Riboflavin Biosynthesis by *Candida robusta*

Part I. Identification of Riboflavin and Several Conditions for Its Production

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During the study on the oxidative sugar metabolism of yeasts, it was found that six strains of *Candida robusta*, isolated from fruits, produced large amounts of yellow pigment in shaking culture and this pigment was identified as riboflavin. Riboflavin production by *C. robusta* has never been reported.

Some notable characteristics of *C. robusta* in riboflavin production were found. As nitrogen sources, ammonium salts and urea were favorable, but nitrate and organic nitrogen sources such as glycine, asparagine and peptone were not utilized for riboflavin production. Riboflavin was not produced in still culture; a highly aerobic condition, as may be obtained by shaking culture, was essential. The addition of excess CaCO_3 was also necessary. Acetic acid, added as the Ca salt in its production as a sole carbon source, was more effective than sugars and optimum concentration of this acid was 7%. Riboflavin were obtained in yields as high as 32 to 34 mg % from the acetate medium after 8 days.

INTRODUCTION

The sugar metabolism of yeast has well been studied in regard to the anaerobic pathway, namely, alcoholic fermentation, but there are fewer reports on the oxidative metabolism including ester or acid productions. However, yeasts are closely related to molds which are concerned with a number of oxidative fermentations. Therefore, when yeasts are cultured under the aerobic condition, it is considered that different kinds of oxidative metabolism will be carried out according to their species as in cases of molds.

From these points, many kinds of yeasts were cultured aerobically to compare their metabolic features and to detect useful products. As a result, some strains of the genus

Candida were discovered, which were able to produce a large amount of gluconic acid from glucose^{1,2)}

During further experiments, it was found that several strains of *Candida robusta* produced large amounts of yellow pigment in their media in shaking culture. This yellow pigment was identified as riboflavin.

It is well known that riboflavin is produced by microorganisms, especially *Eremothecium ashbyii*³⁾, *Ashbya gossypii*^{3,4)}, *Clostridium acetobutylicum*^{5,6)}, *Candida guilliermondii*⁷⁾, *Candida flaveri*⁸⁾, *Mycocandida riboflavina*⁹⁾,

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

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

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

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

7) P.R. Burkholder, *Proc. Natl. Acad. Sci. U.S.*, **29**, 166 (1943).

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

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

1) Y. Sasaki and S. Takao, *J. Agr. Chem. Soc. Japan*, **32**, 576 (1958).

2) S. Takao, *ibid.*, **32**, 872 (1958); **34**, 214, 218, 406 (1960).

and some of them are employed for commercial production of riboflavin.

However, riboflavin production by *C. robusta* has never been reported. In addition to its remarkable riboflavin biosynthetic capacity, this species of yeast has some quite different production characteristics from other riboflavin-producing microorganisms. This report deals with some conditions and peculiarities for the production of riboflavin by *C. robusta*.

MATERIALS AND METHODS

Microorganisms.

One hundred and seven strains belonging to fifty species of yeasts used for the oxidative sugar metabolism were all obtained from the Laboratory of Applied Microbiology, Hokkaido University.

Among them, six strains of *Candida robusta*, No. 1 to 6, capable of producing riboflavin were isolated from fruits and identified by Sasaki and Yoshida¹⁰⁾ at this laboratory.

Cultivation of Organisms.

All strains were grown on potato glucose agar slants containing 5% of malt root extract for 3 days at 30°C. They were inoculated in 50 ml of the following medium in the 500-ml shake flasks, then cultured on a reciprocating shaker at 30°C for 3 days; 10% glucose, 0.2% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5% CaCO_3 , and 0.09% NH_4Cl or 0.2% peptone.

Paper chromatography.

The following solvent systems¹¹⁾ were used for paper chromatography of the yellow pigment: A) *n*-butanol : acetic acid : water (4:1:5), B) *n*-butanol : formic acid : water (77:10:13), and C) *n*-butanol : phenol : water (3:16:10).

Isolation of Yellow Pigment.

The yellow pigment was isolated from the broth of *C. robusta* in the manner described by Saito and Takata¹²⁾.

Determination of Riboflavin.

The riboflavin content of the broth was determined

fluorophotometrically by using Hitachi photoelectric spectrophotometer.

RESULTS

1. Production of Yellow Pigment by *C. robusta*.

During the study of the oxidative sugar metabolism of yeasts using one hundred and seven strains, it was found that the culture media containing NH_4Cl as a nitrogen source inoculated with six strains of *C. robusta* became increasingly yellow with a greenish yellow fluorescence.

The broths of six strains of *C. robusta* were then chromatographed on paper in solvent A. When the chromatogram was observed under ultra violet light, the R_F value of this yellow pigment was very similar to those obtained from the riboflavin solution and the broth of *Eremothecium ashbyii*. This pigment was, therefore, presumed to be riboflavin.

2. Some Fundamental Conditions for the Yellow Pigment Production.

In the previous experiment, the yellow pigment was produced in the medium containing NH_4Cl , but not produced with peptone. Accordingly, nitrogen sources which appeared to be a very important factor for the pigment production, were examined. The concentrations of all nitrogen sources corresponded to a nitrogen level of 0.09% of NH_4Cl . At the same time, glucose was tested at three concentrations: 2, 5 and 10%. Five per cent of CaCO_3 was added in some cases. The media in flasks were inoculated with one strain, *C. robusta* No. 6, which seemed to be the most superior pigment producer in the previous experiment, and then shaken for 9 days.

As shown in Table I, ammonium salts and urea were favorable, but nitrate and organic nitrogen sources, such as glycine, asparagine and peptone, were not utilized for the pigment production. When CaCO_3 was absent from the medium, no pigment was produced;

10) Y. Sasaki and T. Yoshida, *J. Fac. Agr. Hokkaido Univ.*, **51**, 194 (1959).

11) K. Yagi, "Furabin no Seikagaku", Kyoritsu Press (Tokyo), 1957, p. 94.

12) K. Saito and R. Takata, *Jozogaku Zasshi (in Japanese)*, **20**, 316 (1942).

TABLE I. EFFECT OF NITROGEN SOURCES ON YELLOW PIGMENT PRODUCTION BY *C. robusta*

Nitrogen source	Glucose %	Rate of pigment production			
		1	3	6	9 (days)
NH ₄ Cl	2	++	++	++	++
	5	++	++	++	++
	10	++	++	++	++
(NH ₄) ₂ SO ₄	2	++	+++	+++	++
	5	++	+++	++	++
	10	+	+++	++	++
NH ₄ NO ₃	2	++	+++	+++	++
	5	++	+++	++	++
	10	++	+++	++	++
NaNO ₃	2	—	—	—	—
	5	—	—	—	—
	10	—	—	—	—
Urea	2	±	±	±	—
	5	++	++	++	++
	10	++	++	++	++
Glycine	2	—	—	—	—
	5	—	—	—	—
	10	—	—	—	—
Asparagine	2	—	—	—	—
	5	—	—	—	—
	10	—	—	—	—
Peptone	2	—	—	—	—
	5	—	—	—	—
	10	—	—	—	—

TABLE II. SOME CONDITIONS FOR PIGMENT PRODUCTION

Carbon source	(NH ₄) ₂ SO ₄ %	CaCO ₃ %	Pigment production		
			1	2	3 (days)
Glucose	0.05	1	—	—	—
		5	++	++	++
	0.1	1	—	—	—
		5	++	+++	+++
	0.2	1	—	—	—
		5	++	+++	+++
Fructose	0.05	1	—	—	—
		5	+	±	±
	0.1	1	—	—	—
		5	+	++	++
	0.2	1	—	—	±
		5	++	++	+++
Sucrose	0.05	1	—	—	—
		5	—	±	±
	0.1	1	—	±	±
		5	++	++	++
	0.2	1	—	±	±
		5	+++	+++	+++

thus this table shows only the results obtained from the medium with CaCO₃.

Then the still culture was carried out with

various volumes of the medium containing 0.1% (NH₄)₂SO₄, 2% glucose and 5% CaCO₃ which had been shown to be a favorable composition in the previous test. In this case, however, pigment was not produced even after an incubation period of 30 days. Therefore, it was known that the production of this pigment required highly aerobic conditions, as may be obtained with the shaking culture.

As a further preliminary experiment, 2% of fructose or sucrose, instead of glucose, in combination with three levels of (NH₄)₂SO₄ in the media were tested. Sucrose was found to be the best for the pigment production as shown in Table II. When 1% of CaCO₃ was added, very little pigment was produced, hence the addition of excess CaCO₃ must be necessary for the pigment production.

3. Isolation and Identification of Riboflavin.

As the production of yellow pigment was enhanced under the above altered conditions, this pigment was then isolated and identified. The pigment could be extracted with chloroform when it was decomposed by light in alkaline solution and then acidified. It was stable to oxidants, such as potassium permanganate. The yellow color and fluorescence were instantaneously discharged upon the addition of sodium hydrosulphite, but they reappeared on shaking. Based on these properties, this pigment was considered to be flavin. Yellow needle crystals isolated according to Saito and Takata's method had absorption maxima at 266, 365, 373 and 442 mμ as measured with the Hitachi spectrophotometer. This absorption spectrum was in good agreement with that of riboflavin.

Although flavinmononucleotide and riboflavinyl glucoside give the same spectrum as riboflavin, two compounds may be separated by paper chromatography¹³⁾. In the solvent

13) L.G. Whitby, *Biochem. J.*, **54**, 437 (1953).

systems of A, B and C, the R_F values of this pigment were 0.42, 0.16 and 0.66, respectively. These paper chromatograms also indicated that this pigment was riboflavin. From the results obtained, this pigment was recognized as riboflavin.

4. Riboflavin Production in the Media Containing Sucrose and $(\text{NH}_4)_2\text{SO}_4$.

C. robusta No. 6 was cultured on the shaker for 3 days in order to determine optimal concentrations of sucrose and $(\text{NH}_4)_2\text{SO}_4$. As shown in Table III, 8 to 9 mg% of riboflavin were produced under suitable conditions and about 7% of sucrose appeared to be most favorable. On the other hand, the riboflavin yield was diminished with increasing concentration of $(\text{NH}_4)_2\text{SO}_4$.

TABLE III. CONCENTRATIONS OF SUCROSE AND $(\text{NH}_4)_2\text{SO}_4$ FOR RIBOFLAVIN PRODUCTION

Sucrose %	$(\text{NH}_4)_2\text{SO}_4$ %	Riboflavin mg %
3	0.3	6.11
	0.5	5.92
	0.7	4.98
	1.0	0
	1.2	0
5	0.3	6.03
	0.5	7.24
	0.7	8.50
	1.0	0
	1.2	0
7	0.3	8.50
	0.5	9.29
	0.7	8.03
	1.0	1.42
	1.2	0
10	0.3	3.20
	0.5	7.75
	0.7	6.25
	1.0	5.12
	1.2	0

5. Effect of Carbon Sources on Yeast Growth and Riboflavin Production.

Fifteen sugars and related substances, 5 alcohols, and 13 organic acids were tested. All of the organic acids were added as Ca salts. Each carbon source was used in 3% concentration in the basal medium containing 0.3% of $(\text{NH}_4)_2\text{SO}_4$. During incubation for

3 days on the shaker, the yeast cells were counted microscopically daily with Thoma's hemacytometer. The resulting data are shown in Fig. 1.

Lactose, dextrin, soluble starch and glycogen were not acceptable for either riboflavin production or yeast growth. On the other hand, maltose, raffinose and inulin were not suitable for the production of riboflavin though they were utilized largely for abundant growth. Sucrose seemed to be the best carbon source and monosaccharides also produced considerable amounts of riboflavin. It was interesting that xylose, which was not available for many microbes, was more suitable than hexose for riboflavin production. Alcohols, except ethanol, were utilized to some extent.

Among the organic acids, formate, butyrate, propionate, tartrate, fumarate, malate, α -ketoglutarate and citrate were not assimilated. Although pyruvate, lactate, succinate and gluconate were found to be utilized for growth, riboflavin was not produced with lactate and succinate, but small amounts were produced from pyruvate and gluconate. Acetate was the most remarkable carbon source, because it was favorable for good growth and was as effective as available sugars for riboflavin production.

6. Optimum Concentration of Acetic Acid for Riboflavin Production.

Acetic acid was added at levels of 3, 6, 9 and 12% as the Ca salt (concentrations of Ca acetate, $\text{Ca}(\text{CH}_3\text{COO})_2 \cdot \text{H}_2\text{O}$, were 4.4, 8.8, 13.2 and 17.6% respectively). In some instances, 5% of CaCO_3 was added to test its necessity for riboflavin production in the acetate-containing medium. Results obtained after 5 days' culture are shown in Table IV. The addition of CaCO_3 was necessary, as in the case of sugar. Six per cent of acetic acid was most effective and over 16 mg% of ribo-

FIG. 1. Effect of Carbon Sources on Yeast Growth and Riboflavin Production

TABLE IV. OPTIMUM CONCENTRATION OF ACETIC ACID (1)

Acetic acid %		Addition of CaCO_3	Riboflavin, mg %
3	{	—	0
		+	4.16
6	{	—	0
		+	16.22
9	{	—	0
		+	5.37
12	{	—	0
		+	0.80

TABLE V. OPTIMUM CONCENTRATION OF ACETIC ACID (2)

Acetic acid %	Rate of riboflavin production					Riboflavin, mg % after 5 days
	1	2	3	4	5 (days)	
3	++	+++	+++	+++	+++	3.86
4	++	+++	+++	+++	+++	5.52
5	+	++	+++	+++	+++	9.08
6	±	++	+++	+++	+++	16.62
7	—	++	+++	+++	+++	23.23
8	—	+	++	++	+++	14.95
9	—	±	++	++	+++	6.00

flavin was produced at this concentration. When riboflavin was obtained in such a high yield many crystals of riboflavin could be

observed in the culture medium.

The results of a further detailed experiment carried out with a range of 3 to 9%

FIG. 2. Optimum Concentration of CaCO₃ in Sucrose Medium

of acetic acid, are shown in Table V. Riboflavin production was initiated early with the lower concentrations of acetate, but the higher concentrations rapidly enhanced its production during the progress of incubation. Finally, 7% of acetic acid gave in the highest yield over 23 mg% of riboflavin after 5 days. From these results, it was considered that acetate was much better than sugar as a carbon source for riboflavin production.

7. Optimum Concentration of CaCO₃.

As shown in Fig. 2, when CaCO₃ concentration was tested in the medium containing 3% of sucrose and 0.3% of (NH₄)₂SO₄, 5% of CaCO₃ was most effective for riboflavin production after 5 days.

On the other hand, there was not a great difference between the riboflavin yields after 8 days in the range from 2.5 to 10% of CaCO₃ in the media containing Ca acetate corresponding to 6 and 7% of acetic acid, as shown in Table VI. In this experiment, however, riboflavin in yields as high as 20 to 24 mg% and 32 to 34 mg% in 6 and 7% concentrations of acetic acid were obtained at the end of

the incubation period.

DISCUSSION

Oxygen is probably an important factor to riboflavin production by microbes, except anaerobes, such as *Cl. acetobutylicum*, because aerobic conditions provided by aeration or shaking are usually very effective, but *E. ashbyii* is able to produce riboflavin even on an agar slant¹²⁾ or in still culture^{3,12,14)}. *A. gossypii*¹⁴⁾ and *C. guilliermondii*^{7,15)} also produce it in still culture.

However, *C. robusta* never produced riboflavin in still culture or on an agar slant even after an incubation period of 30 days. Only in a shake culture was riboflavin produced, so that an intense oxygen requirement appears to be one of the characteristics of riboflavin production by *C. robusta*.

E. ashbyii, *A. gossypii*, *C. guilliermondii* and *C. flarerii* do not need CaCO₃ for riboflavin production. In a case of *Cl. acetobutylicum*, solvent yield is decreased and considerable amounts of riboflavin are produced when 1 to 3% of CaCO₃ is added to the medium⁵⁾, but this bacterium can also produce it without adding CaCO₃ if favorable media are used^{16,17)}. *C. robusta*, however, produces much riboflavin only when excess CaCO₃ is added. A reason of its necessity will be ex-

TABLE VI. EFFECT ON RIBOFLAVIN PRODUCTION OF CaCO₃ CONCENTRATION IN ACETATE MEDIUM

CaCO ₃ %	Riboflavin, mg % after 8 days	
	Acetic, 6%	Acetic, 7%
2.5	20.75	31.50
5.0	24.25	32.75
7.5	23.50	34.38
10.0	24.25	33.25

14) A. Raffy and M. Fontaine, *C.R. Acad. Sci. Paris*, **205**, 100 (1937).

15) P.R. Burkholder, *Arch. Biochem.*, **3**, 121 (1943).

16) Y. Obata and M. Tomoeda, *Vitamins (in Japanese)*, **5**, 55 (1952).

17) K. Imai, *J. Agr. Chem. Soc. Japan*, **29**, 567 (1955).

plained later elsewhere.

Fermentative sugars are well known to be available usually for riboflavin production, but pentose indicates different utilities according to the microbe. *E. ashbyii* produces a small amount of riboflavin from arabinose¹²⁾, but xylose is not available^{12,18)}. *A. gossypii* can not utilize both of them¹⁹⁾. They are available for good growth by *C. guilliermondii*, but not suitable for riboflavin production¹⁵⁾. The addition of small amounts of xylose in a whey-containing medium remarkably stimulates the riboflavin production by *Cl. acetobutylicum*²⁰⁾. However, *C. robusta* differs from those microbes, because xylose is a favorable carbon source, though arabinose is not so available.

The most notable characteristic of *C. robusta* is the ability to synthesize riboflavin from acetate. It has never been reported that riboflavin was produced from acetic acid as a sole carbon source. *E. ashbyii* is unable to utilize acetate for its production^{12,18,21)}. Fur-

thermore, riboflavin production by this mold is inhibited even with a small amount of acetate in the glucose medium²¹⁾. *A. gossypii* is able to oxidize acetate²²⁾, but there is no report on its riboflavin production. Plaut^{23,24)} reported that acetic acid was incorporated into riboflavin molecule by using radioactive acetic acid, but in his experiment acetic acid was added with glucose in the medium.

It is an interesting fact that *C. robusta* is able to synthesize completely riboflavin from media containing simple compounds, such as acetate and $(\text{NH}_4)_2\text{SO}_4$.

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22) M.N. Mickelson and M.N. Schuler, *J. Bact.*, **65**, 297 (1953).

23) G.W.E. Plaut, *J. Biol. Chem.*, **208**, 513 (1954).

24) G.W.E. Plaut, *ibid.*, **211**, 111 (1954).

18) K. Minoura, *Vitamins (in Japanese)*, **6**, 914 (1953).

19) F.W. Tanner, C. Vojnovich and J.M. Van Lanen, *J. Bact.*, **58**, 737 (1949).

20) R.E. Meade, N.E. Rodgers and H.L. Pollard, *U.S. Patent*, 2,433,232 (1947).

21) E.G. Brown, T.W. Goodwin and S. Pendlington, *Biochem. J.*, **61**, 37 (1955).