Utilization of Hydrocarbons by Microorganisms

Part III. Effects of Organic Nutrients, Mineral Salts and Other Factors on the Accumulation of Vitamin B₂[†]

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The accumulation of vitamin B₂ by *Pichia guilliermondii* Wickerham grown on hydrocarbon was investigated. Addition of the following materials stimulated vitamin B₂ production: metal ions such as ferrous, cobalt, manganese, and calcium ions; organic nutrients such as yeast extract and casamino acid; amino acids such as proline and arginine; vitamins such as B₁, nicotinic acid, inositol, and *p*-aminobenzoic acid. Optimal aeration rate for vitamin B₂ production was obtained in a 500-ml shaking flask containing 75 ml of the medium.

It is well known that riboflavin is produced by microorganisms, especially *Eremothecium ashbyii*,¹⁾ *Ashbya gossypii*,^{1,2)} *Clostridium acetobutylicum*,^{3,4)} *Candida guilliermondii*,⁵⁾ *Candida flareri*,⁶⁾ *Mycocandida riboflavina*,⁷⁾ and *Candida rubusta*,⁸⁾ and some of them are employed for commercial production of riboflavin.

Recently, it was reported that riboflavin could also be produced by several microorganisms such as *Eremothecium ashbyii*,⁹⁾ *Pichia* species¹⁰⁾ in hydrocarbon fermentation.

In the previous paper,¹¹⁾ it was reported that *Pichia guilliermondii* Wickerham isolated from soil produced much riboflavin in a

hydrocarbon medium, and the effect of culture conditions on the growth of this yeast and its vitamin B₂ production were also reported.

In this paper, a remarkable ability of *Pichia guilliermondii* Wickerham for vitamin B₂ production, and the effect of various culture conditions on the growth and vitamin B₂ production are reported.

MATERIALS AND METHODS

Microorganism. The organism used in this study was *Pichia guilliermondii* Wickerham isolated from soil by the authors. This organism was maintained on hydrocarbon agar slant.

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1) A. Guilliermond, M. Fontaine and A. Raffy, *G. R. Acad. Sci. Paris*, **201**, 1077 (1935).

2) L. J. Wickerham, M. H. Flickinger and R. M. Johnston, *Arch. Biochem.*, **9**, 95 (1946).

3) I. Yamasaki and W. Yoshitome, *Biochem. Z.*, **297**, 398 (1938).

4) I. Yamasaki, *ibid.*, **300**, 160 (1939).

5) P. R. Burkholder, *Proc. Natl. Acad. Sci. U. S.*, **29**, 166 (1938).

6) F. W. Tanner, C. Voinovich and J. M. Van Lanen, *Science*, **101**, 180 (1945).

7) J. E. McClarey, *U. S. Patent*, **2**, 537, 148 (1951).

8) S. Takao, *Agr. Biol. Chem.*, **28**, 559 (1964).

9) T. Sato, A. Maekawa, T. Suzuki and Y. Sahashi, *Vitamins (Japan)*, **34**, 542 (1966).

10) Y. Suzuki, T. Matsubayashi and T. Mochizuki, Abstract of papers, Annual Meeting of the Agricultural Chemical Society of Japan, p. 204 (1966).

11) N. Nishio and T. Kamikubo, *J. Ferment. Technol.*, **48**, 1 (1970).

Cultivation methods. Cells, grown on hydrocarbon-agar-slant at 30°C for 4 days, were inoculated into a test tube containing 10 ml of medium (Table I), and then cultured on a shaker (190 strokes/min) at 27°C for 2 days. After centrifugation, the cells were suspended in sterilized water, and transmittance of the cell suspension was adjusted to approximately 14% at 670 m μ . Two ml of the suspension was inoculated into 100 ml of medium in a 500 ml shaking flask and cultured on a reciprocal shaker (115 strokes/min) at 27°C.

TABLE I. COMPOSITION OF BASAL MEDIUM USED

Hydrocarbon (Paraffin mix.)	75 g
NH ₄ H ₂ PO ₄	3.75 g
(NH ₄) ₂ SO ₄	3.75 g
KH ₂ PO ₄	2.5 g
K ₂ HPO ₄	2.5 g
MgSO ₄ ·7H ₂ O	1.0 g
Tween 60	0.5 g
Tap water	1,000 ml
pH	6.5

Hydrocarbon. A hydrocarbon mixture containing *n*-undecane, 1.4%; *n*-dodecane, 7.9%; *n*-tridecane, 47.8%; and *n*-tetradecane, 40.8% by weight was supplied from Idemitsu Kosan Co. and used sole carbon source.

Hydrocarbon concentration in the culture medium was 7.5% (w/v) unless otherwise stated.

Analysis. Riboflavin was estimated fluorophotometrically as described in the previous paper. Other analytical procedures were also as in the previous paper.

RESULTS

Effects of various factors on the growth and vitamin B₂ production were examined.

1) Metal ions

Four kinds of metal ions in a form of sulfate hydride were tested. The results obtained after 10 day culture are shown in Table II. Ferrous ion seems to be necessary for the better growth and somewhat effective for promoting riboflavin production at con-

TABLE II. EFFECT OF METAL IONS ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Metal ions	Amt. added (μ g/liter)	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
			Super-natant (mg/liter)	Cells (μ g/g dry cells)
Fe ²⁺ (FeSO ₄ ·7H ₂ O)	2000	6.17	1.6	2
	200	6.55	83.3	8
	20	4.41	93.3	69
	2	4.10	88.4	60
	0.2	4.18	64.0	31
Co ²⁺ (CoSO ₄ ·7H ₂ O)	10000	2.46	81.8	268
	1000	3.78	117.3	95
	100	6.61	123.8	148
	10	4.36	57.4	89
Mn ²⁺ (MnSO ₄ ·4H ₂ O)	20000	4.83	58.2	9
	2000	4.11	84.2	12
	200	6.36	125.6	14
	20	4.26	120.0	96
Ca ²⁺ (CaSO ₄ ·2H ₂ O)	50000	5.35	151.0	18
	5000	5.20	148.4	49
	500	3.39	67.8	103
	50	3.78	72.8	97
Blank A ^{a)}	—	6.71	192.0	16
Blank B ^{b)}	—	6.21	57.0	16

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

^{a)} Tap water was used in A.

^{b)} Deionized water was used in B.

The conc. of hydrocarbon was 3.75% (w/v).

centration of 2~200 μ g per liter, but the addition of iron at 2 mg per liter gave an inhibitory effect on riboflavin production. Cobalt, manganese, and calcium ions stimulated the vitamin production and their optimal concentrations were 0.1, 0.2, 50 mg per liter, respectively. However, in each case, the vitamin production was lower than in Blank A, in which tap water was used in stead of deionized water.

2) Organic nutrients

Since the composition of medium used in these studies was simple, it was expected that a certain organic substance would have a good effect on the growth of yeast as well as vitamin B₂ production. As shown in Table III, 0.05~0.1% of yeast extract and casamino acid stimulated the vitamin production, but

TABLE III. EFFECT OF ORGANIC NUTRIENTS ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Organic nutrients	Amt. added (g/liter)	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
			Super-natant (mg/liter)	Cells (μg/g dry cells)
Yeast extract	0.1	7.58	114.9	42
	0.5	7.89	187.0	33
	1.0	8.10	200.0	9
Casamino acid	0.1	7.66	105.0	11
	0.5	7.57	252.0	18
	1.0	7.29	280.5	51
Poly peptone	0.1	7.41	91.0	15
	0.5	8.46	172.6	12
	1.0	8.63	80.0	9
Malt extract	0.1	7.10	86.7	5
	0.5	7.41	84.0	16
	1.0	7.38	105.0	11
Meat extract	0.01	7.64	103.2	18
	0.1	7.74	126.0	8
None	—	7.14	150.0	109

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

The conc. of hydrocarbon was 3.75% (w/v).

other organic nutrients showed no effect on the vitamin production, although these stimulated the growth of yeast.

3) Amino acids

Since yeast extract and casamino acid showed some stimulating effect on the vitamin production as mentioned above, the effect of some organic substances on the production of vitamin B₂ was investigated. In this study, various amino acids were added to the medium at a relatively low concentration (1 mmole/liter). As shown in Table IV, arginine and proline slightly stimulated the vitamin production.

4) Vitamins

Among eight water-soluble vitamins tested, B₁, nicotinic acid, inositol, and *p*-aminobenzoic acid showed a good effect on vitamin B₂ production by *Pi. guilliermondii*, as shown in Table V, whereas vitamin B₆, biotin, pantothenic acid, and folic acid were ineffective.

TABLE IV. EFFECT OF AMINO ACIDS ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Amino acid	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
		Super-natant (mg/liter)	Cells (μg/g dry cells)
L-Glycine	7.00	33.9	28
L-Leucine	6.86	62.4	20
D,L-Isoleucine	6.71	125.8	40
L-Cystein	6.02	118.0	22
L-Cystine	6.05	38.5	12
D,L-Methionine	7.07	180.1	17
L-Tyrosine	6.68	84.1	10
L-Arginine·HCl	6.12	247.2	60
L-Histidine·HCl·H ₂ O	6.02	79.5	65
L-Hydroxyproline	6.45	222.2	14
D,L-Tryptophan	6.17	76.2	29
L-Glutamic acid	7.01	73.8	14
L-Asparagine	7.60	48.7	13
D,L-Alanine	6.57	171.9	49
D,L-Valine	6.31	145.2	48
None	6.42	213.9	53

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

Each amino acid was added at 1 mmole/liter.

The conc. of hydrocarbon was 3.75% (w/v).

TABLE V. EFFECT OF WATER SOLUBLE VITAMINS ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Vitamin	Amt. added (μg/liter)	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
			Super-natant (mg/liter)	Cells (μg/g dry cells)
Vitamin B ₁	50	5.82	200.0	17
Vitamin B ₆	50	7.33	130.0	39
Nicotinic acid	50	6.79	174.2	54
Biotin	1	7.27	133.3	17
Ca-pantothenate	50	7.12	111.1	10
PABA	50	6.74	158.2	16
Folic acid	1	7.24	112.5	18
Inositol	5000	7.36	167.9	47
All factors	—	6.57	170.1	40
None	—	6.77	117.3	36

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

The conc. of hydrocarbon was 3.75% (w/v).

TABLE VI. EFFECT OF INORGANIC SALTS ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Inorganic salts				Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
KH ₂ PO ₄	K ₂ HPO ₄	Na ₂ HPO ₄	NaH ₂ PO ₄		Supernatant (mg/liter)	Cells (μg/g dry cells)
Amounts of added (g/liter)						
2.5	—	—	—	7.72	123.5	18
5.0	—	—	—	9.44	160.4	40
—	2.5	—	—	7.83	118.0	43
—	5.0	—	—	8.70	202.8	67
—	—	2.5	—	0.98	0.2	112
—	—	5.0	—	0.99	0.1	111
—	—	—	2.5	1.19	0.2	294
—	—	—	5.0	1.23	0.2	400
2.5	2.5	—	—	9.51	171.4	7
4.0	1.0	—	—	9.19	105.6	46
1.0	4.0	—	—	9.04	162.2	44
2.5	—	2.5	—	8.62	82.6	8
4.0	—	1.0	—	9.41	152.0	74
1.0	—	4.0	—	7.75	111.1	14
—	2.5	2.5	—	7.69	17.7	8
—	4.0	1.0	—	9.10	159.2	25
—	1.0	4.0	—	7.16	131.3	22

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

5) Inorganic salts

Four kinds of salts were tested to see if there might be the influence of buffer action of pH due to inorganic salts in the medium on vitamin B₂ production. As shown in Table VI, maximal vitamin B₂ production was obtained when dipotassium hydrophosphate was added at the concentration of 5 g per liter.

6) Substances related to nucleic acids

It is suggested that a large amount of the vitamin production is concerned with abnormality of nucleic acid metabolism. Therefore, it was expected that addition of a purine base would stimulate vitamin B₂ production. The results are shown in Table VII. Addition of 20 mg per liter of every substance tested, especially inosine, stimulated the vitamin production.

7) Aeration

In hydrocarbon fermentation, aeration is an

TABLE VII. EFFECT OF PURINES ON THE GROWTH AND VITAMIN B₂ PRODUCTION

Purines	Amt. added (mg/liter)	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
			Super- natant (mg/liter)	Cells (μg/g dry cells)
Adenine	5	9.05	140.3	25
	20	8.60	154.1	40
	50	8.86	137.5	56
Xanthine	5	9.20	128.4	50
	20	8.18	153.8	45
	50	8.61	137.1	50
Inosine	5	9.03	186.7	45
	20	7.69	246.8	42
	50	9.21	180.4	34
None	—	8.73	142.8	35

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

The conc. of hydrocarbon was 3.75% (w/v).

important factor for microbial growth as well as emulsification of the substrate. Growth and vitamin B₂ synthesis were also affected by aeration, as shown in Table VIII.

TABLE VIII. RELATIONSHIP BETWEEN OXYGEN SUPPLY AND VITAMIN B₂ PRODUCTION

Amt. of medium in 500 ml flask (ml)	Kd ($\times 10^{-6}$)	Cell yield (g dry wt./liter)	Vit. B ₂ produced in	
			Super-natant (mg/liter)	Cells (μ g/g dry cells)
25	11.0	7.28	132.6	12
50	8.4	8.01	132.0	23
75	6.5	7.22	223.3	53
100	4.7	8.43	166.3	38
125	3.4	8.06	15.8	27,698
150	3.0	4.31	10.9	29,510

Cultivation was carried out on a reciprocal shaker for 10 days at 27°C.

Vitamin B₂ content in the supernatant fraction was maximal in the case of a 500-ml flask containing 75 ml of the medium, and it extremely decreased when the volume of medium was more than 100 ml although the vitamin per cell was remarkably increased.

8) Relationship between dry cell weight and the concentration of growing cells

Concentrations of growing cells indicated as unit of optical density (abbreviated as UOD) was determined as follows: optical density of the cell suspension was adjusted to approximately 0.3 at 570 m μ by diluting

FIG. 1. Relationship between Dry Cell Weight and Cell Concentration.

with water. Subsequently, UOD was determined by the product of diluted volume and OD. As shown in Fig. 1, a linear relation was obtained between cell yield and UOD of the culture.

9) pH control

Figure 2 shows the time course of vitamin B₂ production and the growth of yeast. Since pH of the culture broth lowered in the log phase, it was adjusted to 6.5 with sodium hydroxide. As shown in Fig. 3, when pH of the broth was adjusted to 6.5, the growth of yeast was extremely increased, while vitamin B₂ production was decreased.

DISCUSSION

Some flavinogenic microorganisms are well-known to be strongly inhibited in respect to

FIG. 2. Time Course of Vitamin B₂ Production and the Growth of Yeast.

●—●, Total vit. B₂ produced; ○—○, Cell concentration; ×—×, pH of broth.

FIG. 3. Effect of pH Control on the Growth and Vitamin B₂ Production.

●—●, Total vit. B₂ produced; ○—○, Cell concentration; ×—×, pH of broth; ↑, pH was adjusted at each time with 30% NaOH.

riboflavin production when iron is present in the media; for example, two genera, *Clostridium* and *Candida*, are quite sensitive to this metal. In hydrocarbon fermentation by *Pichia*

guilliermondii Wickerham, it was observed that riboflavin production was sharply reduced with increasing concentration of iron.

In hydrocarbon fermentation, aeration is an important factor for the microbial growth as well as emulsification of the substrate. Growth and vitamin B₂ synthesis of *Pi. guilliermondii* Wickerham were also affected by aeration as shown in Table VIII. Vitamin B₂ content in the supernatant fraction was maximal in the case of a 500-ml flask containing 75 ml of the medium and extremely decreased when the volume of medium was more than 100 ml, although the vitamin per cell was extremely increased in the latter case. Probably, when the volume of medium was increased, the growth of yeast was so slow that almost all vitamin B₂ kept in the cells during 8 day culture.

pH of the culture broth became lower in the log phase, and adjusting pH to 6.5 increased the growth of yeast. Yeast could be grown at relatively low pH as compared with bacteria, but at an extremely low pH, the growth activity of the yeast must be reduced. The adjustment of pH to 6.5, however, significantly decreased vitamin B₂ production in hydrocarbon fermentation by *Pi. guilliermondii* Wickerham.

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