

# Effect of the Concentrations of Nicotinic Acid and Nicotineamide in the Medium on the Content of Pyridine Nucleotide Coenzymes in the Cells of *Candida utilis*

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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<sup>1)</sup>. Takebe and Kitahara<sup>2)</sup> 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 utilis* 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<sub>2</sub>PO<sub>4</sub>, 0.5g MgSO<sub>4</sub>·7H<sub>2</sub>O, 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<sup>3)</sup> and was calculated as NAD<sup>1)</sup>, 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<sup>4)</sup>. Cytochrome c was determined by the method proposed by Paul after being extracted with cetyltrimethyl ammonium bromide<sup>5)</sup>. Glucose was determined by Somogyi method modified by Kobayashi and Tabuchi<sup>6)</sup>.

## 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 Nicotinamide on the Content of Pyridine Nucleotides

| Exptl. No. | Addition of nicotinic acid or nicotinamide, mg/ml | Culture period hrs. | Final pH | Glucose consumed mg/ml | Growth (cell-weight) mg/ml | Pyridine nucleotides |                |                       | Contents of ( $\mu$ g/g cells) |     |               |
|------------|---------------------------------------------------|---------------------|----------|------------------------|----------------------------|----------------------|----------------|-----------------------|--------------------------------|-----|---------------|
|            |                                                   |                     |          |                        |                            | in cells mg/g        | out cells mg/g | total mg/60 ml medium | FAD (as riboflavin)            | FMN | 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-glutamine 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 | PN mg | Contents of ( $\mu$ g cells) |             |                       |
|----------------------------------|-------------------|----------|------------------------|----------------------------|-------|------------------------------|-------------|-----------------------|
|                                  |                   |          |                        |                            |       | FAD $\mu$ g                  | FMN $\mu$ g | cytochrome c. $\mu$ g |
| —                                | 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<sup>1)</sup>. 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<sup>7)</sup>. 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 increased 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<sup>1)</sup>, 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<br>Composition<br>of medium | 6 hrs.                  |            |           | 14 hrs.                 |            |           | Per cent of<br>the cells<br>stained<br>with Mb<br>% |
|--------------------------------------------|-------------------------|------------|-----------|-------------------------|------------|-----------|-----------------------------------------------------|
|                                            | Cell<br>weight<br>mg/ml | PN         |           | Cell<br>weight<br>mg/ml | PN         |           |                                                     |
|                                            |                         | in cells   | out cells |                         | in cells   | out cells |                                                     |
|                                            |                         | mg/g cells |           |                         | mg/g cells |           |                                                     |
| 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 contained 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<br>mg/ml | Addition of ZnSO <sub>4</sub> and FeSO <sub>4</sub><br>μg/ml | Culture period<br>hrs. | Final pH | Glucose consumed<br>mg/ml | Growth (cell-weight)<br>mg/ml | Per cent of the cells stained with Mb. | in cells (mg/g dried cells) | PN out cells | total mg/60ml medium | Contents of (μg/g dried cells) |     |              |
|-------------------------------------|--------------------------------------------------------------|------------------------|----------|---------------------------|-------------------------------|----------------------------------------|-----------------------------|--------------|----------------------|--------------------------------|-----|--------------|
|                                     |                                                              |                        |          |                           |                               |                                        |                             |              |                      | FAD                            | FMN | cytochrome c |
| —                                   | ZnSO <sub>4</sub> ·7H <sub>2</sub> O 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 <sub>4</sub> ·7H <sub>2</sub> O 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<sup>1)</sup> 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  $\text{KH}_2\text{PO}_4$ , 0.5g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and  $0.5\mu\text{g}$  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 mainly NAD and its reduced form.

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