STUDIES ON DEXTRANSUCRASE

II. Factors Affecting the Formation of Riboflavinylglucoside in Growing Cultures of *Leuconostoc mesenteroides*

Yukio SUZUKI

Riboflavinylglucoside was isolated at first by Whitby (1950, 1952, 1954) from the incubation mixture of riboflavin with homogenates of rat liver and with aqueous extracts of acetone dried powder of rat liver. Not only riboflavinylglucoside, but also various kinds of sugar compounds of riboflavin were obtained by Katagiri, Tachibana and Yamada with enzyme preparations from a mutant of *Aspergillus oryzae* (Katagiri and Tachibana, 1953; Tachibana and Katagiri, 1955), *Escherichia coli* (Katagiri, Imai and Yamada, 1954, 1957) and from *Clostridium acetobutyricum* (Tachibana, 1955).

In the previous paper (Suzuki and Katagiri, 1964), it was found that *Leuconostoc mesenteroides* produced a remarkable amount of riboflavinylglucoside in addition to dextran, when it was grown in a medium containing sucrose and riboflavin. The present paper deals with cultural conditions on the formation of riboflavinylglucoside by the bacterium.

METHODS

Two strains of *Leuc. mesenteroides*, IFO. 3426 and L. 20, were used. The stock cultures were maintained by frequent transfers on agar slants (A) and (B). The compositions of the agar slants are as follows; (A) is a malt-extract agar containing 1% glucose, 0.1% yeast extract, 2% $CaCO_3$ and 2% agar-powder and adjusted pH to 7.0, (B) is a surose agar containing 4% sucrose, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 2 mg% $FeSO_4 \cdot 7H_2O$, 2mg% $MnSO_4$, 0.06% $(NH_4)_2SO_4$, 0.25% yeast extract, 0.05% polypeptone, 4% $CaCO_3$ and 2% agar-powder and adjusted pH to 7.0. To prepare the inoculum, one loopful of the bacterium from an agar subculture was suspended in 10 m*l* of a sterilized seed medium and incubated at 30°C for one day under stationary conditions. About 2 m*l* of this seed culture were used as the inoculum. The estimation of riboflavin-compounds was carried out by the method reported previously (Suzuki and Katagiri, 1964).

RESULTS

1. *Effect of Components of Seed Media*

Two kinds of seed media were used in the preparation of the inoculum and

their components were similar to the stock cultures (A) and (B), but both calcium carbonate and agar-powder were omitting. Fifty m*l* of a medium containing 10% sucrose, 0.5% KH_2PO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.03% $(NH_4)_2SO_4$, 0.05% polypeptone, 0.15% yeast extract and 40 mg% riboflavin adjusted pH to 7.0 with sodium hydroxide were inoculated with 2 m*l* of a 24-hours' culture of *Leuc. mesenteroides* L. 20, grown in the seed medium and then incubated at 30°C for 2 days. At certain intervals portions (5 m*l*) of fermentation liquid were withdrawn and the amount of riboflavinylglucoside produced was determined. It will be seen in Table 1 that the seed medium (B) containing sucrose was much

TABLE 1

Effect of components of seed media

(I). After 6 hours' incubation

Condition	Type of seed medium #	Growth O. D. at 530 mμ	B_2 present (mg/50m*l*)		B_2-glucoside produced (mg/50m*l*)	B_2-oligo saccharides produced (mg/50m*l*)
			initial	final		
Shaking	(B)	0.185	20.0	11.5	6.5	0
Stationary	(B)	0.170	20.0	11.4	7.5	0
Shaking	(A)	0.176	20.0	18.8	+	0
Stationary	(A)	0.169	20.0	18.7	+	0

(II). After 18 hours' incubation

Condition	Type of seed medium #	Growth O. D. at 530 mμ	B_2 present (mg/50m*l*)		B_2-glucoside produced (mg/50m*l*)	B_2-oligo saccharides produced (mg/50m*l*)
			initial	final		
Shaking	(B)	0.311	20.0	2.9	15.6	0.3
Stationary	(B)	0.300	20.0	1.2	14.8	2.9
Shaking	(A)	0.287	20.0	11.8	6.1	1.0
Stationary	(A)	0.268	20.0	11.2	5.8	1.9

(III). After 48 hours' incubation

Condition	Type of seed medium #	Growth O. D. at 530 mμ	B_2 present (mg/50m*l*)		B_2-glucoside produced (mg/50m*l*)	B_2-oligo saccharides produced (mg/50m*l*)
			initial	final		
Shaking	(B)	0.390	20.0	0.5	13.7	4.6
Stationary	(B)	0.379	20.0	0	10.9	7.7
Shaking	(A)	0.388	20.0	+	15.1	3.8
Stationary	(A)	0.392	20.0	+	12.7	6.0

(A): malt-extract medium
(B): sucrose medium

favorable for the formation of riboflavinyl glucoside than the malt-extract medium (A), since with the medium (A) some lag periods were necessary before the formation of riboflavinylglucoside.

2. *Effect of Cultural Condition*

Fifty m*l* of 10% sucrose medium with riboflavin mentioned above were taken in 200 m*l* shaking flask or in 100 m*l* Erlenmeyer flask. After inoculation, the flask was incubated at 30°C for 22 hours under shaking or stationary conditions. Table 2 shows a comparison of yields of sugar compounds of riboflavin. No remarkable difference in the yield of riboflavinylglucoside was detected between shaking and stationary conditions, however the amount of riboflavin-compounds of oligosaccharides was found to be somewhat higher on the latter condition.

TABLE 2
Effect of cultural condition

Condition	Shaking	Stationary
Type of seed medium	Sucrose	Sucrose
Growth (O. D. at 530 mμ)	0.324	0.312
B_2 present (mg/50 m*l*)		
{ initial	20.0	20.0
{ final	3.4	0.9
B_2-glucoside produced (mg/50m*l*)	16.1	16.2
B_2-oligosaccharides produced (mg/50 m*l*)	0.5	2.9

3. *Effect of Temperature*

Leuc. mesenteroides, L. 20, was inoculated on the 10% sucrose medium with riboflavin and incubated for 2 days at various temperatures. As is shown in Table 3, higher yield of riboflavinylglucoside was attained at 25—30°C. At higher temperature as 37°C, both the riboflavin consumption and riboflavinylglucoside production were decreased.

TABLE 3
Effect of temperature

Temperature (C°)	25°		30°		37°	
Condition	Shaking		Stationary		Stationary	
Type of seed medium*	(B)	(A)	(B)	(A)	(B)	(A)
Growth (O. D. at 530 mμ)	0.337	0.316	0.394	0.380	0.192	0.188
B_2 present (mg/50m*l*)						
{ initial	25.0	25.0	25.0	25.0	25.0	25.0
{ final	2.9	3.8	0.9	1.8	11.8	13.8
B_2-glucoside produced (mg/50 m*l*)	14.7	16.0	13.9	15.5	10.4	9.1
B_2-oligosaccharides produced (mg/50 m*l*)	5.5	2.6	9.4	5.2	0.9	+

* (A): malt-extract medium
(B): sucrose medium

4. *Effect of Concentration of Sucrose*

Various amounts of sucrose were added to solutions containing 0.5% KH_2PO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.03% $(NH_4)_2SO_4$, 0.05% polypeptone, 0.15% yeast extract, and 20—30 mg% riboflavin, as to get its final concentrations to 0.5%, 1%, 2%, 3%, 10% and 50%. All solutions were dispensed in 50 m*l* quantities into 100 m*l* Erlenmeyer flasks and sterilized. Two strains of *Leuc. mesenteroides*, L. 20 and IFO. 3426, were used and cultured at 23°C under stationary conditions. Almost the same yield of riboflavinylglucoside was obtained despite the concentrations of sucrose in the range of 2—10%, but the yield was decreasing at such low concentration of sucrose less than one percent, as will be seen in Table 4. And also a decrease in the yield was observed at such high level of sucrose as 50 percent.

TABLE 4
Effect of concentration of sucrose

(I). After 7 hours' incubation

Strain	Concentration of sucrose (%)	Growth O. D. at 530 mμ	B_2 present (mg/50 m*l*)		B_2-glucoside produced (mg/50 m*l*)	B_2-oligo saccharides produced (mg/50 m*l*)
			initial	final		
IFO. 3426	0.5	0.188	10.0	8.8	0.7	0
	2.0	0.184	10.0	8.1	1.3	(+)
	10.0	0.190	10.0	7.8	1.6	(+)
L. 20	0.5	0.178	10.0	8.4	0.9	0
	2.0	0.180	10.0	7.4	2.0	+
	10.0	0.179	10.0	7.2	2.1	+

(II). After 14 hours' incubation

Strain	Concentration of sucrose (%)	Growth O. D. at 530 mμ	B_2 present (mg/50 m*l*)		B_2-glucoside produced (mg/50 m*l*)	B_2-oligo saccharides produced (mg/50 m*l*)
			initial	final		
IFO. 3426	1.0	0.235	15.0	11.0	3.6	+
	3.0	0.257	15.0	8.7	5.9	+
	10.0	0.267	15.0	8.4	6.1	+
	50.0	0.135	15.0	13.0	1.7	−
L. 20	1.0	0.237	15.0	10.0	4.6	0.4
	3.0	0.274	15.0	8.1	6.2	0.7
	10.0	0.241	15.0	7.2	7.0	0.8
	50.0	0.120	15.0	10.5	4.0	−

5. *Effect of Kinds of Carbon Source*

The media containing 0.03% $(NH_4)_2SO_4$, 0.15% polypeptone, 0.15% yeast extract, 30 mg% riboflavin, other mineral matters and various kinds of carbon sources described in Table 5 were inoculated with the malt-extract culture of *Leuc.*

TABLE 5
Effect of carbon source

Carbon source (10%)	pH	Growth (O. D. at 530 mμ)	B_2-glucoside produced (mg/50m*l*)
Glucose	4.4	0.387	(±)
Fructose	4.4	0.379	0
Galactose	5.8	0.222	0
Xylose	4.6	0.354	0
Glycerol	6.8	0.010	0
Sucrose	4.2	0.394	21.3
Lactose	5.0	0.173	(±)
Maltose	4.4	0.380	(±)
Soluble starch	6.8	0.010	0
Dextran	6.8	0.010	0
*Glucose + Fructose	4.4	0.377	(±)

* mixture of 5% glucose + 5% fructose

mesenteroides, L. 20, and incubated at 30°C for 2 days under stationary conditions. Relations of the kinds of carbon source to the formation of riboflavinylglucoside in a growing culture were examined by the determination of bacterial growth, final pH and the amount of riboflavin-compounds as are shown in Table 5. It was found that sucrose was the only active source of carbon for the formation of riboflavinylglucoside, while D-glucose, D-fructose and their mixture or other carbon sources were ineffective.

6. *Fluctuation of the Amounts of Riboflavin and of Its Sugar Compounds During the Fermentation*

The fermentations were carried out at 30°C with the same 10% sucrose medium after inoculation with 3 m*l* of a 24-hours' culture of *Leuc. mesenteroides*, IFO. 3426. grown on the seed media (B) and (A). Aliquots were removed at the requisite periods of fermentation and the amounts of riboflavin and of its sugar compounds were examined, and the results are illustrated in Figures 1 and 2. The amount of riboflavinylglucoside attained a ma-

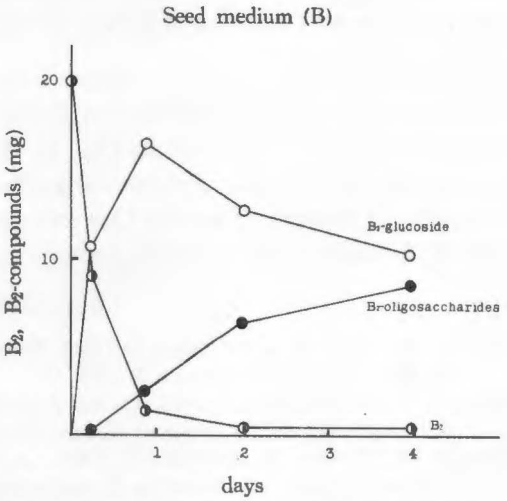

Fig. 1. Fluctuation of riboflavin and its sugar compounds in a stationary culture.

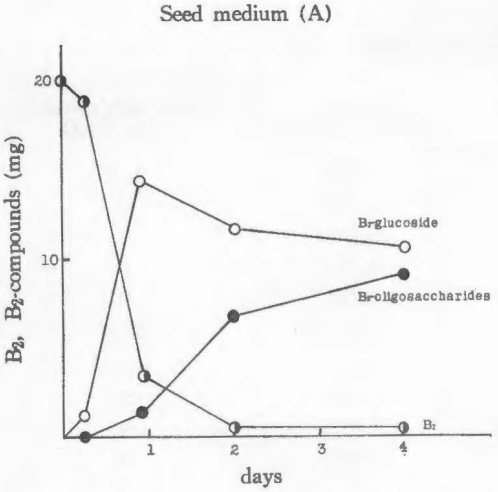

Fig. 2. Fluctuation of riboflavin and its sugar compounds in a stationary culture.

ximum value in the earlier stage of incubation and then gradually decreased, while riboflavin-compounds of oligosaccharide were successively increasing during the decrease of riboflavinylglucoside. These fluctuations seemed to show that riboflavinylglucoside once produced was consumed gradually in order to form riboflavin-compounds of oligosaccharides by receiving glucosyl group again from sucrose; that is to say, riboflavinylglucoside played the role as the second acceptor in the successive glucosyl transfer, as was already demonstrated with maltose and riboflavin by the action of enzymes of *Asp. oryzae*, *E. coli* and of *Cl. acetobutyricum*.

SUMMARY

Leuconostoc mesenteroides was found to produce a remarkable amount of riboflavinylglucoside when grown on sucrose medium containing riboflavin, but not on any other kinds of sugar media. The formation of riboflavinylglucoside was observed to depend largely on the kinds of seed culture and on the temperature of fermentation and also on the concentration of sucrose. Riboflavin-compounds of oligosaccharides were observed to be formed by successive transfers of D-glucosyl group of sucrose to riboflavin during the fermentation.

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