FACTORS AFFECTING RIBOFLAVIN PRODUCTION BY ASHBYA GOSSYPII[1]

F. W. TANNER, JR.,[2] C. VOJNOVICH, AND J. M. VAN LANEN[3]

Northern Regional Research Laboratory,[4] Peoria, Illinois

Received for publication August 8, 1949

The methods that are presently employed for the microbiological production of riboflavin on a commercial scale are based upon the remarkable biosynthetic capacities of two species, viz., *Clostridium acetobutylicum* and *Eremothecium ashbyii*, for this vitamin. With *C. acetobutylicum* riboflavin appears concurrently during the anaerobic fermentation of various carbohydrates to butanol, acetone, and ethanol. As early as 1927 Weyer and Rettger observed that a yellow pigment accumulates in cereal mashes fermented by this organism. They attributed it to zein dissolved from corn by the solvent. It was not until several years later (Yamasaki and Yositome, 1938; Miner, 1940) that this pigmented substance was identified as riboflavin. Subsequent studies on the butyl anaerobes by Arzberger (1943), Meade *et al.* (1945), and others have revealed the critical relationship that exists between the iron concentration of the medium and riboflavin biosynthesis. By controlling this variable, either through adding or restricting iron as dictated by the materials fermented, processes have been developed that are applicable to whey and cereal grain mashes.

The production of a greenish-yellow pigment by *Eremothecium ashbyii* cultivated upon certain solid media was reported in 1935 by Guilliermond *et al.* The identity of this substance with riboflavin was established by the later studies of Raffy (1937) and Mirimanoff and Raffy (1938*a*,*b*). Under submerged aerobic conditions unusually high concentrations of riboflavin are formed by *E. ashbyii*, and most of the biologically produced vitamin is made with this organism. Several processes involving *E. ashbyii* combined with specific media and submerged aerobic techniques have been patented within the past few years (Rudert, 1945; Piersma, 1946; Mayer, 1946; Foster, 1947).

In addition to the two organisms described above, the elaboration of substantial amounts of riboflavin by other yeasts and bacteria is not uncommon. Examples of such include yeasts of the genus *Candida* (Burkholder, 1943; Tanner *et al.*, 1945), *Mycobacterium smegmatis* (Mayer and Rodbart, 1946), and *Aerobacter aerogenes* (Novak, 1948). Because these species synthesize lesser amounts of riboflavin or because they require carefully controlled environments

[1] Presented before the 47th General Meeting, Society American Bacteriologists, Philadelphia, Pennsylvania, May 13–17, 1947.

[2] Present address: Chas. Pfizer and Company, Brooklyn, New York.

[3] Present address: Hiram Walker and Sons, Peoria, Illinois.

[4] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

for maximum production, they hold less promise of industrial utilization than the two types now in commercial use.

Guilliermond *et al.* (1935) observed that under the conditions suitable for riboflavin accumulation by *Eremothecium ashbyii*, the closely related species *Ashbya gossypii* formed only traces of this vitamin. Confirmation of this feature distinguishing the two species may be found also in the reports of Schopfer (1944) and Deseive (1947). Nevertheless, Wickerham *et al.* (1946) found that *A. gossypii* was capable of substantial synthesis of riboflavin when selected media and cultural conditions were provided. For example, a medium composed of glucose, Difco peptone, and Difco yeast extract inoculated with *A. gossypii* and aerated by introducing sterile air gave culture liquors within an incubation period of 8 days which assayed as high as 381 μg per ml. These yields, although somewhat below those reported for *E. ashbyii*, indicated the industrial potentialities of this organism and justified a more intensive study.

In the present investigation the effects of various factors upon riboflavin synthesis by *A. gossypii* have been investigated. Particular attention has been paid to available low-cost media, cultural conditions, inoculum development, and sterilization procedures, since these were found to have an important bearing upon the riboflavin productivity of this organism. Pilot-plant studies have been undertaken utilizing the more favorable conditions developed herein, and these will be reported separately.

METHODS

At the beginning of this study, *A. gossypii* was maintained on agar slants containing 1 per cent glucose, 0.5 per cent peptone, 0.3 per cent yeast extract, and 1.8 per cent agar. Later this medium was supplemented with 0.3 per cent Difco malt extract. Malt extract appeared to stimulate the rate of pigment formation on agar media, but there was no evidence that this beneficial influence was transmitted to the liquid media subsequently used in the principal fermentation.

To study riboflavin biosynthesis in submerged fermentations, liquid media was dispensed in 100-ml amounts in 500-ml Erlenmeyer flasks. These were autoclaved 15 minutes at 121 C, cooled, and inoculated with a liquid culture propagated under submerged aerobic conditions. All flasks were incubated at 28 ± 1 C on a reciprocating shaker having 92 three-inch strokes per minute. At suitable intervals, samples were withdrawn for routine analyses. Riboflavin was determined with a photofluorometer after hydrolysis of the sample by autoclaving for 30 minutes either in the presence of 0.1 N HCl or 0.123 M acetate buffer, pH 4.7. Unlike many natural materials, the riboflavin in *A. gossypii* cultures was readily liberated by water extraction. However, the acid or buffer provided the necessary stabilization of riboflavin during the hydrolysis of culture liquors which had reached a definitely alkaline reaction. Fluorometric analyses compared very well with those obtained microbiologically and spectrophotometrically.

RESULTS AND DISCUSSION

Several factors that modify riboflavin synthesis were studied in turn and more or less independently, and accordingly the importance of each factor was not fully appreciated at the time some of the data were recorded. Especially was this true during the early part of the investigation before it was fully recognized that a number of conditions were capable of deleteriously affecting growth or riboflavin production. Some of these are pointed out below; others are as yet incompletely understood. Consequently, most of the tabular data are indicative primarily of the significance of a particular variable rather than of the maximum yield obtainable under each set of conditions.

Temperature. Although *A. gossypii* grows abundantly over a rather wide temperature range, the greatest riboflavin accumulation occurred within fairly

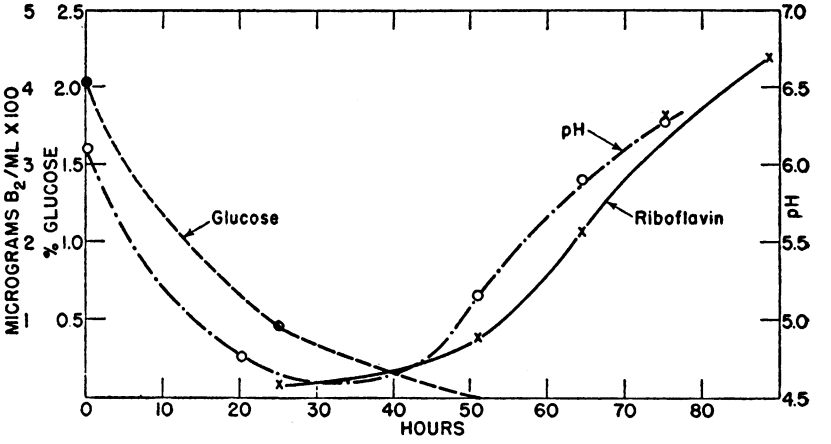

Figure 1. The fermentor was charged with 2,470 grams of corn steep liquor, 617.5 grams of Difco peptone, 5,000 grams of glucose monohydrate, and approximately 51 gallons of tap water. The pH was adjusted to 6.57 with sodium hydroxide before sterilization. The medium was sterilized by injecting steam and maintaining a pressure of 15 pounds for 35 minutes. After cooling, to 28 C, the medium received 6 liters of 24-hour inoculum and was aerated at a rate of one-fourth volume of air per volume of medium per minute.

narrow limits. The highest yields were obtained when cultures were incubated at 26 to 28 C, which is below the optimum for growth. Incubation at temperatures higher than 28 C resulted in sharply decreased yields, whereas low temperatures were less critical but required longer fermentation periods to obtain equivalent potencies.

Reaction of the medium. The best yields of riboflavin were obtained when the initial reaction of the medium was above pH 5.5, and preferably in the range of pH 6.0 to 7.0. When the initial reaction was between pH 4.5 and 5.5, good and rapid multiplication followed, but the amount of riboflavin produced was less than when the foregoing preferred range was used. Media initially adjusted to pH 4.0 gave little growth or riboflavin.

Data from a tank fermentation which are presented in figure 1 illustrated

the course of a typical fermentation. It may be seen that the fermentation is comprised of two rather distinct phases. In the first, glucose is dissimilated and the medium becomes acid, generally reaching about pH 4.7 with an occasional minimum of pH 4.5. This change occurs within the first 24 to 36 hours. When glucose consumption is substantially complete, there is a gradual rise to an alkaline reaction occasionally reaching pH 8.5. Only negligible amounts of riboflavin appear in the medium before the carbohydrate is metabolized, the bulk of it being formed during the second phase in which a neutral or alkaline reaction develops. Fermentations in shaken flasks follow a similar pattern, but generally require longer incubation periods, probably owing to less efficient aeration under such conditions.

TABLE 1

Riboflavin synthesis by Ashbya gossypii in relation to initial glucose supply*

INITIAL GLUCOSE	pH			RIBOFLAVIN		
	4 days	6 days	9 days	4 days	6 days	9 days
per cent				*μg/ml*		
0	8.0	8.1	8.2	0.4	0.4	0.5
0.25	8.3	8.3	8.5	10.2	10.5	11.7
0.50	8.3	8.3	8.2	23	23	23
1.0	8.1	8.1	8.3	35	35	34
2.0	6.8	8.2	8.3	125	159	274
3.0	6.1	6.8	8.4	210	340	365
4.0	6.2	6.2	7.7	297	339	352
5.0	5.4	6.2	6.1	208	301	344

* Flasks inoculated with 1.0 per cent of 24-hour liquid culture grown on 2.0 per cent glucose, 0.5 per cent peptone, and 0.3 per cent yeast extract.

Carbohydrate sources and concentration. The amount of riboflavin formed was found to be closely related, within certain limits, to the quantity of fermentable sugar supplied; in a basal medium containing 0.5 per cent corn steep liquor and from 0.25 to 3.0 per cent glucose, riboflavin appeared to increase with the amount of sugar supplied. The difference between 3.0 and 4.0 per cent glucose was slight, and levels above 4.0 per cent were not beneficial although 10 per cent sugar was found in other trials to have no detrimental effect. When sugar was supplied in concentrations below 0.25 per cent, apparently it was utilized largely for growth and an alkaline reaction developed rapidly without appreciable riboflavin production. When the available carbohydrate was increased, the characteristic pH changes took place and were accompanied by a much greater synthesis of riboflavin.

A. gossypii possesses little or no saccharifying power; thus, starch or modified starches fail to serve adequately as carbohydrate sources. Pentoses such as xylose or arabinose, likewise, are not metabolized. Sucrose and maltose may be employed in place of glucose if supplied in relatively pure form, but attempts to utilize cane molasses, beet molasses, or hydrol (molasses from corn sugar

manufacture) in this fermentation were unsuccessful although they supported abundant growth.

Inoculum development. The influence of the age and volume of inoculum was explored, and pertinent results on the former are shown in figure 2. Liquid cultures were prepared from agar slants on successive days to provide inocula varying in age from 24 to 120 hours. Each of these was then employed to seed media of the same composition at a rate of 1 per cent by volume. The results show that, though the age of the inoculum did not markedly influence the time required to initiate riboflavin synthesis, considerably greater production was obtained from inocula incubated for only 24 hours. The young vigorous cells seemingly induced a rapid and more prolonged period of synthesis, whereas cultures made from older preparations produced riboflavin at a relatively slow

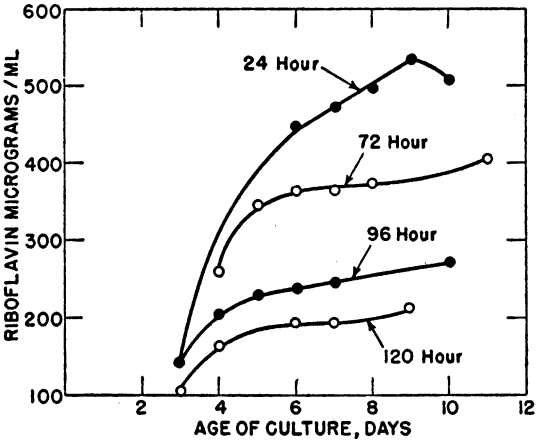

Figure 2. **Influence of the age of *Ashbya gossypii* on the synthesis of riboflavin.**

rate and for periods of short duration. A similar effect was observed with respect to the age of the slant culture from which liquid inocula were developed, transfers from young slants tending to be more productive. Because of the critical nature of this step, the practice of making daily transfers of both liquid and agar cultures was adopted in all subsequent experiments. In addition to the beneficial effect of employing young inocula, better riboflavin yields were generally secured when the volume of inoculum was equivalent to 0.5 to 1.0 per cent of the volume of medium. Levels appreciably above 1.0 per cent were less favorable, and a pronounced reduction in yield generally resulted with 10 per cent of inoculum.

Medium composition. In the light of the observations of Wickerham *et al.* (1946) it must be concluded that the failure of earlier workers to observe more than traces of riboflavin in cultures of *A. gossypii* was due largely to the media employed. Gorodkowa and Sabouraud media (Guilliermond *et al.*, 1935), and malt extract media (Stelling-Dekker, 1931) gave only slight quantities of riboflavin even over extended incubation periods. Likewise, *A. gossypii* grows readily without producing visible amounts of riboflavin in a medium composed of thiamine, biotin, inositol, glucose, asparagine, and potassium nitrate and other

inorganic salts (Robbins and Schmidt, 1939). On the other hand, Wickerham and co-workers noted that both agar and liquid media containing carbohydrate, peptone, and yeast extract led to substantial vitamin synthesis. They did observe differences in the effectiveness of various lots of medium ingredients, particularly the yeast extract. Our efforts were directed, therefore, to the development of uniformly good media from available crude proteinaceous materials.

Corn steep liquor was found to be a very satisfactory substitute for yeast extract, and since it is available in abundance at low cost, it was incorporated into most of the test media. Little effort was expended in exploring other substitutes for yeast extract; however, distillers' solubles showed promise as an alternative material.

Replacement of the commercial peptone was somewhat more difficult. Materials such as soybean meal, linseed meal, cottonseed meal, corn gluten, and wheat gluten were tested. These gave lower and generally more variable results and none produced yields consistently comparable to peptone plus corn steep liquor. Materials of animal origin were considerably more effective replacements than plant products. Among those tested and found to support good riboflavin production in combination with glucose and corn steep liquor were animal stick liquor,[5] tankage, and meat scraps. Frequently, even greater riboflavin yields were obtained by subjecting these supplements to digestion with proteolytic enzymes prior to incorporation into the medium. Papain and trypsin were about equally effective. Products such as liver tankage, liver meal, and blood meal gave lower riboflavin synthesis than stick liquor and were not substantially improved by proteolysis. The one sample of fish stick liquor tested was inactive.

Examples of the yields obtained with several of these supplements are shown in table 2. Two media, each composed of 4.0 per cent glucose and 0.5 per cent corn steep liquor but with 0.25 per cent peptone in the one case and 0.5 per cent peptone in the other, were used as standards of comparison. The yields from 50 trials with these standard media averaged 392 and 395 μg of riboflavin per ml, respectively. Although the variations between successive runs were considerable, the higher level of peptone generally gave superior results. By taking advantage of the preferred conditions, as was possible during the 10 latest experiments, yields were less variable and averaged 575 and 555 μg per ml, respectively. In these later trials, more significance should be attached to the increase in average yields than to the influence of peptone concentration, since during this period several different lots of supplements were evaluated. The improvement of yields by modifying the type and ratio of medium ingredients suggests also that further studies along this line might be fruitful. This is strongly indicated by the highest unconfirmed single yield of 1,050 μg per ml obtained in a shake flask culture

[5] Stick liquor is a by-product of wet rendering which is obtained by suspending packinghouse wastes in water and heating under pressure to release the fat. The fat and other insolubles are then separated and the liquid evaporated. Stick liquor is the condensed liquid phase of this digestion.

and 1,060 μg per ml and 1,420 μg per ml obtained in parallel 30-liter fermentations.

Sterilization of media. Prolonged autoclaving of media was observed to have a marked adverse effect on riboflavin production. In table 3 are presented results

TABLE 2

The suitability of various animal proteinaceous materials as substitutes for peptone in the production of riboflavin

FOUR PER CENT GLUCOSE PLUS	AGE OF CULTURE	
	8 days μg/ml	
0.5% CSL* + 0.25% peptone	392†	
0.5% CSL + 0.50% peptone	395†	
	5 days	7 days
0.5% CSL + 0.25% beef scraps	186	296
0.5% CSL + 0.50% beef digester tankage	270	356
0.5% CSL + 0.25% beef stick liquor	178	304
0.5% CSL + 0.50% beef stick liquor	164	312
0.5% CSL + 0.10% fish stick liquor	37	38
0.5% CSL + 0.25% fish stick liquor	13	14
Corn stillage + 0.25% peptone (dil. 1:1 H_2O)	72	128
Corn stillage + 0.50% peptone	60	238
Corn stillage + 0.75% peptone	62	256

* Corn steep liquor.

† These are the average yields from 50 separate trials.

TABLE 3

Influence of sterilizing conditions on riboflavin yields

STERILIZATION TREATMENT	AGE OF CULTURE	
	8 days	10 days
	μg/ml	
Seitz filtration	678	676
Autoclave 15 minutes	648	700
Autoclave 30 minutes	680	700
Autoclave 45 minutes	494	586
Autoclave 60 minutes	308	346
Autoclave 75 minutes	288	360
Autoclave 90 minutes	248	324

of a representative experiment to show the critical nature of this step. It may be noted that yields were sharply reduced when the autoclaving time exceeded 30 minutes at 121 C. After 90 minutes at this temperature, potencies were lowered from one-half to one-third those formed in the controls autoclaved for 15 minutes. This factor becomes of considerable importance when large volumes of medium are to be sterilized, as for example, in pilot-plant or commercial-scale operations,

and when the use of flash sterilizing techniques is indicated. The difficulty also was overcome to a considerable extent by sterilizing the medium ingredients separately.

ACKNOWLEDGMENT

The authors are greatly indebted to Dr. Harlow H. Hall for his helpful criticism and extensive aid in the preparation of the manuscript.

SUMMARY

Ashbya gossypii was found capable of synthesizing large amounts of riboflavin when it was propagated in certain media under conditions of submerged aerobic cultivation. Several factors were found to influence riboflavin production, the most important of which were concerned with the type and concentration of the medium constituents. Crude proteinaceous nitrogen sources were required, and combinations of corn steep liquor with certain materials of animal origin, e.g., animal stick liquor and tankage or meat scraps, were most satisfactory. These, with fermentable sugar, constituted a satisfactory commercial medium. Glucose, sucrose, or maltose served as adequate carbohydrate sources, but pentoses were not assimilated.

The use of small quantities of young inocula, a minimum sterilization time, and an efficient means of aeration were additional factors of importance.

The data indicate that a fermentation method for the commercial preparation of riboflavin either in concentrate or pure form might be based upon the use of *Ashbya gossypii*.

REFERENCES

ARZBERGER, C. F. 1943 Production of riboflavin by butyl alcohol producing bacteria. U. S. Patent 2,326,425.

BURKHOLDER, P. R. 1943 Influence of some environmental factors upon the production of riboflavin by a yeast. Arch. Biochem., **3**, 121–129.

DESEIVE, E. 1947 Zur kenntnis der Lactoflavinbildung durch *Eremothecium ashbyii*. Die Milchwissenschaft, **3**, 141–148.

FOSTER, J. W. 1947 Improvement in or relating to the production of riboflavin and materials containing the same. British Patent 593,027.

GUILLIERMOND, A., FONTAINE, M., AND RAFFY, A. 1935 Sur l'existence dans l'*Eremothecium ashbyii* d'un pigment jaune se rapportant au groupe des flavins. Compt. rend., **201**, 1077–1080.

MAYER, R. L. 1946 Procédé de préparation de vitamine B_2. French Patent 913,165.

MAYER, R. L., AND RODBART, M. 1946 The production of riboflavin by *Mycobacterium smegmatis*. Arch. Biochem., **11**, 49–63.

MEADE, R. E., POLLARD, H. L., AND RODGERS, N. E. 1945 Process for manufacturing a vitamin concentrate. U. S. Patent 2,369,680.

MINER, C. S. 1940 Production of composition containing components of the vitamin B complex. U. S. Patent 2,202,161.

MIRIMANOFF, A., AND RAFFY, A. 1938*a* Extraction et cristallisation de la flavine d'un ascomycete: *Eremothecium ashbyii*. Bull. soc. chim. biol., **20**, 1166–1174.

MIRIMANOFF, A., AND RAFFY, A. 1938*b* Obtention de flavin à partir d'un ascomycete *Eremothecium ashbyii*. Helv. Chim. Acta, **21**, 1004–1006.

NOVAK, A. F. 1948 Synthesizing vitamins in stillage. U. S. Patent 2,447,814.

PIERSMA, H. D. 1946 Production of riboflavin. U. S. Patent 2,400,710.

RAFFY, A. 1937 Propriétés vitaminique de la flavine d'*Eremothecium ashbyii*. Compt. rend. soc. biol., **126**, 875–877.

ROBBINS, W. J., AND SCHMIDT, M. B. 1939 Preliminary experiments on biotin. Bull. Torrey Botan. Club, **66**, 139–150.

RUDERT, F. J. 1945 Production of riboflavin by biochemical methods. U. S. Patent 2,374,503.

SCHOPFER, W. H. 1944 La biotine, l'aneurine et le *meso*-inositol, facteurs de croissance pour *Eremothecium ashbyii* Guilliermond. La biosynthèse de la riboflavin. Helv. Chim. Acta, **27**, 1017–1032.

STELLING-DEKKER, N. M. 1931 Die Hefesammlung des "Centraalbureau voor Schimmelcultures." I. Tiel. Die sporogenen Hefen. Amsterdam.

TANNER, F. W., JR., VOJNOVICH, C., AND VAN LANEN, J. M. 1945 Riboflavin production by *Candida* species. Science, **101**, 180–181.

WEYER, E. R., AND RETTGER, L. F. 1927 A comparative study of six different strains of the organism commonly concerned in large-scale production of butyl alcohol and acetone by the biological process. J. Bact., **14**, 399–424.

WICKERHAM, L. J., FLICKINGER, M. H., AND JOHNSTON, R. M. 1946 The production of riboflavin by *Ashbya gossypii*. Arch. Biochem., **9**, 95–98.

YAMASAKI, I., AND YOSITOME, W. 1938 Über die Bildung des Vitamin B_2-Komplexes aus Cerealien bei der Garung von Aceton-Butyl-Alkoholbacterien. Biochem. Z., **297**, 398–411.