.10	4.47	—
Aspartic acid	7.97	8.47	8.53	—
Glutamic acid	14.82	9.09	9.06	—

Amino acid grams/16 grams nitrogen

bacteria belonging to the genus *Flavobacterium* as described in Bergey's Manual of Determinative Bacteriology (8th Edition). This strain is similar to *F. ferrugineum* in its G.C. content in DNA, but it differs from *F. ferrugineum* with respect to hydrolyzed gelatin and starch and formation of acid from sugars. This strain is characterized by its optimum growth temperature of 37°C. There are 4 kinds of strain, *F. meningosepticum*, *F. ferrugineum*, *F. lutescens* and *F. rigense*, belonging to the genus *Flavobacterium* which are capable of growth at 37°C. However, SB-1701 differs from those strains in its mycologic characteristics. From the above facts, SB-1701 has been identified as a new strain belonging to the genus *Flavobacterium*.

3.3 Analysis of Bacteria

The results of elementary analysis of SB-1701 are shown in Table 3. The biomass contains a high protein concentration similar to other SCP's.

The amino acid composition of this strain and that of typical SCP's are shown in Table 4. The amino acid distribution of this strain is nearly comparable with other SCP's except for sulfur-containing amino acids.

3.4 Growth Characteristics

The optimum growth temperature of SB-1701 at pH 6.1 was 35~40°C, and the growth rate decreases considerably at 45°C (Fig. 2). The optimum pH range at 37°C was 5.7~6.7, and at pH 4.8 no growth was observed (Fig. 3). The maximum specific growth rate of the isolate under optimum growth conditions was 0.12 hr⁻¹.

One of the major problems in SCP production

Fig. 2 Effect of Growth Temperature in Batch Culture at pH 6.1 2l Jar Fermenter, 20% Propane-Air Mixture, 1 vvm.

Fig. 3 Effect of pH on Growth in Batch Culture at 37°C 2l Jar Fermenter, 20% Propane-Air Mixture, 1 vvm.

Table 5 Growth characteristics of typical propane and butane utilizing bacteria

Organism	Temperature (°C)	Specific Growth Rate (hr ⁻¹)	Reference
<i>Nocardia paraffinica</i> KY 4334 (<i>n</i> -butane)	30	0.077	3)
<i>Corynebacterium hydrocarbonclastus</i> (propane)	30	0.046	4)
<i>Arthrobacter</i> P4 (<i>n</i> -butane)	30	0.173	5)
<i>Flavobacterium</i> SB-1701 (propane and butane)	35~40	0.116	

from hydrocarbons is the removal of the large amount of heat evolved during fermentation because of the small temperature differential found between the fermentation medium and the cooling water. One means of overcoming this problem is to grow the microorganisms at higher temperatures. **Table 5** shows the growth temperatures of representative LPG utilizing bacteria. It can be said from this that SB-1701 has a higher growth temperature than other bacteria, thus it seems to be more effective than the others for the removal of heat of fermentation.

3.5 Medium

A comparison of SB-1701 grown with medium A and with medium B is shown in **Fig. 4**. Experiments were carried out in a 20-liter fermenter.

Fig. 4 Comparison of Medium in Batch Culture 20 l Jar Fermenter, pH 6.1, Temperature 37°C, 20% Propane-Air Mixture, 0.5 vvm

The exponential growth of medium A could not be maintained beyond about O.D. 20 and the final cell density was about O. D. 50. However, in the case of medium B, the exponential growth was longer (O.D. 50), and the final cell concentration reached O. D. 200 with an increase in the maximum productivity from 0.5 to 2.0 g cell/l·hr. Trace quantities of metallic salts did not accelerate the growth rate but they are necessary for obtaining high cell concentration.

3.6 Continuous Culture

The results of continuous culture with a 20-liter fermenter are shown in **Fig. 5**. The continuous culture was maintained at a steady state for more than 400 hours at a dilution rate of 0.06 hr⁻¹, and a cell concentration of 23~24 g cell/l. Productivity was 1.4 g cell/l·hr, and the yield based on propane was 0.95 g cell/g propane and that based on oxygen was 0.41 g cell/g oxygen.

4 Conclusion

n-Butane-utilizing SB-1701 was isolated from soils. The following results were obtained from batch culture, continuous culture and taxonomical experiments.

- (1) It grew on propane and butane as its sole carbon-energy source at 35~40°C with a specific growth rate of 0.12 hr⁻¹.
- (2) Continuous culture was maintained at a steady state for 400 hours, productivity was 1.4 g cell/l·hr and yield based on propane was 0.95 g cell/g propane.
- (3) Crude protein content of SB-1701 was 65%.
- (4) SB-1701 was identified as a new strain of the genus *Flavobacterium*.

Fig. 5 Course of the Growth of SB-1701 in the Continuous Culture 20l Jar Fermenter, pH 6.1, Temp. 37°C, 20% Propane-Air Mixture, 0.5 vvm

Acknowledgement

The authors express their thanks to Mr. K. Maruyama and Mr. K. Fukuda for their assistance in this experimental work.

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