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The levels of pyridine nucleotide coenzymes in yeast, and the accumulation of the coenzymes in *Candida utilis* were already reported by Hayano, Takebe and Kitahara[1]. Takebe and Kitahara[2] also observed that the levels of nicotineamide adenine dinucleotide (NAD) in *Lactobacillus plantarum* are to be regulated by systematically varying the quantity of nicotinic acid in the medium.

It was ascertained that some increase of pyridine nucleotide coenzymes (PN) of *Candida utillis* is to be brought forth by the addition of nicotinic acid or its amide to the medium. The present paper deals with some of the results obtained.

Materials and Methods

Candida utilis IAM 2415 was used throughout this experiment. Organism was grown in 60 ml medium in 300 ml Erlenmeyer flask on a shaker at 30°. The basal medium was composed of by the following prescription; 30g glucose, 4g urea, 1.5g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$, and 0.5 μg biotin in 1 liter of distilled water (pH 5.0).

Dry-weight determination: The dry-weight of the cells was determined by drying the cells at 105°. Dry-weight of yeast cells was, moreover, calculated on the previously prepared standard-curve of optical density of cell suspension at 660 mμ.

Analytical methods: PN was determined fluorometrically by a modification of the method of Bassham et al[3] and was calculated as NAD[1], without making any distinction of NAD, nicotineamide adenine dinucleotide phosphate and their reduced forms.

Flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD) were determined by the method proposed by Burch[4]. Cytochrome c was determined by the method proposed by Paul after being extracted with cetyltrimethyl ammonium bromide[5]. Glucose was determined by Somogyi method modified by Kobayashi and Tabuchi[6].

Results and Discussion

The effect on PN synthesis of nicotinic acid and its amide added to the medium. *Candida utilis* was grown in the media of different concentrations of nicotinic acid and nicotineamide. After the harvesting of cells by centrifugation, the PN-contents of the cells and in the supernatant, and in addition, the contents of FMN, FAD and cytochrome c of the cells were measured. The amount of PN in the supernatant was expressed as the

Table 1. Effect of Nicotinic Acid and Nicotineamide on the Content of Pyridine Nucleotides

Exptl. No.	Addition of nicotinic acid or nicotineamide mg/ml	Culture period hrs.	Final pH	Glucose consumed mg/ml	Growth (cell-weight) mg/ml	Pyridine nucleotides in cells mg/g	Pyridine nucleotides out cells mg/g	Pyridine nucleotides total mg/60 ml medium	Contents of (μg/g cells) FAD (as riboflavin)	Contents of (μg/g cells) FMN (as riboflavin)	Contents of (μg/g cells) cytochrome c.
1	—	24	3.8	10	2.3	3.5	0.5	0.55	27	12	140
2	—	36	3.8	—	4.4	5.2	0.4	1.57	—	—	—
3	—	40	5.0	26	4.8	6.8	0.7	2.16	33	15	125
4	N* 0.2	24	3.8	13	1.9	6.2	0.9	0.81	28	13	205
5	N 0.2	40	3.6	25	3.9	9.1	1.9	2.57	35	14	—
6	N 0.4	40	4.2	29	4.4	12.2	1.4	3.59	31	14	170
7	N 1.0	40	3.6	29	4.3	10.4	1.5	3.07	26	15	220
8	N 1.0**	40	5.5	30	5.0	14.4	0.7	4.53	54	23	230
9	A* 0.4	24	4.0	15	3.7	3.4	2.0	1.20	22	15	110
10	A 0.4	40	4.2	29	5.2	4.9	4.0	2.78	26	18	130
11	L-glutanine only 3 mg	24	4.0	19	5.2	1.2	1.6	0.87	8	10	70

* N: nicotinic acid, A: nicotine amide

** Adenine 0.3 mg and L-glutamine 0.3 mg per ml medium were added together with nicotinic acid.

Table 2. Effect of Peptone and Yeast Extract on PN-Content

Addition of nicotinic acid mg/ml	Cultural age hrs.	Final pH	Glucose consumed mg/ml	Growth (cell-weight) mg/ml	Contents of (/g cells) PN mg	FAD μg	FMN μg	cytochrome c. μg
—	24	4.2	29	8.3	3.1	32	19	640
0.2	24	4.2	29	7.9	4.1	30	17	530
—	40	4.0	30	11.2	2.9	32	19	670
0.2	40	4.0	30	9.7	3.6	31	18	650

Medium: peptone 1% and yeast extract (Difco) 0.2% were added to the basal medium.

amount leaked from lg dried cells.

As shown in Table 1, the PN-content of the cells increased not only in the presence of nicotinic acid, but also with the length of culture-period. By the addition of adenine and L-glutamine, the assumed precursors of PN, together with nicotinic acid, further increase in PN-content was observed. By the addition of only L-glutamine, however, the content of PN was rather decreased. In the presence of nicotineamide, the similar results were obtained to nicotinic acid, excepting that the leakage of PN from the cells increased.

High content of PN was observed in the yeast cells grown in an aerobic condition as described by Hayano, Takebe and Kitahara[1]. Any addition of nicotinic acid or its amide had no effect on the contents of FMN, FAD and cytochrome c, which are known as the substances of respiratory system. Hence, the increase of PN in the cells by the presence of nicotinic acid may be attributed to the sufficient supply of nicotinic acid which may serve as a precursor of PN.

The boiling water extract of the cells obtained in the presence of nicotinic acid or its amide was evaporated in vacuo, and the medium was further condensed by carbon absorption method[7]. Then, paper chromatography was carried out with the solvent of pyridine-water (1 : 1, V/V). Detection of any fluorescent spot was not possible in alkaline condition, with the exception of NAD and its reduced form. This confirmation was made also in the experiment of secondary culture which is going to be described below. It was clarified that NAD incresed by the addition of nicotinic acid to the medium.

The addition of peptone and yeast extract to the basal medium stimulated the growth, and increased the content of cytochrome c, but it decreased the content of PN in the cells (Table 2). The observation agrees with the result of Hayano, Takebe and Kitahara[1], who observed that the content of PN in the cells grown in natural medium, such as koji-extract, was lower than that of the cells grown in the synthetic medium.

Accumulation of PN in secondary culture. Using the cells grown in the basal medium for 40 hours (the experimental NO. 3 in Table 1), the effects of nicotinic acid, adenine, L-glutamine and glucose upon the accumulation of PN in the cells in secondary shaking

Table 3. Accumulation of PN in Secondary Shaking Culture

Culture period	6 hrs.			14 hrs.			
Composition of medium	Cell weight mg/ml	PN in cells mg/g cells	PN out cells mg/g cells	Cell weight mg/ml	PN in cells mg/g cells	PN out cells mg/g cells	Per cent of the cells stained with Mb %
G.N.A. L-G	8.8	7.8	0.14	9.5	11.6	0.36	8
G.N.A	8.5	7.5	0.15	9.4	9.9	0.34	10
G.N	8.2	6.5	0.16	9.0	4.5	0.20	17
N	6.9	6.3	0.14	6.1	7.7	0.18	32

Secondary culture: The cells shown in experimental No. 3 of Table 1 were used for this experiment. The incubation media cnotained each substance indicated in the Table in 0.43 M phosphate buffer (pH 5.5). Initial concentration of cells was 7.9 mg dried cells per ml.
Temperature; 32° C.
G; glucose 600 mg, N; nicotinic acid 20 mg,
A; adenine 20 mg, L-G; L-glutamine 20 mg.

Table 4. Effect of Zinc and Iron in the Medium

Addition of nicotinic acid mg/ml	Addition of $ZnSO_4$ and $FeSO_4$ μg/ml	Culture period hrs.	Final pH	Glucose consumed mg/ml	Growth (cell-weight) mg/ml	Per cent of the cells stained with Mb.	PN in cells (mg/g dried cells)	PN out cells (mg/g dried cells)	total mg 60ml medium	Contents of (γg/g dried cells) FAD	FMN	cyto-chrome c
—	$ZnSO_4 \cdot 7H_2O$ 0.1	24	3.6	16	3.8	—	1.0	2.0	0.68	10	4	50
0.2	〃	24	3.6	15	4.2	—	4.0	2.4	1.60	36	12	200
—	〃	40	6.2	27	4.0	100	0.7	2.9	0.86	5	1	0
0.2	〃	40	5.8	28	5.0	100	0.5	5.4	1.77	4	1	0
—	$FeSO_4 \cdot 7H_2O$ 0.5	24	4.0	14	5.6	—	1.3	1.7	1.01	10	6	120
0.2	〃	24	4.0	17	5.7	—	1.8	3.1	1.68	9	5	115
—	〃	40	5.2	29	9.1	—	1.8	4.3	3.33	9	4	200
0.2	〃	40	5.0	29	7.7	—	2.5	5.3	3.60	10	8	170

culture were studied. The data summarized in Table 3 show that a large quantity of PN was formed in the presence of nicotinic acid, adenine and glucose in 0.43 M phosphate buffer (pH 5.5). When the cells were incubated for 14 hours in the medium containing nicotinic acid and glucose, the content of PN was decreased.

Effect of zinc and iron in the medium. Hayano, Takebe and Kitahara[1] observed that the content of PN in the cells of *Candida utilis* was lowered by the addition of inorganic salts, especially zinc, when the organism was grown aerobically in the synthetic medium. In the present study, the addition of zinc and iron to the basal medium seems to be ineffective on the synthesis of PN itself, though the leakage of PN from the cells is markedly stimulated. The leakage of PN must be caused by the injury in the permeability of the cells, because a large percentage of the cells grown in the presence of zinc was stained by methylene blue (Table 4). The low content of FMN and FAD in the cells grown in the presence of zinc and iron may be caused by their leakage from the cells, too.

Summary

When *Candida utilis* is aerobically grown in the medium containing 30g glucose, 4g urea, 1.5g KH_2PO_4, 0.5g $MgSO_4 \cdot 7H_2O$ and 0.5μg biotin in 1 liter of distilled water, the addition of nicotinic acid or its amide increases the content of pyridine nucleotide coenzymes to 10.4 mg per g dried cells. Further addition of adenine and L-glutamine to the medium, moreover, increases PN content to 14.4 mg per g dried cells.

In case the culture was carried out in the presence of zinc or iron the effect of nicotinic acid on PN content was also observed. Large amount of PN was leaked, however, from the cells during the culture by the addition of zinc or iron. The phenomenon seems to be caused by the abnormal growth of the cells.

When secondary culture is carried out in phosphate buffer containing nicotinic acid, glucose, adenine and L-glutamine which are assumed to be precursors of PN, the content of PN in the cells increases to 11.6 mg from 6.8 mg per g dried cells during the incubation period of 14 hours. In addition, it was confirmed that PN which was increased by the addition of nicotinic acid is mainely NAD and its reduced form.

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