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Production of Pyrroloquinoline Quinone by Using Methanol-Utilizing Bacteria

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A large number of methanol-utilizing bacteria were screened for extracellular production of pyrroloquinoline quinone (PQQ) by using methanol as the carbon and energy sources. Of the bacteria selected, *Hyphomicrobium* sp. strain TK 0441 was examined for PQQ production by using a jar fermentor. The amount of PQQ in the broth and the level of methanol dehydrogenase activity in the cells were increased by simply decreasing the amount of Fe added to the medium. On the other hand, extracellularly produced protein which interfered with the purification of PQQ was decreased by simply increasing the amount of Mg added to the medium. A suitable medium that contained 1 μg of Fe per ml, 150 μg of Mg per ml, and trace elements was developed. In this medium, the production of PQQ reached approximately 1 mg/ml and protein formation was low.

In 1979, pyrroloquinoline quinone (PQQ) was purified and crystallized as a cofactor of methanol dehydrogenase, which was produced by methanol-utilizing bacteria (35). PQQ was found in several bacterial enzymes, including glucose dehydrogenase (14), alcohol dehydrogenase (13), aldehyde dehydrogenase (2), glycerol dehydrogenase (5), polyethylene glycol dehydrogenase (26), and polyvinyl alcohol dehydrogenase (37). Furthermore, Ameyama et al. (3, 4, 6) reported the stimulating effect of PQQ on the growth of bacteria and yeasts, and Shimao et al. (36, 38) isolated a PQQ-requiring bacterium.

PQQ has also been found in such eucaryotes as fungi, plants, and mammals (7, 16, 19, 30, 33, 48–51). However, Janes et al. (24) reported 6-hydroxydopa instead of PQQ at the active site of bovine serum amine oxidase (30) and Kumazawa et al. (28) failed to verify the presence of PQQ in bovine plasma amine oxidase. At present, whether PQQ is a cofactor in eucaryotes is questionable. However, disease caused by a deficiency of PQQ in mice and rats has been reported by Killgore et al. (27); furthermore, PQQ has been detected in rat tissues and human body fluids (29). In addition, therapeutic uses of PQQ for mammals have been reported, including use as a scavenger (20) and as an inhibitor of liver disease (54) and cataract formation (34).

Ameyama et al. (1) showed that some methylotrophs were most favorable for study of PQQ production and that more than 10 μg of PQQ per ml was produced in broth after 2 days of incubation. McIntire and Weyler (32) reported the production of PQQ by the methylotrophic bacterium W3A1 during growth on methylamine, and van Kleef and Duine (53) reported that a large number of bacteria, including methylotrophs, extracellularly produced PQQ in microgram amounts per 1 ml of broth. Furthermore, van Kleef and Duine (52) and Houck et al. (22, 23) showed glutamate and tyrosine to be biosynthetic precursors of PQQ. However, bacterial production of PQQ is not economical. An organic chemical synthesis of PQQ has been reported by Corey and Tramontano (12). However, the synthesis requires many steps taking a very long time until completion and requires difficult steps for the removal of isomers and various other byproducts. At present, the yield of chemical synthesis of PQQ is low.

This article deals with the screening of bacteria which produced PQQ extracellularly and the establishment of optimum culture conditions for the production of PQQ by *Hyphomicrobium* sp. strain TK 0441 by using a jar fermentor.

MATERIALS AND METHODS

Screening of PQQ-producing bacteria. For the seed culture, bacterial cells were inoculated into 10 ml of medium in a test tube (18 by 165 mm) for 2 to 4 days at 30°C with shaking (220 rpm). Medium B was used for strains of the genera *Methylobacillus* (41), *Methylophilus* (25), *Methylobacterium* (11, 17, 18), *Ancylobacter* (40), *Hyphomicrobium* (21, 44), *Xanthobacter* (43), *Thiobacillus* (43), and *Mycobacterium* (39, 47) and for *Paracoccus denitrificans* strains (46). Medium C was used for strains of the genus *Acidomonas* (45). Medium D was used for *Paracoccus alcaliphilus* strains (46). Medium E was used for strains of the genus *Methylophaga* (42). Medium B contained 3.0 g of $(NH_4)_2SO_4$, 1.4 g of KH_2PO_4, 3.0 g of Na_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 30 mg of $FeC_6H_5O_2 \cdot XH_2O$, 30 mg of $CaCl_2 \cdot 2H_2O$, 5.0 mg of $MnCl_2 \cdot 4H_2O$, 5.0 mg of $ZnSO_4 \cdot 7H_2O$, 0.5 mg of $CuSO_4 \cdot 5H_2O$, 1.0 ml of a vitamin solution, 8 ml of CH_3OH, and 1,000 ml of distilled water; the pH of this medium was adjusted to 7.0. The vitamin solution used contained 2 mg of biotin, 400 mg of calcium pantothenate, 400 mg of pyridoxine hydrochloride, 400 mg of thiamine hydrochloride, 200 mg of *p*-aminobenzoic acid, 2 mg of folic acid, 2 g of inositol, 400 mg of nicotinic acid, 200 mg of riboflavin, and 1,000 ml of distilled water. Medium C contained medium B but with 4.0 g of KH_2PO_4 instead of 1.4 g and with no Na_2HPO_4; the pH of this medium was adjusted to 4.5 with 1 N HCl. Medium D was medium B adjusted to pH 9.0 with a 10% Na_2CO_3 solution. Medium E was medium B supplemented with 3%

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NaCl and 10 μg of vitamin B_{12} per liter. For culture, 2 ml of the seed culture broth was transferred into 200 ml of medium in a 1-liter Erlenmeyer flask and incubated at 30°C with shaking (220 rpm) until the methanol in the culture broth decreased to below 10 ppm. The concentration of methanol in the broth was analyzed with a Shimadzu model 8A gas chromatograph (FID) (Shimadzu, Kyoto, Japan) and a glass column (3 mm by 2 m) on Porapack PS (80 to 100 mesh). The column temperature was 160°C, and the injection temperature was 250°C. For estimation of the production of PQQ in the supernatants, the culture broth was centrifuged at 10,000 × *g* for 10 min. Quantitative analysis of PQQ in the supernatants was done with D-glucose dehydrogenase of *Escherichia coli* K-12 (15).

Culture system. *Hyphomicrobium* sp. strain TK 0441 (44), which was selected on the basis of its production of PQQ and growth rate, was used for study of the production of PQQ. Batch cultivation was done with a 30-liter jar fermentor (working volume, 15 liters) operated at an aeration rate of 15 liters/min (1 vvm) and an agitation rate of 500 rpm. The fermentor used in this study is made from stainless steel, but the amount of Fe contributed to the medium by the fermentor during cultivation seems to be less than the amount of Fe added to the medium. The dissolved oxygen concentration in the fermentor was kept between 3 and 8 ppm. Unless otherwise stated, the culture temperature was controlled at 30°C and the culture pH was controlled at 6.8 to 7.0 by adding aqueous ammonia. The composition of the basal medium was as follows: methanol, 2 g; $(NH_4)_2SO_4$, 1.0 g; KH_2PO_4, 3.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; trace element solution, 10 ml. The total weight was adjusted to 1,000 g by the addition of distilled water, and the pH of this medium was adjusted to 7.0 by adding aqueous ammonia. The trace element solution consisted of 3 g of $CaCl_2 \cdot 2H_2O$, 3 g of $FeC_6H_5O_2 \cdot XH_2O$ (the content of Fe was 20% by weight, which was determined by using a Hitachi model P-5200 ICP radiance analyzer [Hitachi, Tokyo, Japan]), 2 g of $ZnSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot 7H_2O$, and 0.1 g of $CuSO_4 \cdot 5H_2O$, all per liter. The total volume was adjusted to 1,000 ml by the addition of distilled water. The concentration of methanol in the culture broth was detected as carbon compounds in the outlet gas by using a Sensortec methanol controller with a semiconductor detector (Sensortec, Shiga, Japan), and it was maintained at between 0.1 and 0.2% by weight.

Analytical methods. The growth of cells was estimated from the optical density at 660 nm. The concentration of methanol in the broth was measured by gas chromatography and the methanol controller, as described above. For estimation of the extracellular production of PQQ and protein and residual Fe and Mg in the broth, the cultures were centrifuged at 10,000 × *g* for 10 min and the supernatants were obtained. The supernatants were diluted with 50 mM potassium phosphate (pH 6.0), and the concentrations of PQQ in the supernatants were measured by using a Shimadzu model LC-6A high-performance liquid chromatograph (Shimadzu) and a column (4.6 by 150 mm) on ODS A-302 (YMC, Kyoto, Japan). The solvent used was acetonitrile-water (1:9, vol/vol) containing 0.1 M KH_2PO_4 and 0.1 M $HClO_4$ (the pH was adjusted to 2.2 with NaOH). The flow rate was 1.5 ml/min. Detections were done with a Shimadzu model SPD-6A UV spectrophotometric detector at 259 nm and a Shimadzu model RF-535 fluorescence high-performance liquid chromatography monitor with 367 nm of excitation wavelength and 485 nm of emission wavelength. The concentrations of protein in the supernatants were measured colorimetrically with Folin phenol solution and albumin as the standards by the method of Lowry et al. as modified by Bensadoun and Weinstein (10). The concentrations of Fe and Mg in the supernatants were measured by using a Hitachi model P-5200 ICP radiance analyzer. For measuring the dry cell weight, bacterial cells were harvested by centrifugation at 10,000 × *g* at 5°C for 10 min, washed twice with distilled water, dried at 105°C for 24 h, and weighed. For determining PQQ and protein production and methanol dehydrogenase activity in the cells, the bacterial cells were washed twice with 50 mM potassium phosphate buffer (pH 7.0) by centrifugation and disrupted by sonication at 5°C for 6 min in the same buffer. The homogenates were then centrifuged at 10,000 × *g* at 5°C for 60 min, and the clear supernatant extracts were stored at −20°C until just before use. The protein concentrations in the extracts were deter-

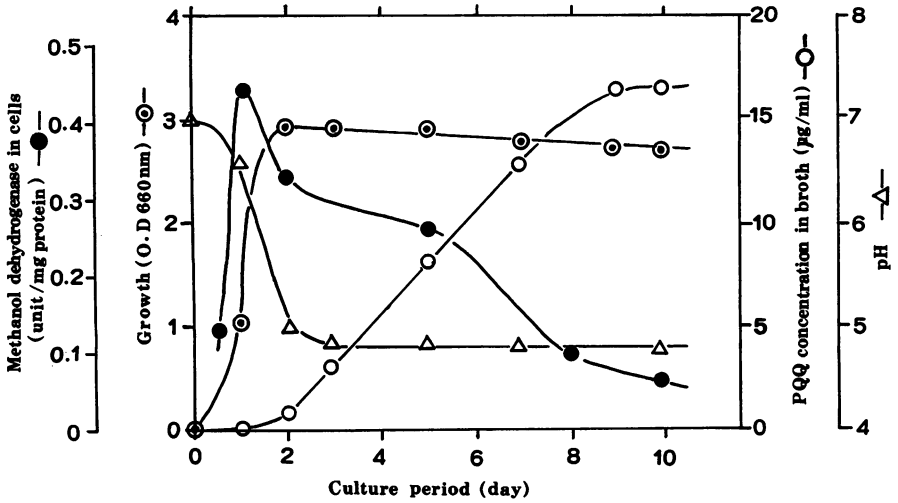

FIG. 1. Time course of growth, production of PQQ, methanol dehydrogenase activity in cells, and pH of culture broth using *Hyphomicrobium* sp. strain TK 0441. The temperature was 30°C, the initial pH was 7.0 (not controlled), and methanol was at 0.1 to 0.2% by weight. $O.D_{660nm}$, optical density at 660 nm.

TABLE 1. Effects of trace elements in the medium on the production of PQQ

Amt of trace element solution added to medium (ml/liter)[a]	Cell concn (OD_{660}[b])	PQQ concn in broth (μg/ml)
10	54.3	5
7	56.4	22
2	38.7	167
1	13.3	154

[a] The trace element solution consisted of $CaCl_2 \cdot 2H_2O$ (3 mg/ml), $FeC_6H_5O_2 \cdot XH_2O$ (3 mg/ml), $ZnSO_4 \cdot 7H_2O$ (2 mg/ml), $MnSO_4 \cdot 7H_2O$ (0.1 mg/ml), and $CuSO_4 \cdot 5H_2O$ (0.1 mg/ml); the solution was added to L medium. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight; cultivation was for 10 days.

[b] OD_{660}, optical density at 660 nm.

mined by the method of Lowry et al. (31), with Folin phenol solution and albumin as the standards. The PQQ concentrations in the extracts were determined by use of D-glucose dehydrogenase from *E. coli* K-12. The methanol dehydrogenase activities in the extracts were determined by the method of Anthony and Zatman (8), except that 1 μM PQQ was added to the reaction solution. A unit of the enzyme is defined as the quantity which catalyzes the reduction of 2,6-dichlorophenol indophenol at the rate of 1 μmol/min at pH 9.0 and 30°C. The molecular extinction coefficient for 2,6-dichlorophenol indophenol at 600 nm was reported to be 1.91×10^7 $cm^2 \cdot mol^{-1}$ (9).

RESULTS

Production of PQQ by methanol-utilizing bacteria. Almost all bacteria tested produced PQQ extracellularly; amounts (in micrograms per milliliter) were as follows: *Methylobacillus* strains, 0.07 to 0.42; *Methylophilus* strains, 0.16 to 0.23; *Methylobacterium* strains, 0.34 to 0.75; *Ancylobacter* strains, 0.6 to 1.0; *Hyphomicrobium* strains, 0.3 to 0.9; *Xanthobacter* strains, 0.65 to 1.0; *Acidomonas* strains, below 0.01; *Paracoccus* strains, 0.25 to 0.9; *Thiobacillus* strain, 2.2; *Methylophaga* strains, 3.0 to 0.6; *Mycobacterium* strains, below 0.01. Three *Hyphomicrobium* strains (TK 0414, TK 0415, and TK 0441), *Thiobacillus novellus*, and *Methylophaga* strains produced large quantities of PQQ. *Hyphomicrobium* sp. strain TK 0441, which was selected on the basis of the production of PQQ and growth rate, was used for the study of PQQ production by using a jar fermentor.

TABLE 2. Effects of trace element components in the medium on the production of PQQ

Amt of each trace element solution added to medium (ml/liter)[a]					Cell concn (OD_{660}[b])	PQQ concn in broth (μg/ml)
Ca	Fe	Zn	Mn	Cu		
10	1	1	1	1	18.6	158
1	10	1	1	1	32.2	9
1	1	10	1	1	26.3	206
1	1	1	10	1	21.5	152
1	1	1	1	10	17.3	127
1	1	1	1	1	13.3	154
10	10	10	10	10	54.3	5

[a] The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. Cultivation was for 10 days. Trace element solutions were as follows: Ca, $CaCl_2 \cdot 2H_2O$ (3 mg/ml); Fe, $FeC_6H_5O_2 \cdot XH_2O$ (3 mg/ml); Zn, $ZnSO_4 \cdot 7H_2O$ (2 mg/ml); Mn, $MnSO_4 \cdot 7H_2O$ (0.1 mg/ml); Cu, $CuSO_4 \cdot 5H_2O$ (0.1 mg/ml).

[b] OD_{660}, optical density at 660 nm.

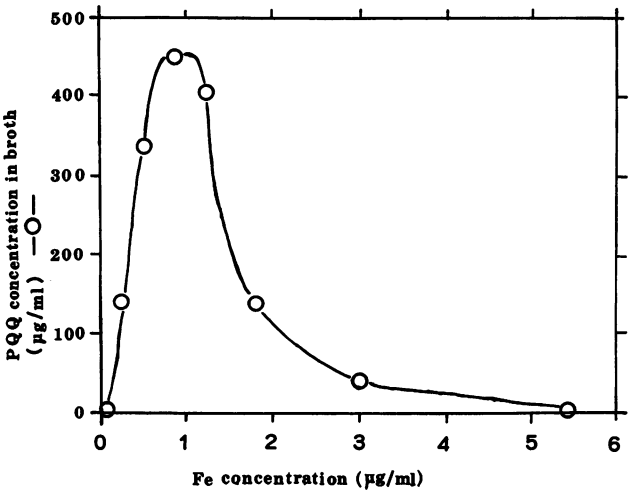

FIG. 2. Effect of Fe concentration on the production of PQQ. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. Cultivation was for 10 days.

Batch cultivation of *Hyphomicrobium* sp. strain TK 0441. Batch cultivation was done with a 30-liter jar fermentor. The temperature of the culture broth was controlled at 30°C and the concentration of methanol in the culture broth was maintained at between 0.1 and 0.2% by weight, but the pH of the culture broth was not controlled. The pH of the broth decreased from 7.0 in proportion to cell growth, and growth was stopped when the pH reached below pH 5.0 (Fig. 1). Cell growth reached an optical density of approximately 3 at 660 nm at 2 days of cultivation, and afterward it decreased gradually. On the other hand, PQQ was produced in the broth linearly from when cell growth was stopped and reached 16 μg/ml at 10 days of cultivation. Methanol dehydrogenase activity in the cells varied during the growth phase, maximized at approximately 0.45 U/mg of protein during exponential growth, and decreased to below 0.1 U/mg of protein at 10 days of cultivation, when the production of PQQ had already stopped.

Effect of the amount of trace elements added to the medium on the production of PQQ. For the purpose of increasing the production of PQQ, the culture conditions were studied. During cultivation with the pH controlled at 6.8 to 7.0, the concentration of cells increased but the amount of PQQ in the broth was approximately 5 μg/ml, as shown in Table 1. On the other hand, when the amount of the trace elements added to the medium was decreased, the concentration of cells decreased but the amount of PQQ increased. After the addition of 1/10 to 1/5 the quantity of the trace elements (1 to 2 ml of the trace element solution per 1 liter of medium), the amount of PQQ reached approximately 150 μg/ml, as shown in Table 1. Cell growth was inhibited by a deficiency in the trace elements, and approximately a quarter of the optical density at 660 nm was reached. The concentrations of PQQ in the cells and extracellularly (broth) increased linearly with the cultivation time and reached approximately 70 μg/g of dry cells and approximately 150 μg/ml of broth, respectively, at 10 days of cultivation. The activity of methanol dehydrogenase in the cells maximized at 2.5 U/mg of protein and

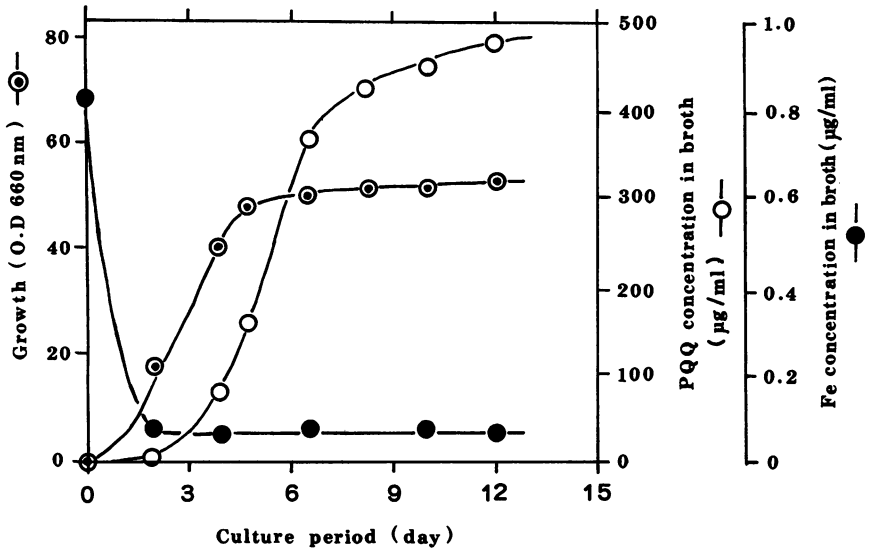

FIG. 3. Production of PQQ by Fe-limiting culture. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. The Fe concentration was 1 μg/ml. $O.D_{660nm}$, optical density at 660 nm.

stayed at approximately 1.5 U/mg of protein at 10 days of cultivation.

Effects of trace element components on the production of PQQ. The amount of PQQ was increased by using the medium containing 1/10 the quantity of the trace elements, and then the effect of each trace element component was studied. Cultivations with medium containing 1/10 the quantities of four of the five components (Ca, Fe, Zn, Mn, and Co) and the full quantity of the other component were done for 10 days. The amount of PQQ was small only with the medium containing the full quantity of Fe, and its value was nearly equal to that obtained with the medium containing the full quantities of all five trace elements, as shown in Table 2. These results clearly show the significance of Fe added to the medium.

Effect of the amount of Fe added to the medium on the production of PQQ. The amount of PQQ increased as the amount of Fe added to the medium was decreased. Therefore, the effect of the amount of Fe added on the production of PQQ was studied. The amount of PQQ maximized at approximately 500 μg/ml after approximately 1 μg of Fe per ml was added, as shown in Fig. 2. The time course of the cultivation with 1 μg of Fe added per ml is shown in Fig. 3. Cell growth was inhibited by the deficiency of Fe and reached an optical density of approximately 50 at 660 nm after 5 days of cultivation. The residual Fe in the broth decreased to below 0.1 μg/ml after 2 days of cultivation. On the other hand, the production of PQQ in the broth commenced at 2 days of cultivation and reached approximately 500 μg/ml after 10 days of cultivation.

Effect of the amount of Mg added to the medium on the production of PQQ and protein. Small amounts of impure substances in the broth are desirable to facilitate purification of PQQ from broth. The effect of the amount of Mg added to

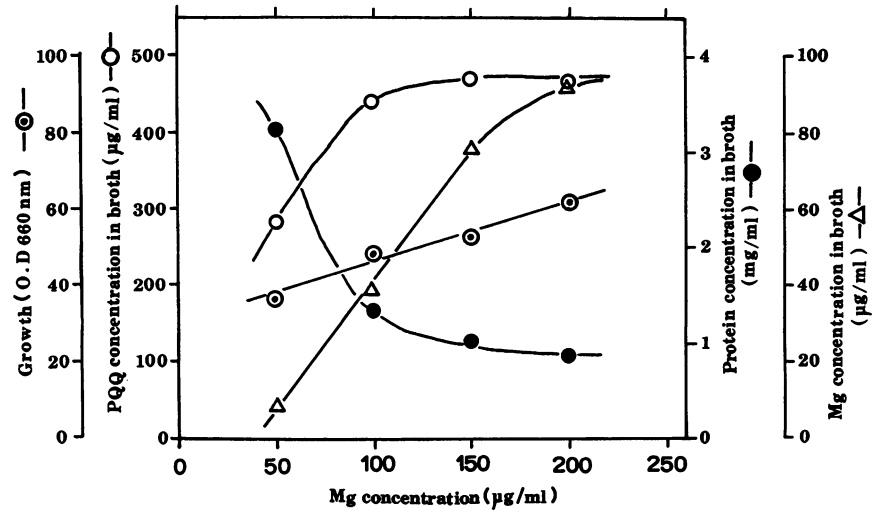

FIG. 4. Effect of Mg concentration on the production of PQQ and protein. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. The Fe concentration was 1 μg/ml, and the cultivation was for 10 days. $O.D_{660nm}$, optical density at 660 nm.

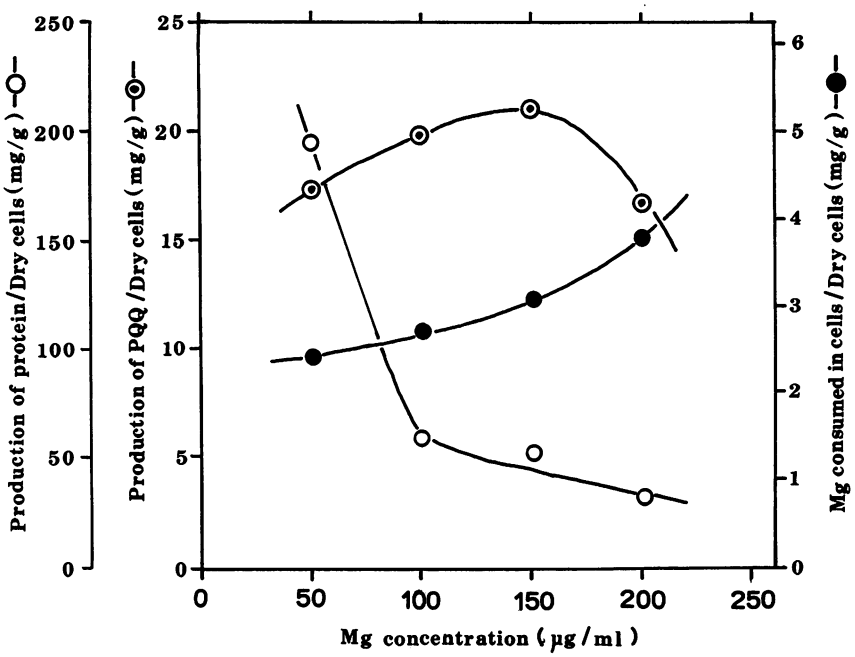

FIG. 5. Effect of Mg concentration on the production of PQQ and protein in cells. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. The Fe concentration was 1 μg/ml, and cultivation was for 10 days.

the medium on the amounts of PQQ and protein in the broth was studied. Cell growth and the amounts of PQQ and protein were affected by the amount of Mg added, as shown in Fig. 4. The amount of PQQ was approximately 500 μg/ml after 100 to 200 μg of Mg was added per ml, but it was approximately 300 μg/ml after 50 μg of Mg was added per ml. On the other hand, the amount of protein decreased after the amount of Mg added to the medium was diminished, and it was approximately 1 mg/ml after 100 to 200 μg of Mg per ml was added. The residual Mg in the broth decreased after the amount of Mg added to the medium was diminished and was below 10 μg/ml after 50 μg of Mg per ml was added. The production of PQQ and protein per gram of dry cells and the consumed Mg in the cells are shown in Fig. 5. The production of PQQ maximized at approximately 20 mg/g of dry cells after 100 to 150 μg of Mg per ml was added, and the production of protein minimized at approximately 50 mg/g of dry cells after 100 to 200 μg of Mg per ml was added. On the other hand, the consumed Mg in the cells increased with the amount of Mg added to the medium. Therefore, the desired amount of Mg added to the medium is 100 to 150 μg/ml on the basis of the production of PQQ and the purification of PQQ from the broth.

Effects of the amounts of other trace elements added to the

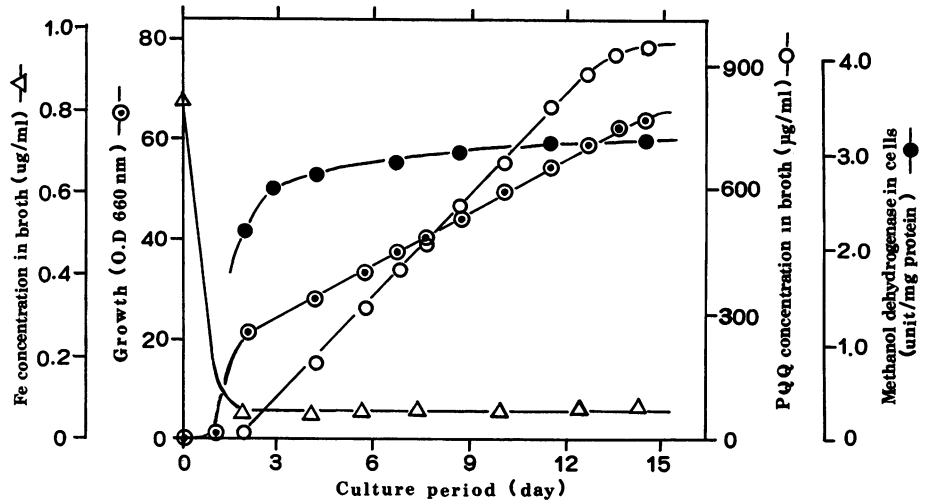

FIG. 6. Time course of growth, production of PQQ, methanol dehydrogenase activity in cells, and Fe concentration in broth with *Hyphomicrobium* sp. strain TK 0441. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. The Fe concentration was 1 μg/ml, and the Mg concentration was 150 μg/ml. $O.D_{660nm}$, optical density at 660 nm.

TABLE 3. Effects of trace element components in the medium on the production of PQQ

Amt of trace element added[a]					Cell concn (OD_{660}[b])	PQQ concn in broth (μg/ml)
Ca	Fe	Zn	Mn	Cu		
1	1/10	1/10	1/10	1/10	18.6	158
1/10	1	1/10	1/10	1/10	32.2	9
1/10	1/10	1	1/10	1/10	26.3	206
1/10	1/10	1/10	1	1/10	21.5	152
1/10	1/10	1/10	1/10	1	17.3	127
1/10	1/10	1/10	1/10	1/10	13.3	154
1	1	1	1	1	54.3	5

[a] 1/10 indicates that 1/10 the full quantity (1) was used. The temperature was 30°C, the pH was 7.0, and methanol was at 0.1 to 0.2% by weight. Cultivation was for 10 days. The trace element solution consisted of $CaCl_2 \cdot 2H_2O$ (30 mg/liter), $FeC_6H_5O_2 \cdot XH_2O$ (30 mg/liter), $ZnSO_4 \cdot 7H_2O$ (20 mg/liter), $MnSO_4 \cdot 7H_2O$ (1 mg/liter), and $CuSO_4 \cdot 5H_2O$ (1 mg/liter).

[b] OD_{660}, optical density at 660 nm.

medium on the production of PQQ. The effects of other trace elements (I, Mo, Co, B, and Na) on the production of PQQ were studied. The medium used contained 0.01 μg of KI per ml, 0.01 μg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per ml, 0.01 μg of $CoCl_2 \cdot 6H_2O$ per ml, 0.02 μg of H_3BO_3 per ml, and 1.0 μg of NaCl per ml. The amount of Fe added was 1 μg/ml (5 μg of $FeC_6H_5O_7 \cdot XH_2O$ per ml), and the amount of Mg added was 150 μg/ml (1.5 mg of $MgSO_4 \cdot 7H_2O$ per ml). Cell growth was inhibited by the deficiency of Fe, increased linearly after 2 days of cultivation, and reached an optical density of approximately 70 at 660 nm after 14 days of cultivation, as shown in Fig. 6. The concentration of residual Fe in the broth decreased to below 0.1 μg/ml at 2 days of cultivation. On the other hand, the production of PQQ in the broth commenced at 2 days of cultivation and reached approximately 1 mg/ml at 14 days of cultivation. The activity of methanol dehydrogenase in the cells was almost constant (approximately 3 U/mg of protein) between days 3 and 14 of cultivation, and the amount of protein in the broth was approximately 1 mg/ml at 14 days of cultivation.

DISCUSSION

All methanol-utilizing bacteria used in this study produced PQQ extracellularly when methanol was used as the carbon and energy sources, but the amounts of PQQ in the broth differed among the bacteria. These bacteria metabolized methanol by using methanol dehydrogenase with PQQ as the cofactor. Of note, three *Hyphomicrobium* strains, *Thiobacillus novellus*, and *Methylophaga* strains produced large amounts of PQQ extracellularly. These amounts were almost equal to those of *Pseudomonas* sp. strain M5 in a study by Ameyama et al. (1). The three *Hyphomicrobium* strains seem to belong to a new species on the basis of morphological and physiological characteristics (21) and DNA-DNA homology (unpublished data). *Hyphomicrobium* sp. strain TK 0441, which was selected on the basis of its production of PQQ and growth rate, was examined for PQQ production by cultivation with a jar fermentor. The amount of PQQ in the broth could be increased by simply decreasing the amount of Fe added to the medium (Table 3). During the culture under this condition, the level of methanol dehydrogenase activity in the cells remained high. The release of regulation in the biosynthesis of PQQ by the deficiency of Fe seems to be the cause of the high production of PQQ. It is not explained how the concentration of Fe salt could effect the biosynthesis of PQQ, but these results seem to be useful for explaining the biosynthesis of PQQ. Moreover, the discrepancy between synthesis of PQQ and methanol dehydrogenase is remarkable. The extracellular protein production, which interfered with the purification of PQQ, was decreased by simply increasing the amount of Mg added to the medium. Suitable medium that contained 1 μg of Fe per ml, 150 μg of Mg per ml, and the trace elements was developed. In this medium, the production of PQQ reached approximately 1 mg/ml and the protein formation was low.

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