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Production of Flavine-Adenine Dinucleotide from Riboflavine by a Mutant of *Sarcina lutea*

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A study was made to develop a new method for the production of flavine-adenine dinucleotide (FAD) from riboflavine and adenine by a mutant of *Sarcina lutea* deficient in the enzyme adenosine deaminase. It was found that this strain could convert exogenously supplemented riboflavine to extracellular FAD. The yields of FAD were increased by addition of D-cycloserine in the culture medium. The culture conditions for FAD production were investigated under the addition of D-cycloserine, and increased production of FAD was observed with the addition of an appropriate amount of thiamine, acetate, and sodium ion. The yield of 0.7 g/liter was obtained in the optimal culture in 5 days. Accumulated FAD was readily isolated by adsorption chromatography and ion-exchange chromatography in a 70% yield.

The use of flavine-adenine dinucleotide (FAD) as a biochemical and nutritional agent has been recently increasing, and the chemical, biochemical, and fermentative methods have been reported for its production. In the past, FAD was produced by extraction from the mycelium of *Eremothecium ashbyii* (10) or by the chemical synthesis from flavine mononucleotide (FMN) and adenosine monophosphate (AMP) (2). We previously showed that a large amount of FAD accumulated in culture fluid when a strain of *Sarcina lutea* was cultured in the medium supplemented with FMN, a well-known precursor of FAD, and adenine (7).

During the course of these studies, it was found that riboflavine was more favorable than FMN as the precursor for FAD production because of its low cost and easy separation from FAD.

This paper deals with the fermentative method for FAD production from riboflavine and adenine.

MATERIALS AND METHODS

Organism. A purine-requiring and adenosine deaminase-less mutant ATCC 21881, which was derived from *Sarcina lutea* IFO 1099, was used for the fermentative experiments.

Fermentation experiments. Unless otherwise noted, fermentations for FAD production were carried out as follows. The compositions of seed medium and fermentation medium are shown in Table 1. Seed media were distributed in 30-ml amounts to 500-ml flasks, sterilized, and inoculated with one loopful of cells of the *S. lutea* mutants. Cultures were incubated at 30 C. After 24 h of incubation, 0.5 ml of the seed medium was combined with 20 ml of the respective fermentation medium in a 500-ml shaking flask. All cultures were incubated at 30 C with reciprocal shaking (120 rpm, 8-cm stroke). After 24 h of incubation, a 2.5-ml solution of 0.5% adenine and 0.5% riboflavine was added, and incubation was continued for an additional 2 to 4 days. Culture broth was heated at 80 C for 3 min and centrifuged. The supernatant was employed in the determination of products.

Methods of analysis. The assay of FAD and FMN was carried out by the manometric method (7) and the fluorometric method (11). Determination of growth was carried out as follows. The culture broth was diluted 80-fold the original volume with water, and the optical density at 660 nm was measured with a photometer (Hitachi EPO-B type). An absorbance of 1.00 represented 1.6 mg of dry cells per ml. Sucrose

TABLE 1. *Components of culture media*

Component	Seed medium[a]	Fermentation medium[a]
Sucrose	10%	10%
Peptone	2%	2%
KH_2PO_4	0.75%	0.75%
$MgSO_4 \cdot 7H_2O$	0.1%	0.1%
$CaCl_2 \cdot 2H_2O$	0.01%	0.01%
$B_1 \cdot HCl$	0	0.8 mg/liter
Pantothenic acid (Ca)	5 mg/liter	5 mg/liter
Yeast extract	1%	0.5%
Adenine	0.02%	0.02%
Guanine	0.02%	0.02%
CH_3COONH_4	0	1.1%
NaOH	0	0.04%

[a] pH was adjusted to 7.0 with KOH.

was measured by the resorcinol method of Kulka (3). Pyruvate was measured by the method of Hirata and Hayaishi (1). Adenine, hypoxanthine, AMP, and cyclic AMP were determined by measuring their optical densities at 260 nm after extracting their spots from paper chromatograms with 0.01 N HCl. Paper chromatography was carried out on Toyo filter paper no. 51A using a solvent system containing isobutyric acid and ammoniacal water (5:3, vol/vol).

Chemicals. FAD was purchased from Boehringer Mannheim (Mannheim, GFR), adenine was purchased from Kohjin Co., Ltd. (Tokyo), and riboflavine came from Tokyo Tanabe Seiyaku Co., Ltd. (Tokyo). All other chemicals used were of the best quality available.

RESULTS

Comparison of FMN and riboflavine as a precursor for FAD production. Studies on the use of *S. lutea* previously demonstrated that FMN was an effective precursor for FAD production (7). However, a large amount of FMN remained unchanged in the final culture broth because the conversion rate of FMN into FAD was low. Not only are the chemical structures of FMN and FAD similar to one another, but their chemical and physical characteristics are quite alike. Therefore, the separation of a large amount of FMN from FAD in the culture broth was difficult. In order to avoid the disadvantage of using FMN, we decided to use riboflavine, a far intermediate in the biosynthetic pathway of FAD, instead of FMN as a precursor for FAD production. Figure 1 shows the results of FAD production from riboflavine or FMN. Fermentations were carried out on basal medium. FAD was almost extracellular at the end of the fermentation. Although the amount of accumulated FAD was reduced by using riboflavine instead of FMN as the precursor, FAD could be easily isolated without separating FMN and FAD since only traces of FMN were formed from riboflavine. Accordingly, it was predicted that riboflavine might be a more favorable precursor for FAD production.

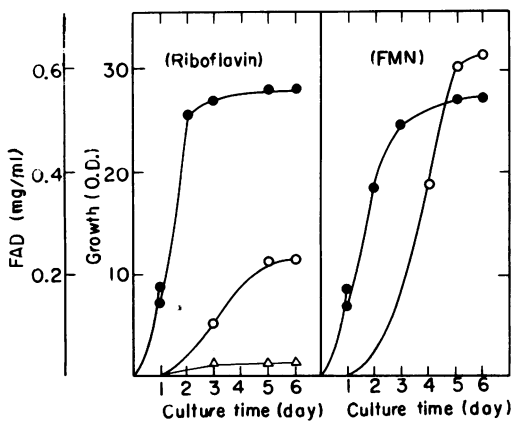

FIG. 1. *FAD production from FMN or riboflavine. Symbols:* ●, *growth (optical density);* ○, *FAD;* △, *FMN.*

Screening of detergents. It was shown that some detergents stimulated fermentative production of amino acids (8) and nucleic acid-related compounds (6). The benefit of adding detergents to the medium is the increase in cell permeability. The results of Fig. 1 show that the rate-limiting step of FAD synthesis may be riboflavine kinase (adenosine 5'-triphosphate [ATP] riboflavine 5'-phosphotransferase; EC 2.7.1.26) by which exogenously supplemented riboflavine is converted to FMN. Therefore, if the permeability barrier of cells could be changed by the addition of detergents, FMN formation would be stimulated and yields of FAD would be substantially increased. Thus, the effect of detergents on FAD overproduction was investigated. Cationic surfactants, such as cetyltrimethylammonium bromide (CTAB) or cetylpyridinium chloride inhibited both growth and FAD production at 0.01% concentration (Table 2). Growth inhibition could be reversed by lowering the concentration of the addition, but FAD production could not be increased. Almost all antibiotics, such as penicillin and streptomycin, showed the same result as ionic surfactants. Overproduction of FAD occurred only when D-cycloserine was added to the culture medium. The stimulatory effect of D-cyclo-

TABLE 2. *Effect of surfactants and antibiotics on FAD production*[a]

Addition to medium (μg/ml)[b]	Addition time (h)	Growth[c]	FMN (μg/ml)	FAD (μg/ml)
CTAB				
100	8	2	0	15
100	24	14	0	63
CPC				
100	8	1	0	4
100	24	9	0	8
Penicillin				
50	8	13	0	25
50	24	17	0	50
Streptomycin				
50	8	9	0	47
50	24	24	7	198
D-Cycloserine				
80	8	14	18	590
80	24	19	29	610
None		29	3	315

[a] The cultures were incubated for 120 h.

[b] CTAB, Cetyltrimethylammonium bromide; CPC, cetylpyridinium chloride.

[c] Optical density.

serine was specific for FAD oversynthesis. The fact that the addition of D-cycloserine inhibited growth showed that FAD overproduction was not due to an effect of D-cycloserine on growth, but was due to some other unknown mechanism. In media with D-cycloserine, the amount of FAD accumulated in the culture broth was twice that accumulated in medium without D-cycloserine.

Effect of addition time of D-cycloserine on FAD production. The correlation between FAD production and addition time of D-cycloserine was studied in detail (Fig. 2). Growth inhibition was observed when D-cycloserine was added within 24 h after inoculation, but FAD production was effectively stimulated. On the other hand, when it was added later, FAD production was appreciably depressed in spite of no inhibition of growth. This result suggests that FAD production is increased under conditions causing growth inhibition. Accordingly, the addition time of D-cycloserine should be at the early stage of FAD fermentation. When D-cycloserine was supplied within 24 h after inoculation, growth obtained was less than half of the maximal growth.

Effect of D-cycloserine concentration on FAD production. The effect of D-cycloserine concentration on FAD production is shown in Fig. 3. Experiments were made to determine an optimal concentration of D-cycloserine at zero-time addition. As seen in Fig. 3, addition of D-cycloserine at the concentration of 80 μg/ml gave the optimal yield of FAD, but growth was nearly 60% of the maximal growth without added D-cycloserine. *S. lutea* was markedly sensitive to D-cycloserine. The addition of 120 μg/ml strongly inhibited growth but that of 40 μg/ml permitted normal growth.

Formation of FAD from FMN or riboflavine by non-growing cells. To examine how D-cycloserine stimulates the accumulation of FAD from riboflavine, activities of FAD formation in the cells grown on the basal medium with D-cycloserine and in the cell supernatant were compared with those grown without D-cycloserine (Table 3). The cells grown on the medium without D-cycloserine formed a small amount of FMN, but not FAD, from riboflavine, while they synthesized a relatively large

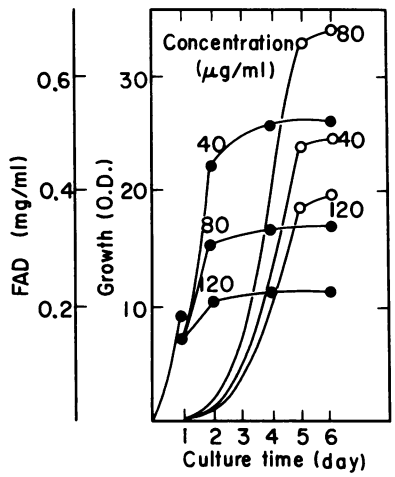

FIG. 3. *Effect of D-cycloserine concentration on FAD production. D-Cycloserine was added at zero-time as indicated. Symbols: ●, growth (optical density); O, FAD.*

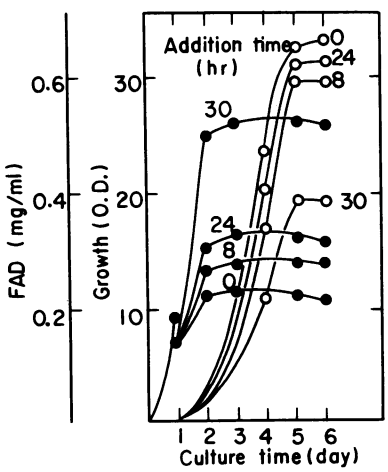

FIG. 2. *Effect of addition time of D-cycloserine on FAD production. D-Cycloserine (80 μg/ml) was added at the indicated time. Symbols: ●, growth (optical density) O, FAD.*

TABLE 3. *Formation of FAD from FMN or riboflavine by resting cells grown on the medium with D-cycloserine*[a]

Addition to medium	Source	Substrate	Formation of:[b] FAD	FMN
D-Cycloserine	Cells	Riboflavine	0.50	0.14
		FMN	0.93	
	Supernatant	Riboflavine	0	0
		FMN	0	
None	Cells	Riboflavine	0	0.10
		FMN	0.18	
	Supernatant	Riboflavine	0	0
		FMN	0	

[a] Reaction mixture contained 1 μmol of ATP, 1 μmol of $MgCl_2$, 0.1 μmol of riboflavine, or FMN, 20 μmol of Tris-hydrochloride buffer (pH 7.5), and 10 mg of dry cells (or the concentrate of supernatant equivalent to 10 mg of dry cells) in a total volume of 1 ml. Reactions were carried out at 37 C for 120 min.

[b] Measured in nanomoles per hour per milligram of dry cells.

amount of FAD from FMN. On the other hand, in the cells grown on the medium supplemented with D-cycloserine, FAD was equally synthesized either from FMN or riboflavine in reasonable yields. The cell supernatant had no activities of FAD formation, regardless of the addition of D-cycloserine to medium. These results suggest that the stimulation of FAD formation from riboflavine may not be caused by the leakage of enzymes from the cells, but by the improvement of permeabilities of substrate or products.

Effect of culture conditions on FAD production. The culture conditions for FAD production were investigated under the addition of D-cycloserine.

(i) Effect of precursors. Various kinds of precursors were added to the culture medium at 24 h after inoculation. As shown in Table 4, it is apparent that this strain has a de novo pathway for FAD formation because a small amount of FAD was made without addition of precursor. Guanine, a well-known precursor of riboflavine, inhibited growth and was not effective for FAD production, whereas adenine and riboflavine stimulated FAD production without inhibiting growth. But separate addition of adenine and riboflavine did not stimulate FAD production compared with their simultaneous addition. Accordingly, it is essential for FAD overproduction that adenine and riboflavine are simultaneously added to culture medium. Growth inhibition by guanine was reversed by adding adenine, but the amount of FAD accumulated was about 120 μg/ml, which was the same amount as that accumulated by the single addition of adenine.

(ii) Effect of aeration. The effect of aeration was studied by changing the volume of medium in the flasks. Maximal production of FAD was obtained with 25 ml of medium or less in a 500-ml flask, which is equivalent to an oxygen absorption rate greater than 2.95 mmol/min (Table 5).

(iii) Effect of thiamine. Table 6 demonstrates the effect of thiamine concentration on FAD production. A low concentration of thiamine supported good growth but not FAD overproduction. There was also a similar tendency with regard to growth and FAD production at a high level of thiamine. The optimal concentration of thiamine was limited to a narrow range which was about 0.5 μg to 2.0 μg/ml.

(iv) Effect of sodium ion. Sodium ion was also found to exert a considerable effect on FAD production. Experiments were carried out to examine the changes during fermentation with or without sodium ion (Fig. 4). When sodium ion was removed from basal medium, growth was enhanced, but FAD production was strongly inhibited. It was estimated that the inhibition of sugar assimilation caused by the removal of sodium ion repressed ATP formation. As the result, FAD production was inhibited.

(v) Effect of acetate. We reported that acetate stimulated FAD formation from FMN (7). A similar result was obtained when using riboflavine (Fig. 5). Omission of acetate from the medium reduced the FAD yield by about 80%. When an optimal concentration of acetate was added, rapid assimilation of sugar and no for-

TABLE 4. *Effect of precursors on FAD production*[a]

Additions to medium (%)			Growth[b]	pH	FAD (μg/ml)	FMN (μg/ml)
Adenine	Guanine	Riboflavine				
0	0	0	28	7.1	52	7
0.1	0	0	26	6.4	118	0
0	0.1	0	19	6.5	59	6
0.1	0.1	0	26	7.3	119	0
0.1	0	0.05	25	7.4	718	14
0.05	0	0.05	22	7.3	745	12
0	0	0.05	26	7.1	196	29

[a] The cultures were incubated for 120 h.
[b] Optical density.

TABLE 5. *Effect of aeration on FAD production*[a]

Vol of medium (ml)	OAR[b]	Growth[c]	pH	Sugar[d] (mg/ml)	FAD (μg/ml)	FMN (μg/ml)
15	3.55	23	7.5	16	643	10
25	2.95	19	6.6	21	695	7
50	1.45	19	6.4	37	480	13
100	1.20	18	6.4	38	390	47
150	1.01	15	6.2	49	230	54

[a] The cultures were incubated for 120 h.
[b] Oxygen absorption rate (millimoles of O_2 per liter per minute).
[c] Optical density.
[d] Residual sugar.

TABLE 6. *Effect of thiamine on FAD production*[a]

Thiamine (μg/ml)	Growth[b]	pH	FAD (μg/ml)	FMN (μg/ml)
0.25	31	6.2	390	0
0.5	26	6.8	758	18
1.0	21	7.1	711	19
2.0	20	7.1	705	26
5.0	27	7.3	466	42

[a] The cultures were incubated for 120 h.
[b] Optical density.

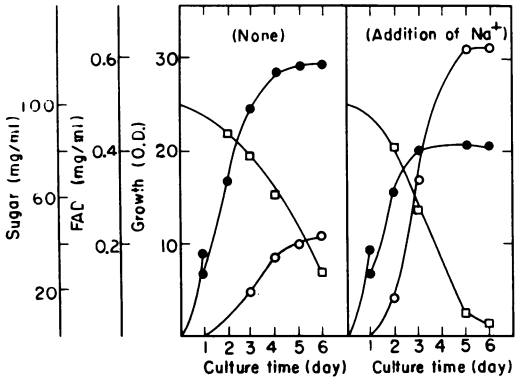

FIG. 4. *Effect of sodium ion on FAD production. Symbols:* ●, *growth (optical density);* ○, *FAD;* □, *sugar.*

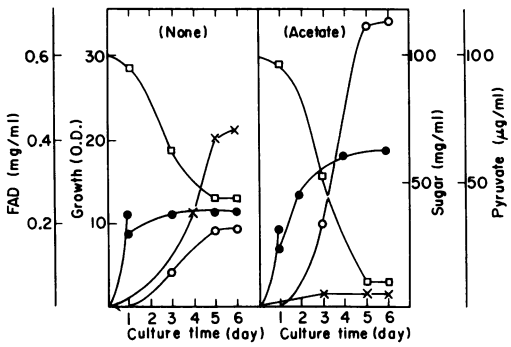

FIG. 5. *Effect of acetate on FAD production. Symbols:* ●, *growth (optical density);* ○, *FAD;* □, *sugar;* ×, *pyruvate.*

mation of pyruvate was observed. The definite mechanism responsible for this phenomenon has not been established.

Changes occurring during fermentation. An example of the chemical changes which occurred during the fermentation of FAD under optimal conditions is given in Fig. 6. After an initial lag period of approximately 12 h, the logarithmic phase proceeded for a long period, and rapid consumption of sucrose was accompanied by growth. As growth slowed down, FAD production started and reached a maximum of about 700 μg/ml at 5 days. The characteristic pH change during the growth phase was a feature of this fermentation process. The rise in pH suggested the start of logarithmic phase, and the pH rose as high as 8.5. Assimilation of acetate and formation of ammonium ion from peptone contributed to the pH rise. After growth ceased, the pH decreased to 6.5 ~ 7.0 and remained constant during the latter part of fermentation. During fermentation, only traces of FMN formed in the medium. Accordingly,

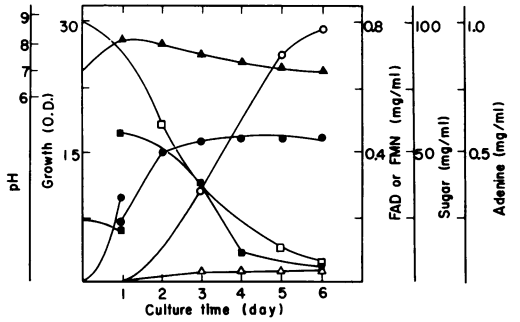

FIG. 6. *Changes during FAD fermentation. Fermentation was carried out with a 10-liter Jar fermentor containing 5 liters of the fermentation medium. Aeration rate, 2.5 liters/min; agitation, 550 rpm. Symbols:* ●, *growth (optical density);* ○, *FAD;* △, *FMN;* □, *sugar;* ■, *adenine;* ▲, *pH.*

FAD in the fermentation broth was easily isolated by an ordinary procedure using Florisil and ion-exchange resins in a 70% yield. Infrared spectrum of the product was identical with that of authentic FAD, and no other fluorescence compound was detected by paper chromatography.

DISCUSSION

Many purine-pyrimidine-related substances are produced by fermentation methods. These include a number of nucleotides, nucleosides, and their analogues. Practical methods for the synthesis of nucleotide derivatives from the corresponding bases were recently reported (5, 9). The synthesis of 5'-inosinic acid, ATP, nicotinamide adenine dinucleotide, coenzyme A, etc., are the examples of these methods. In a previous paper the production of FAD by a microorganism was reported; i.e., a large amount of FAD was produced by *S. lutea* from FMN and adenine in a medium containing sucrose and salts (7). But this previous method had some disadvantages. Among them are the high cost of FMN and the difficulty of separating FMN from FAD during the isolation procedure of FAD. To overcome these disadvantages, we attempted to use riboflavine instead of FMN as a precursor for FAD production. Riboflavine was an inferior precursor in comparison to FMN, because the ability to convert riboflavine into FMN was low in *S. lutea*. In general, fermentative production of nucleotides is markedly affected by the cellular permeability of the microorganism used. Therefore, studies were performed on the removal of the permeability barrier of *S. lutea*. The permeability barrier is removed by controlling the levels of trace nutrients or metals, or by the addition of the agents

affecting cellular permeability such as surfactants, antibiotics, etc. As described above, FAD production from riboflavine was stimulated only by the addition of D-cycloserine. An example of using D-cycloserine was shown for fermentative production of 5'-inosinic acid by Nara et al. (6). They reported that the most important condition was to add the antibiotics at a very early stage of fermentation. A similar observation was made on FAD production. The addition of D-cycloserine at later than 24 h of incubation resulted in a marked reduction of FAD yields. It was estimated that the stimulation with the addition of D-cycloserine was caused by the improvement of the permeability barrier through the change of cell wall because D-cycloserine is known as a compound which affects the structure of microbial cell wall (4). This was confirmed by the experiments shown in Table 3. The change of cell permeability caused by D-cycloserine allowed for a more rapid conversion of riboflavine into FMN, the most immediate precursor of FAD. But it was desirable for production and isolation of FAD that the accumulation of FMN was not accelerated since accumulated FMN was easily converted to FAD by FAD pyrophosphorylase (ATP:FMN adenyltransferase; EC 2.7.7.2) in this strain. From a practical point of view, the present method has considerable advantages over any other microbial process previously reported (7, 10). One of the advantages is that a high concentration of FAD is accumulated in culture fluid and another is that only traces of FMN are formed. These characteristics allow for the purification of product in a high yield without tedious procedure. Thus, the method presented here is considered to be a very advantageous one for FAD production.

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