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Isolation and Cultivation of Methanol-Utilizing Bacteria

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A large number of methanol-utilizing bacteria were isolated from a wide variety of natural sources in Japan. Isolates BNK-84, B-185 and BNS-25 which showed interesting characters of high growth rate, high optimum growth temperature, or low optimum growth pH were used for this study. Continuous cultures of these strains were carried out, and the feasibility of single cell protein production from methanol was studied.

Optimum cultural conditions of strain BNK-84 were 38°C, pH 6.2–6.8 and a dilution rate of 0.2–0.6 h^{-1}, those of strain B-185 were 40–42°C, pH 6.5–7.0 and a dilution rate of 0.1–0.33 h^{-1}, and those of strain BNS-25 were below 32°C, pH 4.0–4.5 and a dilution rate of 0.10–0.17 h^{-1}. Of these strains, strain BNK-84 seems to be the most suitable for commercial production of bacterial SCP from methanol in the cell yield, the growth rate, the contents of crude protein and amino acids, and the composition of amino acids. Strain B-185 showed an interesting character of high growth temperature, and strain BNS-25 exhibited an optimum growth pH of as low as 4.5. Strain BNK-84 showed the maximum specific growth rate, 0.60 h^{-1}; a cell yield, 45 wt%; a content of crude protein, 86 wt%; a content of amino acids, 70 wt%; and the maximum content of nucleic acids, 20 wt%. The methanol consumption rate per unit cell mass for maintenance, m, was about 0.04 g methanol/g cell·h.

Protein and anthrone-positive substances (APS) were detected in the broth supernatant of the three strains. Especially strain B-185 produced a large amount of protein, and its productivity was decreased by an increase of the dilution rate and the cultural temperature.

Single cell protein (SCP) would be useful for preventing the anticipated world protein shortage. Methanol is one of the desirable carbon sources for production of SCP on the basis of quantity of production, purity, and water solubility. At first, *Bacillus methylicus* was reported as a methanol-utilizing bacterium in 1892 by Loew,[1] and since then, *Bacillus extorquens* in 1914[2] was reported. In the last ten years the production of microbial protein from methanol was extensively studied by a large number of scientists and corporations.[3–17] Currently the Imperial Chemical Industry (*I.C.I*) is producing SCP commercially using methanol as a carbon source.[18]

In any processes in SCP production, it is very important to know and optimize the substrate yield which is defined as the weight of dry cells produced per g of carbon-containing substrate consumed. The three parameters of fermentations that influence substrate yield are the dilution rate, the cultural temperature and the cultural pH.[5,6,9,11,13,17,19] The choice of optimum values for these three parameters can only be made by considering the effects they may have on both the substrate yield and the cell composition. Variations in the dilution rate, the cultural temperature and the cultural pH would influence the intracellular contents of protein, nucleic acids, lipids and polysaccharide, and the extracellular content of carbon compounds.[11,17,20–23]

This paper deals with the isolation of methanol-utilizing bacteria and the optimum operating conditions for continuous cultures.

Materials and Methods

Isolation of methanol-utilizing bacteria Methanol-utilizing bacteria were isolated by the en-

richment culture technique at 30 or 37°C from a wide variety of natural sources in Japan.[24] Media A and C were used for the isolation. Medium A was composed of $(NH_4)_2HPO_4$, 3.0 g; KH_2PO_4, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $MnCl_2 \cdot 4H_2O$, 2.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; vitamin solution, 0.1 ml; CH_3OH, 10 ml, and distilled water 1,000 ml, the pH was adjusted to 7.0. Medium C was composed of $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 20 mg; $FeSO_4 \cdot 7H_2O$, 20 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg; vitamin solution, 0.1 ml; CH_3OH, 10 ml, and distilled water 1,000 ml, the pH was adjusted to 4.5. The vitamin solution was composed of biotin, 2 mg; calcium pantothenate, 400 mg; pyridoxine-HCl, 400 mg; thiamine-HCl, 400 mg; P-aminobenzoic acid, 200 mg; folic acid, 2 mg; inositol, 2 g; nicotinic acid, 400 mg; riboflavin, 200 mg; and distilled water 1,000 ml. Pure cultures were obtained by repeated plating on medium A or C solidified with 2% agar. According to this procedure, 144 strains were isolated. Isolates BNK-84, B-185 and BNS-25 showed interesting characters of high growth rate, high optimum growth temperature, or low optimum growth pH, and were used for subsequent experiments.

Culture system Continuous cultivations were carried out in a 10 *l* jar fermentor operated at an aeration rate of 5 *l*/min and an agitation rate of 1,000 rpm as previously reported.[25,26] The dissolved oxygen concentration in the fermentor was kept between 2 to 8 ppm during cultivation. The pH was controlled by adding aqueous ammonia. The composition of the medium used was as follows; CH_3OH, 10 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 1.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 30 mg; $FeC_6H_5O_7 \cdot XH_2O$, 30 mg; $MnCl_2 \cdot 4H_2O$, 5.0 mg; $ZnSO_4 \cdot 7H_2O$, 5.0 mg; $CuSO_4 \cdot 5H_2O$, 0.5 mg, and distilled water 1,000 ml. Medium supplemented with calcium panthotenate, 0.4 mg/*l*, was used for strain BNS-25.

Methods of analysis The cell yield on methanol (grams of dry cell weight produced per gram of methanol consumed) in a continuous culture was determined for a number of steady state conditions after 30 h or more cultivation at varying dilution rates. For the estimation of the dry cell weight, samples of cell suspensions were centrifuged at $10,000 \times g$ for 10 min. The pellets were washed twice with distilled water, dried at 105°C for 24 h, and weighed. The concentration of methanol was measured by gas chromatography. The content of crude protein was estimated from the total nitrogen content measured by the micro-Kjeldahl method. The content of amino acids was estimated by assaying with an automatic amino acid analyzer. Samples were prepared by hydrolysing whole cells in 6N-HCl for 22 h at 110°C under vacuum. Tryptophan was estimated separately by a colorimetric method.[27] The content of nucleic acids was measured according to the method of Schneider.[28] The maintenance coefficient (m) which represents the energy expended in processes other than those directly involved in synthesizing the biomass was estimated by the method of Abbott *et al.*[20] The content of extracellular carbon compounds in the broth supernatant was determined as chemical oxygen demand (COD) by the method in the previous report.[26] The content of true protein in the broth supernatant was measured colorimetrically by the method of Lowry *et al.*,[29] with Folin-phenol solution and albumin as standards. The content of anthrone-positive substances (APS) in the broth supernatant was assayed by a colorimetric procedure with anthrone reagent and expressed as glucose.[30]

Results

Continuous cultures of strains BNK-84, B-185 and BNS-25

(1) *Influence of dilution rate on the cell yield, the specific rate of methanol consumption, and the composition of cells* Strains BNK-84, B-185 and BNS-25 were cultivated in a chemostat under methanol-limiting conditions at different dilution rates. Strain BNK-84 was cultivated at 38°C and pH 6.5, strain B-185 at 42°C and pH 6.5, and strain BNS-25 at 32°C and pH 4.5. The cell yield, and the contents of crude protein, amino acids and nucleic acids, at the different dilution rates are shown in Figs. 1, 2 and 3.

The cell yield increased with the dilution rate, and then decreased. The contents of crude protein and amino acids were constant for all the dilution rates tested. The content of nucleic acids increased in parallel with the dilution rate up to the maximum dilution rate. The specific consumption rates of methanol at various dilution rates are shown in Fig. 4. The methanol consumption rates per unit cell mass for the maintenance, m, of strains BNK-84, B-185 and BNS-25 were estimated to be about 0.04 g methanol/g cell·h by extrapolation.

(2) *Influence of cultural temperature on the growth rate, the cell yield, and the composition of cells* Aside from the cost of the carbon

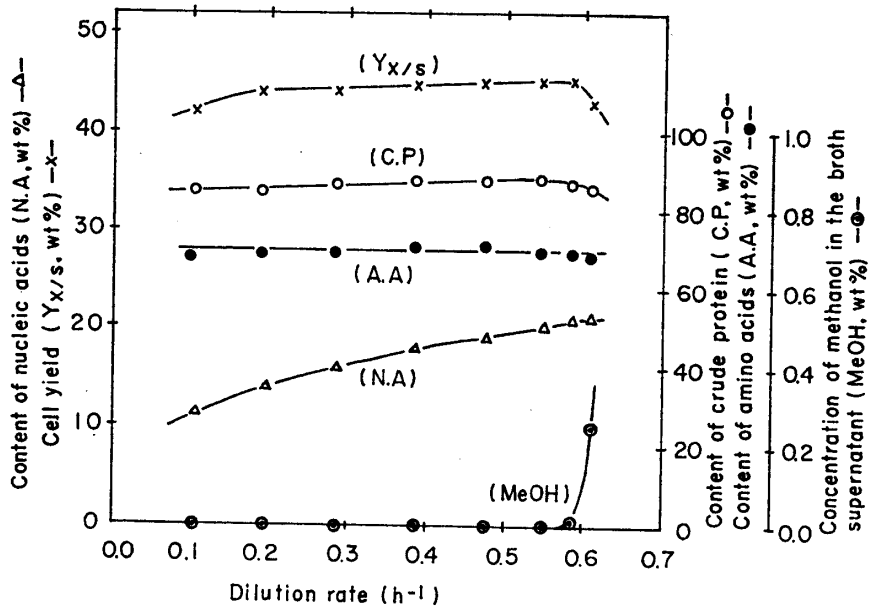

Fig. 1. Influence of dilution rate on the cell yield and the composition of cells (Strain BNK-84, temperature: 38°C, pH 6.5).
Cell yield ($Y_{X/S}$, wt%) –×–, content of crude protein (C. P, wt%) –○–, content of amino acids (A. A, wt%) –●–, content of nucleic acids (N. A, wt%) –△–, concentration of methanol in the broth supernatant (MeOH, wt%) –⊙–

source for the production of SCP, one of the major factors concerning capital and operating costs is the removal of heat of the fermentation. For this reason, strains which show a higher optimum temperature for growth are desired.

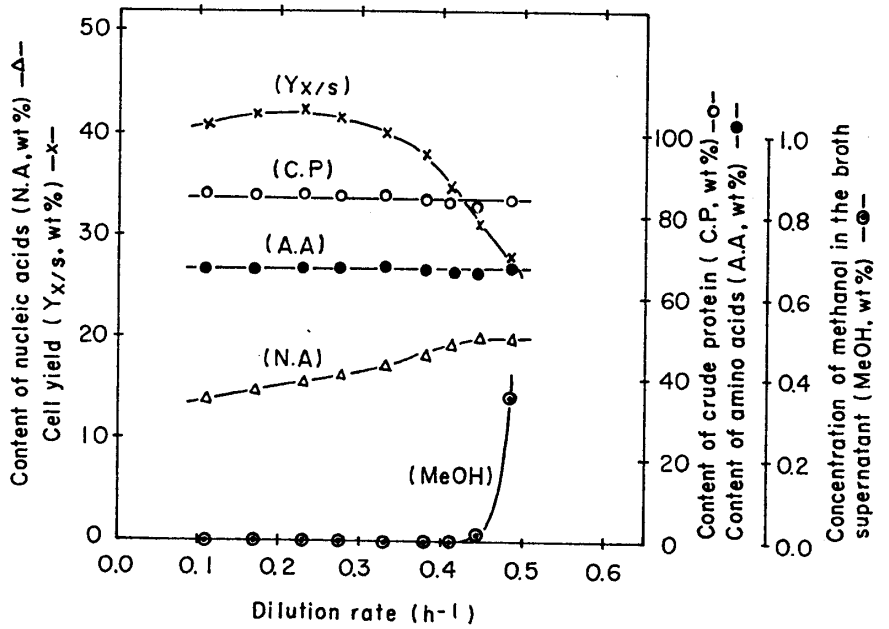

Fig. 2. Influence of dilution rate on the cell yield and the composition of cells (Strain B-185, temperature: 42°C, pH 6.5).
Cell yield ($Y_{X/S}$, wt%) –×–, content of crude protein (C. P, wt%) –○–, content of amino acids (A. A, wt%) –●–, content of nucleic acids (N. A, wt%) –△–, concentration of methanol in the broth supernatant (MeOH, wt%) –⊙–

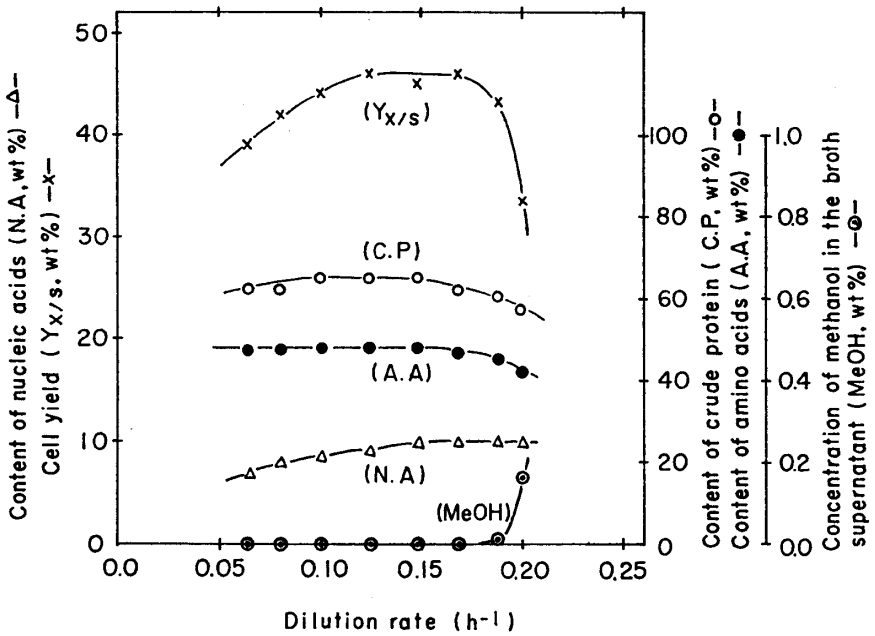

Fig. 3. Influence of dilution rate on the cell yield and the composition of cells (Strain BNS-25, temperature: 32°C, pH 4.5).
Cell yield ($Y_{X/S}$, wt%) -x-, content of crude protein (C. P, wt%) -○-, content of amino acids (A. A, wt%) -●-, content of nucleic acids (N. A, wt%) -△-, concentration of methanol in the broth supernatant (MeOH, wt%) -⊙-

The maximum specific growth rate, the cell yield, and the contents of crude protein and amino acids of strains BNK-84, B-185 and BNS-25 at different temperatures are shown in Figs. 5, 6 and 7. The pH of the cultures for strains BNK-84 and B-185 was controlled at pH 6.5, and that for strain BNS-25 at pH 4.5. The cell yield decreased with the

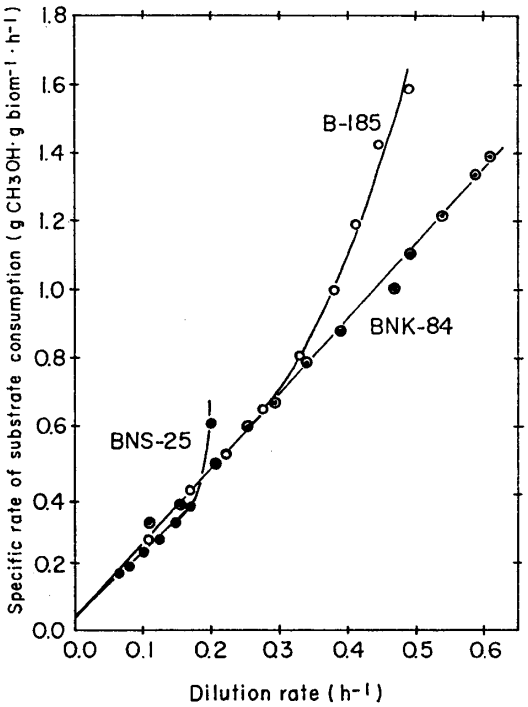

Fig. 4. Influence of dilution rate on the specific rate of methanol consumption in chemostat cultures.
-⊙- Strain BNK-84, temperature 38°C, pH 6.5, -○- Strain B-185, temperature 42°C, pH 6.5, -●- Strain BNS-25, temperature 32°C, pH 4.5.

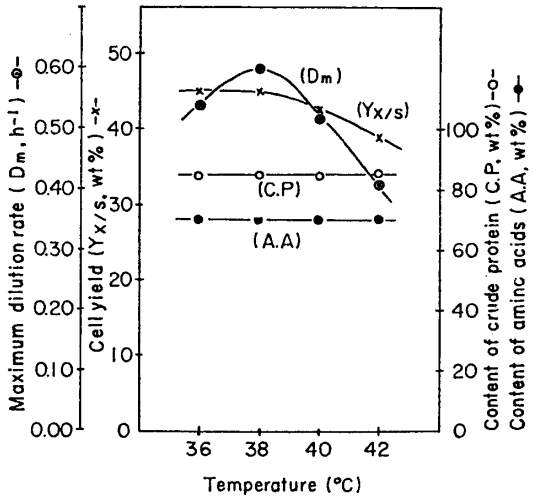

Fig. 5. Influence of cultural temperature on the maximum growth rate, the cell yield, and the composition of cells (Strain BNK-84, pH 6.5).
Maximum dilution rate (D_m, h^{-1}) -⊙-, at dilution rate: D=0.40 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -x-, Content of crude protein (C.P, wt%) -○-, Content of amino acids (A.A, wt%) -●-.

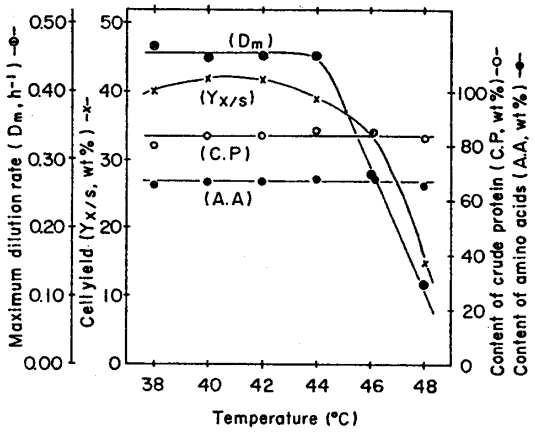

Fig. 6. Influence of cultural temperature on the maximum growth rate, the cell yield, and the composition of cells (Strain B-185, pH 6.5).
Maximum dilution rate (D_m, h^{-1}) -⊙-.
at dilution rate: D=0.22 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -×-, Content of crude protein (C. P, wt%) -○-, Content of amino acids (A.A, wt%) -●-.

increase of the cultural temperature. The optimum temperature of strain BNS-25 was below 32°C, that of strain BNK-84 was about 38°C, and that of strain B-185 was about 40 to 42°C for the cell yield, the growth rate, and contents of crude protein and amino acids. Strain B-185 was able to grow sufficiently at 46°C.

(3) *Influence of cultural pH on the growth rate, the cell yield, and the composition of cells* In-

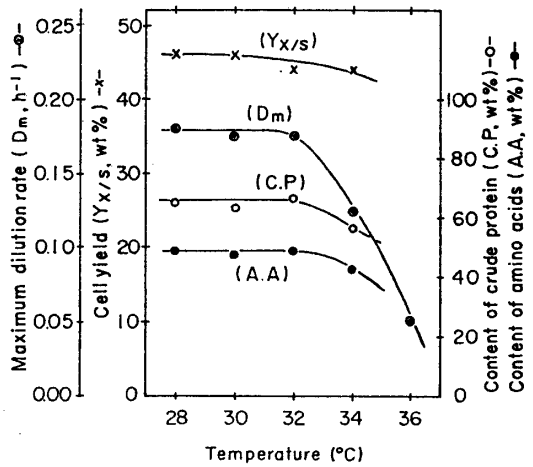

Fig. 7. Influence of cultural temperature on the maximum growth rate, the cell yield, and the composition of cells (Strain BNS-25, pH 4.5).
Maximum dilution rate (D_m, h^{-1}) -⊙-.
at dilution rate: D=0.125 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -×-, Content of crude protein (C.P, wt%) -○-, Content of amino acids (A.A, wt%) -●-.

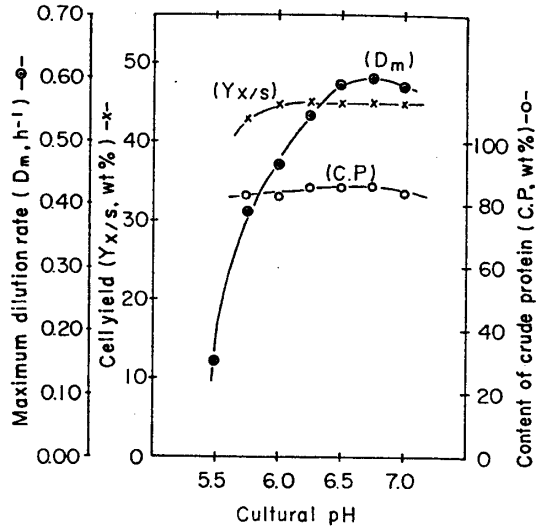

Fig. 8. Influence of cultural pH on the maximum growth rate, the cell yield, and the composition of cells (Strain BNK-84, 38°C).
Maximum dilution rate (D_m, h^{-1}) -⊙-.
at dilution rate: D=0.40 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -×-, Content of crude protein (C.P, wt%) -○-.

fluence of cultural pH on the growth of strains BNK-84, B-185 and BNS-25 was studied. The maximum specific growth rate, the cell yield, and the content of crude protein of strains BNK-84, B-185 and BNS-25 at different cultural pHs are shown in Figs. 8, 9 and 10. The cultural temperature of strain BNK-84 was controlled at 38°C, that of strain

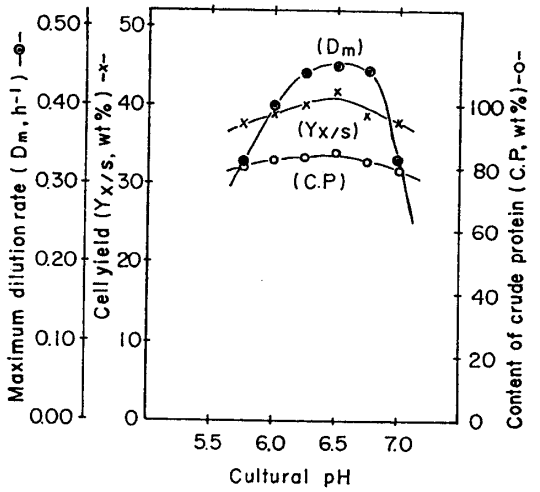

Fig. 9. Influence of cultural pH on the maximum growth rate, the cell yield, and the composition of cells (Strain B-185, 42°C).
Maximum dilution rate (D_m, h^{-1}) -⊙-.
at dilution rate: D=0.22 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -×-, Content of crude protein (C.P, wt%) -○-.

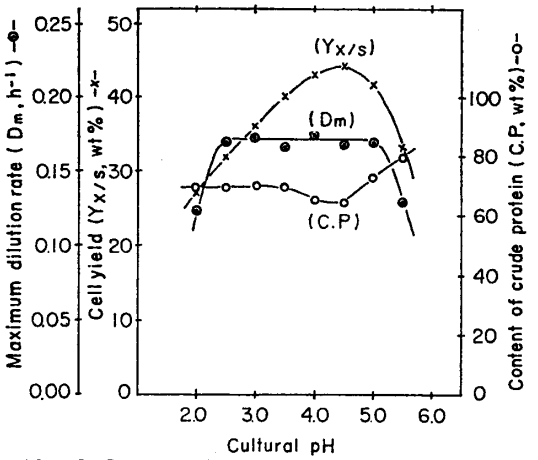

Fig. 10. Influence of cultural pH on the maximum growth rate, the cell yield, and the composition of cells (Strain BNS-25, 32°C).
Maximum dilution rate (D_m, h^{-1}) -⊙-,
at dilution rate: D=0.125 h^{-1}, Cell yield ($Y_{X/S}$, wt%) -×-, Content of crude protein (C.P, wt%) -○-.

B-185 at 42°C, and that of strain BNS-25 at 32°C.

The optimum pH for the growth of strain BNS-25 was approximately 4.0 to 5.0, that of strain BNK-84 was approximately 6.5 to 7.0, and that of strain B-185 was approximately 6.2 to 6.8. Strain BNS-25 grew at a pH as low as 2.0, but did not grow at pH 6.0.

(4) *Influence of the dilution rate and cultural temperature on the production of extracellular carbon compounds* The contents of extracellular carbon compounds in the broth supernatant of strains BNK-84, B-185 and BNS-25 cultivated at different dilution rates, different temperatures and different pHs were studied. Strains BNK-84 and BNS-25 produced a small amount of extracellular carbon compounds, and the content was about 100 to 500 ppm as COD. On the other hand, strain B-185 produced a large number of extracellular carbon compounds approximately 1,400 ppm as COD at dilution rates below 0.3 h^{-1}, 38°C and pH 6.5. The content decreased with increases of the dilution rate and cultural temperature, and it was below 500 ppm as COD at dilution rates of more than 0.3 h^{-1} or temperatures of more than 42°C. The contents of protein and anthrone-positive substances (APS) in the

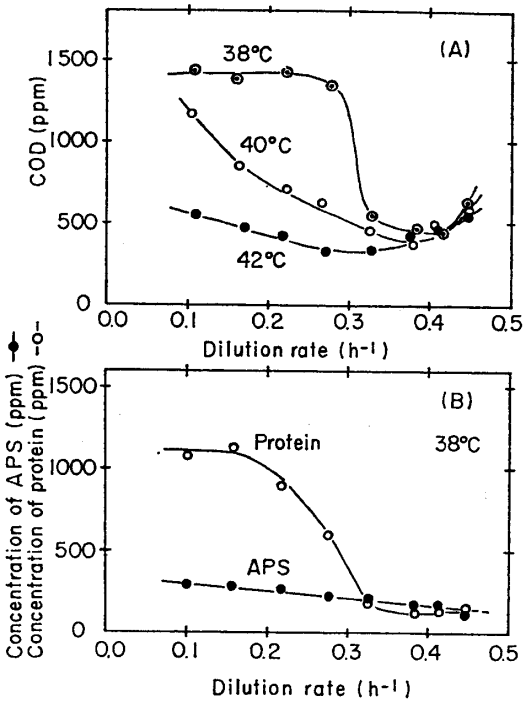

Fig. 11. Extracellular carbon compounds in the broth supernatant of strain B-185 (pH 6.5).
(A) Influence of the dilution rate and cultural temperature. -○- 38°C, -○- 40°C, -●- 42°C.
(B) Concentration of protein and anthrone-positive substances (38°C). -○- protein, -●- APS.

broth supernatant cultivated at 38°C, and pH 6.5, are shown in Fig. 11. The change of the protein content with the dilution rate resembled the COD change, and the maximum content of protein was approximately 1,100 ppm. The content of APS was changed little by the dilution rate, it was approximately 100 to 300 ppm. From these results, the majority of the extracellular carbon compounds were presumed to be proteinaceous.

Strain B-185 produced the slime in the continuous culture at 38°C and pH 6.5. The slime decreased with the increase of the dilution rate and the cultural temperature like the contents of extracellular carbon compounds, and were scarcely found at dilution rates of more than 0.42 h^{-1}, or at temperatures of more than 40°C. On the other hand, strains BNK-84 and BNS-25 did not produce the slime in all the continuous culture conditions tested.

Comparison of strains for production of SCP from methanol Results of continuous cultures of strains BNK-84, B-185

Table 1. Summarized data of continuous cultures of strains BNK-84, B-185 and BNS-25.

	BNK-84	B-185	BNS-25
Optimum growth pH	6.5-7.0	6.2-6.8	4.0-5.0
Optimum growth temperature (°C)	38	40-42	~32
Maximum specific growth rate (h^{-1})	0.60	0.45	0.20
Optimum dilution rate (h^{-1})	0.20-0.60	0.10-0.33	0.10-0.17
Cell yield (wt%)	45	42	45
Content of crude protein (wt%)	86	84	64
Content of amino acids (wt%)	70	67	48
Content of nucleic acids (wt%)	14-20	14-17	8-10

and BNS-25 are summarized in Table 1. The amino acid compositions are shown in Table 2. Compared with the standard composition proposed by the FAD (Food and Agricultural Organization), the content of methionine in strain BNS-25 was low. But, the contents of other amino acids in strain BNS-25, and the contents of all amino acids in strains BNK-84 and B-185, in terms of essential amino acids, were very or fairly high. Of these strains, strain BNK-84 seems to be the most suitable for commercial production of bacterial SCP from methanol on the basis of the cell yield, the growth rate, the contents of crude protein and amino acids, and the composition of amino acids.

Optimum cultural conditions for strain BNK-84 were 38°C, pH 6.2–6.8, and a

Table 2. Comparison of the compositions of cells and the FAD standard.

Component	Strain BNK-84		Strain B-185		Strain BNS-25		FAD standard
	g/100 g of biomass	g/100 g of protein	g/100 g of biomass	g/100 g of protein	g/100 g of biomass	g/100 g of protein	g/100 g of protein
Crude protein ($N\times6.25$)	88.8		84.0		64.9		
Amino acids	70.8	100.0	67.0	100.0	48.6	100.0	100.0
(Essential amino acids)							
Lysine	4.4	6.2	4.6	7.0	2.6	5.3	4.2
Threonine	3.5	4.9	3.3	4.9	2.6	5.3	2.8
Valine	5.0	7.1	4.3	6.4	3.5	7.2	4.2
Methionine	1.9	2.7	1.7	2.5	0.7	1.4	2.2
Isoleucine	4.1	5.8	3.7	5.5	2.4	4.9	4.2
Leucine	6.4	9.0	5.7	8.6	4.2	8.6	4.8
Phenylalanine	3.6	5.1	3.3	4.9	2.1	4.3	2.8
Tryptophan	1.6	2.3	1.3	1.9	0.9	1.9	1.4
(Non-essential amino acids)							
Alanine	5.8	8.2	5.4	8.1	4.4	9.1	
Arginine	3.6	5.1	4.1	6.1	3.9	8.0	
Aspartic acid	7.9	11.2	7.2	10.7	5.1	10.6	
Cysteine	0.6	0.8	0.6	0.9	0.4	0.8	
Glutamic acid	8.4	11.8	7.7	11.6	5.5	11.4	
Glycine	4.8	6.8	4.5	6.7	3.3	6.8	
Histidine	1.2	1.7	1.3	1.9	1.1	2.3	
Proline	2.7	3.8	3.3	4.9	2.3	4.7	
Serine	2.7	3.8	2.5	3.7	1.8	3.7	
Tyrosine	2.6	3.7	2.5	3.7	1.8	3.7	
Nucleic acids	19.3		15.7		10.0		

Strain BNK-84: 38°C, pH 6.5, $D=0.472\ h^{-1}$
Strain B-185: 42°C, pH 6.5, $D=0.220\ h^{-1}$
Strain BNS-25: 32°C, pH 4.5, $D=0.148\ h^{-1}$

Table 3. Comparison of methanol-utilizing bacteria for production of bacterial SCP.

Strains	Optimum temperature (°C)	Optimum pH	Maximum dilution rate (h^{-1})	Cell yield (wt%)	Content of crude protein (wt%)	Content of amino acids (wt%)	Maintenance coefficient (m, g methanol/g cell/h)	Reference
Pseudomonas methylotropha	37	6.8	>0.23	44				(4)
Methylomonas sp.	30	7.3	0.25	40	71.5			(9)
Methylomonas methanolica M13V	35	6.7	0.50	39				(10)
Mixed MSI	34	6.85	0.48	44	78.6		0.08	(11)
Methylomonas clara	39	6.8	0.50	50	80-85	69-73	2.2	(12)
Methylomonas sp. C-1012	40	7.0	0.30	48	72.5	60.5		(14)
Pseudomonas sp. A	37	6.5-7.0	0.45	44.8	76	68	0.02	(16)
Strain BNK-84	38	6.5	0.60	45	86	70	0.04	This work
Strain B-185	42	6.5	0.45	42	84	67	0.04	//
Strain BNS-25	32	4.5	0.20	45	64	48	0.04	//

dilution rate of 0.2–0.6 $h.^{-1}$ The maximum specific growth rate was approximately 0.60 h^{-1}, the cell yield was approximately 45 wt%, the content of crude protein was approximately 86 wt%, the content of amino acids was approximately 70 wt%, and the maximum content of nucleic acids was approximately 20 wt%.

Discussion

Miura *et al.*[23] reported that the content of nucleic acids increased with the dilution rate but the content of protein stayed constant, and Goto *et al.*[17] reported that the contents of crude protein and nucleic acids decreased with an increase of cultural temperature, but the content of amino acids stayed constant. These results were confirmed by the present study. Protein and APS were detected in the broth supernatant of continuous cultures as reported by Goto *et al.*[17] Especially, strain B-185 produced a large amount of protein, and its productivity was decreased by an increase of the dilution rate and the cultural temperature.

The characteristics of strains BNK-84, B-185 and BNS-25 were compared with those of other methanol-utilizing bacteria [4,9–12,14,16] as shown in Table 3. Abbott and Clamen[19] have demonstrated the importance of the maintenance coefficient (m) in the economics of the SCP production process and the importance of choosing microorganisms having a low maintenance coefficient. The maintenance coefficients of strains BNK-84, B-185 and BNS-25 were lower than other methanol-utilizing bacteria. Strain BNK-84 was presumed to one of the most suitable strains for the production of SCP on the basis of the growth rate, the cell yield, the content of crude protein, and the optimum growth temperature. Strain B-185 showed an interesting character of a high growth temperature, and strain BNS-25 exhibited an optimum growth pH of as low as 4.5. Almost all of the strains used for the production of SCP were so-called obligated methanol-utilizing bacteria, and fixed formaldehyde by the ribulose phosphate pathway.[31] This supported the results of van Dijken and Harder.[7]

A continuous culture of strain BNK-84 was conducted utilizing the pilot plant provided with an air-lift fermentor of which reports were presented at the second and the third international symposia on microbial growth on C_1-compounds.[32,33]

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