Production of Nicotinamide Adenine Dinucleotide by *Saccharomyces carlsbergensis*[†]

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The accumulation of NAD was studied by culturing yeast in the presence of NAD precursors. Among the strains tested, *Saccharomyces carlsbergensis* showed the highest ability for the accumulation of NAD. Additions of pantothenate, inositol, zinc ion and fatty acids were effective for the accumulation of NAD. Under the optimal culture condition, NAD level in *Saccharomyces carlsbergensis* reached 42 mg per gram dry cells. Surfactants belonging to alkyl sulfate were effective on the leaking of the intracellular NAD, and about 75 mg of NAD per 100 ml was accumulated.

There have been some reports dealing with the pyridine nucleotide level in microorganisms.^{1~5)} In these studies, the relation between the pyridine nucleotide level and the environmental conditions of microorganism was discussed. The results of these studies suggest that, generally, in microorganisms, the pyridine nucleotide level is not so high, and the *de novo* synthesis of pyridine nucleotide is regulated physiologically by certain mechanisms. This is the major reason why the advantageous fermentative method for the production of pyridine nucleotides could not be settled.

For a long time, NAD has been prepared from baker's yeast by extraction and purification.

Recently, utilizing the salvage synthesizing ability of bacteria, Nakayama *et al.*⁶⁾ succeeded in the fermentative production of NAD.

The authors attempted the production of NAD by using microorganisms. During the course of research, it was found that some microorganisms belonging to yeast accumulated large amounts of NAD when they were cultured in the presence of adenine and nicotin-

amide. Accumulated NAD could be leaked out by the addition of surfactant.

METHODS AND MATERIALS

Organisms. The yeasts used in this study were supplied from the stock culture in this laboratory.

Cultural condition. The screening test of NAD accumulating yeast was carried out as follows: The 500-ml flasks each containing 45 ml of the screening medium were inoculated with one loopful of yeast and incubated for 3 days on a reciprocal shaker (140 rpm, 8.5-cm stroke), at 30°C. After then, each 2.5 ml of 6% of adenine and 6% of nicotinamide were added, and incubation was continued for 3 more days.

The fermentation tests for the accumulation of NAD by *S. carlsbergensis* were carried out as follows: The 500-ml flask containing 45 ml of the fermentation medium was inoculated with 1 ml of the seed which was incubated for 48 hr in the medium shown in Table I and incubated for 24 hr on a shaker. Adenine and nicotinamide were added to make the final concentration of each 0.3%.

Reagents. All the reagents were obtained from commercial sources.

Assay of pyridine nucleotide. Pyridine nucleotide in yeast was extracted with water at 85°C for 5 min and determined by the paper chromatographic method⁷⁾ or enzymatic method.⁸⁾

RESULTS AND DISCUSSION

I. Screening of pyridine nucleotide accumulating yeast

[†] Production of Nicotinamide Adenine Dinucleotide. Part I.

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TABLE I. COMPOSITION OF MEDIA FOR THE SCREENING AND THE FERMENTATION TEST

Compound	Screening medium (g/liter)	Fermentation medium (g/liter)
Glucose ^{b)}	50	50
Casein hydrolysate	15	15
NaCl	5	—
KH ₂ PO ₄	1	5
K ₂ HPO ₄	1	5
MgSO ₄ ·7H ₂ O	0.3	5
ZnSO ₄ ·7H ₂ O	—	1.2 ^{a)}
Ca-pantothenate	—	10 ^{a)}
Thiamine-HCl	—	5 ^{a)}
Inositol	—	100 ^{a)}
Yeast extract	15	—
pH	5.0	5.0

^{a)} Milligram per liter.^{b)} Autocleaved separately.

As shown in Table II, the NAD content in most of the yeasts increased remarkably when they were cultured in the presence of adenine and nicotinamide than when they were cultured in the absence of these substances.

Debaryomyces rini, *Nematospora coryli*, *Saccharomyces carlsbergensis*, *Saccharomyces fermentati*, *Saccharomyces logos*, *Saccharomyces marxianus*, *Saccharomyces peka*, *Saccharomyces capsularis* and *Zygosaccharomyces soyae* accumulated relatively large amounts of NAD in the cells but not in the broth. Generally the content of NADP was much smaller than that of NAD.

II. Accumulation of NAD by *S. carlsbergensis*

Effect of vitamins. As mentioned above, *S. carlsbergensis* accumulated large amounts of

TABLE II. ACCUMULATION OF PYRIDINE NUCLEOTIDES IN VARIOUS YEASTS

Yeast	Growth (mg dry cells/ml)	Intracellular pyridine nucleotides accumulated ^{a)} (mg/g dry cells)	
		NAD	NADP
<i>Ashbya gossypii</i>	2.9	— (—)	—
<i>Brettanomyces anomalus</i>	4.5	— (—)	—
<i>Candida guilliermondii</i>	11.6	— (—)	—
<i>Candida krusei</i>	14.1	— (0.58)	—
<i>Candida mycoderma</i>	22.9	1.05(1.73)	—
<i>Candida parapsilosis</i>	23.7	— (0.96)	—
<i>Candida rugosa</i>	7.6	— (0.31)	—
<i>Candida utilis</i>	14.1	— (0.63)	—
<i>Cryptococcus albidus</i>	10.0	— (0.95)	—
<i>Cryptococcus neoformans</i>	6.1	— (—)	—
<i>Debaryomyces hansenii</i>	1.2	— (—)	—
<i>Debaryomyces japonicus</i>	4.1	— (—)	—
<i>Debaryomyces vini</i>	19.6	0.60(2.63)	—
<i>Endomycopsis fibuliger</i>	11.8	— (—)	—
<i>Eremothecium ashbyii</i>	3.0	— (—)	—
<i>Hanseniaspora valbyensis</i>	5.9	— (0.82)	—
<i>Hansenula anomala</i>	8.7	— (1.19)	—
<i>Kloeckera africana</i>	4.5	— (—)	—
<i>Kloeckera apiculata</i>	0.8	— (—)	—
<i>Lipomyces starkeyi</i>	11.2	— (0.75)	—
<i>Nematospora coryli</i>	12.5	1.90(2.30)	—
<i>Pichia farinosa</i>	10.6	— (0.96)	—
<i>Pichia polymorpha</i>	12.0	— (—)	—
<i>Rhodotorula minuta</i>	7.2	— (—)	—
<i>Rhodotorula texensis</i>	17.5	— (—)	—
<i>Saccharomyces carlsbergensis</i>	18.3	2.90(8.50)	—
<i>Saccharomyces cerevisiae</i>	19.8	1.70(2.45)	—

TABLE II. ACCUMULATION OF PYRIDINE NUCLEOTIDES IN VARIOUS YEASTS (Continued)

Yeast	Growth (mg dry cell/ml)	Intracellular pyridine nucleotides accumulated ^{a)} (mg/g dry cells)	
		NAD	NADP
<i>Saccharomyces ellipsoideus</i>	9.8	— (—)	—
<i>Saccharomyces exiguus</i>	18.3	1.50(1.68)	—
<i>Saccharomyces fermentati</i>	21.2	1.46(2.37)	—
<i>Saccharomyces fragilis</i>	5.1	— (—)	—
<i>Saccharomyces logos</i>	20.4	2.50(3.78)	—
<i>Saccharomyces marxianus</i>	21.2	3.35(4.08)	—
<i>Saccharomyces pastorianus</i>	2.4	— (—)	—
<i>Saccharomyces peka</i>	16.3	2.21(2.39)	—
<i>Saccharomyces rouxii</i>	11.3	— (1.00)	—
<i>Saccharomyces sake</i>	1.3	— (—)	—
<i>Saccharomyces marxianus</i>	14.9	— (—)	—
<i>Saccharomycodes ludwigii</i>	6.1	— (—)	—
<i>Saccharomycopsis capsularis</i>	20.0	2.29(3.50)	—
<i>Schizosaccharomyces liquefaciens</i>	20.5	— (—)	—
<i>Torula lactis</i> var. <i>japonica</i>	10.2	— (0.54)	—
<i>Torulopsis candida</i>	19.3	— (1.22)	—
<i>Torulopsis colliculosa</i>	16.9	1.20(1.24)	—
<i>Torulopsis lactis</i>	5.7	— (—)	—
<i>Trichosporon beigeli</i>	19.3	0.59(1.27)	—
<i>Trigonopsis variabilis</i>	18.0	1.50(1.69)	—
<i>Zygosaccharomyces japonicus</i>	6.3	1.32(1.61)	—
<i>Zygosaccharomyces major</i>	5.5	1.36(1.45)	—
<i>Zygosaccharomyces soya</i>	14.3	— (2.37)	—

—: less than 0.5 mg/g dry cells

In parenthesis are shown the values of NAD accumulated when added adenine and nicotinamide.

^{a)} Pyridine nucleotide was not accumulated in the broth.

TABLE III. EFFECT OF INDIVIDUAL VITAMIN ON GROWTH

In addition to the vitamins, all media contained 5% glucose, 1.5% casein hydrolysate, 0.1% KH_2PO_4 , 0.1% K_2HPO_4 , 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1% guanine, 0.07% xanthine, 0.07% cytosine and 0.07% uracil, pH 5.0.

Vitamin ($\mu\text{g/ml}$)		Growth (mg dry cells/ml)	
		Single addition	Single omission ^{a)}
None		0	7.30
Thiamine-HCl	0.6	0	7.59
Riboflavin	0.6	0	6.78
Pyridoxal	2.0	0	7.10
Ca-pantothenate	1.0	3.39	0
Biotin	0.02	0	8.20
Niacin	0.1	0	7.41
Inositol	70.0	0	6.22

^{a)} Single omission from vitamin mixture listed above.

NAD, so the authors attempted to use the microorganism for the production of NAD and investigated the suitable conditions for its accumulation.

S. carlsbergensis is known as the yeast which requires pantothenic acid, biotin, pyridoxine and inositol for the growth and is used for the quantitative determination of pyridoxine.

The strain required only pantothenic acid for the growth under the stated cultural condition (Table III). In the fermentation test performed by the authors, the strain grew even in the medium without addition of pantothenic acid because it was carried from the seed cultural medium.

Table IV shows the effect of some vitamins on the growth and the accumulation of NAD. It indicates that inositol is effective on both. In a separate investigation, the effect of inositol

TABLE IV. EFFECT OF INDIVIDUAL VITAMIN ON GROWTH AND NAD ACCUMULATION

Basal medium was the same as that in Table III except the addition of Ca-pantothenate (10 μ g/ml). Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

Vitamin (μ g/ml)	Single addition				Single omission ^{a)}		
	Growth (mg/ml)	NAD accumulated		Growth (mg/ml)	NAD accumulated		
		(mg/g cells)	(mg/100 ml medium)		(mg/g cells)	(mg/100 ml medium)	
None	5.2	12.8	6.6	13.4	30.5	40.9	
Thiamine-HCl	5.0	6.5	23.0	12.4	31.4	38.9	
Riboflavin	5.0	1.0	4.4	12.9	30.1	38.8	
Pyridoxal	5.0	4.4	16.4	12.0	34.9	41.9	
Biotin	0.1	4.5	18.6	11.8	33.6	39.7	
Inositol	100	12.3	31.4	3.4	31.0	10.5	

^{a)} Single omission from vitamin mixture listed above.

on NAD accumulation was studied. From the results of the study, the optimal concentration of inositol was determined as 100 μ g/ml. Since thiamine was also effective on the growth, the relation of the growth and the thiamine concentration of the medium was studied. The optimal thiamine-HCl concentration was found to be around 5 μ g/ml, and this concentration of thiamine-HCl was added to the medium in the following investigations.

Figure 1 shows the relationship between the concentration of pantothenic acid and the growth or the accumulation of NAD. These

data indicate the effectiveness of pantothenic acid on both. The optimal concentration of calcium pantothenate for the growth and accumulation was found to be 5 μ g/ml and 10 μ g/ml, respectively. Ten microgram per milliliter of calcium pantothenate was used in the following investigations.

Effect of metal ions. The effect of some metal ions was also investigated. Among the tested metal ions, only Zn^{2+} was effective on the growth (Table V).

Previously, Miwa and Ueyama^{9,10)} studied the effect of Zn^{2+} on the growth of yeast using *S. sake* and *C. utilis* and reported that the growth of the yeasts were enhanced by Zn^{2+} . Moreover, they also suggested that the growth promoting effect of Zn^{2+} depended on the promotion of the biosynthesis of the respiration system in the yeast.

On the other hand, there have been reports which suggest a relationship between Zn^{2+} and pyridine nucleotide. Namely, Williams *et al.*,¹¹⁾ Vallee *et al.*,¹²⁾ and Vallee and Hoch¹³⁾ reported that various kinds of dehydrogenase which are dependent on pyridine nucleotide contain Zn^{2+} .

Nason *et al.*⁴⁾ reported that the tryptophan (a precursor of pyridine nucleotide) synthetase level in *Neurospora crassa* fell when cultured in a Zn^{2+} -deficient medium.

These facts suggest that Zn^{2+} is related to the pyridine nucleotide level in the micro-

FIG. 1. Effect of Pantothenate.

Basal medium contained 5% glucose, 1.5% casein hydrolysate, 0.5% KH_2PO_4 , 0.5% K_2HPO_4 , 0.5% $MgSO_4 \cdot 7H_2O$, 5 μ g/ml thiamine-HCl, 100 μ g/ml inositol, Adenine (2 mg/ml) and nicotinamide (2 mg/ml) were added at a 28 hr culture period.

●—●: Growth ○—○: NAD accumulated (mg/100 ml medium), Δ — Δ : NAD (mg/g dry cells).

TABLE V. EFFECT OF METAL IONS

In addition to metal ions, all media contained 5% glucose, 1.5% casein hydrolysate, 0.5% KH_2PO_4 , 0.5% K_2HPO_4 , 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g/ml}$ Ca-pantothenate, 5 $\mu\text{g/ml}$ thiamine-HCl, 100 $\mu\text{g/ml}$ inositol, pH 5.0. Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

Metal ion ($\mu\text{g/liter}$)	Growth (mg/ml)	NAD accumulated	
		(mg/g cells)	(mg/100 ml medium)
None	0	10.9	27.0
Al^{3+}	20	9.11	26.5
	100	10.9	23.0
	200	9.63	24.3
	200	9.63	23.4
Ba^{2+}	20	7.59	12.1
	100	9.04	23.0
	200	8.81	10.6
Ca^{2+}	20	7.16	29.6
	100	7.41	35.8
	200	7.30	33.3
Co^{2+}	20	5.33	39.4
	100	9.88	10.4
	200	7.91	27.8
Cu^{2+}	20	9.67	22.1
	100	8.72	27.0
	200	7.68	30.9
Fe^{3+}	20	9.74	19.9
	100	8.88	25.6
	200	9.74	27.4
Fe^{2+}	20	7.82	33.2
	100	9.11	30.9
	200	11.0	23.4
Mn^{2+}	20	7.03	26.5
	100	7.03	34.0
	200	6.85	27.0
Ni^{2+}	20	7.55	31.8
	100	11.1	20.8
	200	9.94	23.4
Zn^{2+}	20	12.4	21.2
	100	12.0	21.2
	200	12.3	29.6

organism.

Furthermore, the authors studied the effect of Zn^{2+} on the accumulation of NAD in *S. carlsbergensis*. Figure 2 shows the effect of the Zn^{2+} level in the medium on the accumulation of NAD. From the results the suitable Zn^{2+} concentration was determined 260 $\mu\text{g/liter}$, and the concentration was applied in the following studies.

FIG. 2. Effect of Zn^{2+} Ion.

Culture condition was as same as that in Table V. ●—●: Growth, ○—○: NAD accumulated (mg/100 ml medium), △—△: NAD (mg/g dry cells)

Effect of the P_i/Mg^{2+} ratio. To obtain the optimal concentration of phosphate (P_i) and Mg^{2+} , investigations were carried out. From the results the optimal concentration was determined as 0.5% of KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, respectively (Table VI).

TABLE VI. EFFECT OF P_i/Mg RATIO

In addition to KH_2PO_4 and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, all media contained 5% glucose, 1.5% casein hydrolysate, 1.2 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g/ml}$ Ca-pantothenate, 5 $\mu\text{g/ml}$ thiamine-HCl and 100 $\mu\text{g/ml}$ inositol, pH 5.0. Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

KH_2PO_4 (%)	$\text{MgSO}_4^{a)}$ (%)	Growth (mg/ml)	NAD accumulated	
			(mg/g cells)	mg/100 ml medium)
0	0	2.51	14.1	3.5
	0.1	11.8	18.6	22.0
	0.5	12.3	17.7	21.8
	1.0	11.5	17.2	19.8
0.2	0	2.58	11.5	3.0
	0.1	11.9	30.1	35.8
	0.5	13.1	31.7	41.5
	1.0	12.9	34.6	44.6
1.0	0	0.9	23.3	2.1
	0.1	13.6	32.6	44.3
	0.5	13.0	35.7	44.4
	1.0	12.2	36.7	44.5
2.0	0	1.0	20.8	2.2
	0.1	10.0	40.9	40.9
	0.5	11.9	33.9	40.3
	1.0	12.6	32.7	41.2

^{a)} $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was used.

TABLE VII. EFFECT OF NITROGEN SOURCES

Besides the below constituents, all media contained 5% glucose, 0.5% KH_2PO_4 , 0.5% K_2HPO_4 , 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.2 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, pH 5.0. Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

Nitrogen source (1.5%)	(A)			(B)		
	Growth (mg/ml)	NAD accumulated		Growth (mg/ml)	NAD accumulated	
		(mg/g cells)	(mg/100 ml medium)		(mg/g cells)	(mg/100 ml medium)
None	1.5	11.4	1.7	2.1	18.4	3.9
$(\text{NH}_4)_2\text{SO}_4$	2.5	2.2	0.6	9.4	10.0	9.4
Casein hydrolysate	2.3	14.4	3.3	11.1	33.4	37.1
Peptone	2.5	5.3	1.3	11.9	27.2	32.4
Yeast extract	13.7	24.2	33.2	13.9	25.7	35.7
Meat extract	3.7	11.9	4.4	6.4	7.5	4.8
Malt extract	2.6	15.2	3.9	2.7	12.0	3.2
C.S.L. ^{a)}	8.7	13.3	11.6	12.3	17.6	21.7
Urea	2.3	9.8	2.2	4.6	29.0	13.3

(A): No addition, (B): Ca-pantothenate (10 $\mu\text{g}/\text{ml}$), thiamine-HCl (5 $\mu\text{g}/\text{ml}$) and inositol (100 $\mu\text{g}/\text{ml}$) were added.

^{a)} Corn steep liqure

Effect of nitrogen sources. Since *S. carlsbergensis* is known as the strain requiring amino acids for the growth, the authors used casein hydrolysate (vitamin free) as the nitrogen source. To obtain a more suitable nitrogen source, effect of various nitrogen sources for the accumulation of NAD were investigated. As the results shown in Table VII, casein hydrolysate was found to be the advantageous nitrogen source not only for the growth but also for the accumulation of NAD. Peptone, yeast extract and corn steep liquor (C.S.L.) were also effective on both the growth and the accumulation of NAD.

Effect of carbon sources. The yeast assimilated raffinose, sucrose, maltose and galactose but lactose, xylose, ribose and dextrin. Sucrose, glucose, maltose, fructose and mannose were effective on the accumulation of NAD (Table VIII). In the following studies glucose was used as carbon source, because glucose was easily available and effective for the growth of the cells and the accumulation of NAD.

Effect of fatty acids. It is well known that NAD is closely connected with the metabolism of fatty acid. Therefore, the effect of fatty

TABLE VIII. EFFECT OF SUGARS

Besides the below constituents, all media contained 1.5% casein hydrolysate, 0.5% KH_2PO_4 , 0.5% K_2HPO_4 , 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g}/\text{ml}$ Ca-pantothenate, 5 $\mu\text{g}/\text{ml}$ thiamine-HCl and 100 $\mu\text{g}/\text{ml}$ inositol, pH 5.0. Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

Sugar (5%)	Growth (mg/ml)	NAD accumulated	
		(mg/g cells)	(mg/100 ml medium)
Raffinose	7.6	6.7	5.1
Sucrose	12.6	25.8	32.5
Maltose	8.5	25.9	21.9
Glucose	12.3	28.8	35.4
Fructose	13.6	25.4	34.6
Mannose	13.3	24.1	32.1
Galactose	8.9	20.7	18.4
Ribose	0.3	—	—
Xylose	0.8	—	—
Dextrin	2.0	—	—

acids on the accumulation of NAD was investigated using various carbon numbers ($\text{C}_{12} \sim \text{C}_{18}$) of fatty acids. The results are shown in Table IX. The fatty acids used in the study were effective except laurate whose carbon number is 12. When lauric acid (carbon number 12) was used, NAD produced was leaked completely to the culture broth.

TABLE IX. EFFECT OF FATTY ACIDS IN THE PRESENCE AND ABSENCE OF SURFACTANT

Besides the below constituents, all media contained 5% glucose, 1.5% casein hydrolysate, 0.5% KH_2PO_4 , 0.5% K_2HPO_4 , 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 mg/liter $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g/ml}$ Ca-pantothenate, 5 $\mu\text{g/ml}$ thiamine-HCl and 100 $\mu\text{g/ml}$ inositol, pH 5.0. Adenine (3 mg/ml) and nicotinamide (3 mg/ml) were added at a 24-hr culture period.

Fatty acid (mg/ml)	(A)		(B)	
	NAD accumulated (mg/100 ml medium)		NAD accumulated (mg/100 ml medium)	
	Intracellular	Extracellular	Intracellular	Extracellular
None	41.0	0	0	48.9
Laurate-Na 0.1	36.7	0	0	11.1
1.0	0	11.2	0	8.9
Myristate-Na 0.1	55.3	0	0	60.8
1.0	62.5	0	0	75.2
Palmitate-Na 0.1	57.0	0	0	68.0
1.0	58.8	0	0	66.5
Stearate-Na 0.1	57.4	0	0	68.9
1.0	55.8	0	0	61.9
Oleate-Na 0.1	53.0	0	0	63.0
1.0	62.0	0	0	65.0

(A) No addition, (B) Two mg/ml sodium cetyl sulfate was added at a 28-hr culture period.

TABLE X. EFFECT OF SURFACTANTS ON NAD ACCUMULATION

Culture condition was as same as that in Table IX. Surfactants were added at a 20, 24, or 28-hr culture period.

Surfactant (0.02 ~ 0.1 %)	Growth inhibition	NAD accumulated	
		Intra-cellular	Extra-cellular
None	—	+	—
Nonionics	—	+	—
Cationics	+	—	—
Anionics			
SLS	+	—	+
SMS	—	—	+
SCS	—	—	+
SSS	—	+	—
Nikkol OTP	—	+	—
" SBL-4N	—	+	—
" DLP-10	+	—	—
" TLP-4	—	+	—
" Sarcocinate	+	—	—
LN			
Amphotherics			
Anon BF	±	+	—
" BT	+	+	—
Lipomin SA	—	+	—
" SH	—	+	—
Rebone 15	—	+	—
" 20	—	+	—

Abbreviation: SLS, sodium lauryl sulfate; SCS, sodium cetyl sulfate; SMS, sodium myristyl sulfate; SSS, sodium stearyl sulfate.

TABLE XI. EFFECT OF ALKYL SULFATE SURFACTANT

Culture condition was as same as that in Table X. Surfactants were added at a 28-hr culture period.

Surfactant (%)	NAD accumulated (mg/100 ml medium)		
	Intra-cellular	Extra-cellular	Total
None	48.2	0	48.2
SLS 0.05	0	31.3	31.3
0.10	0	29.9	29.9
0.20	0	24.3	24.3
0.40	0	25.4	25.4
SMS 0.05	0	47.9	47.9
0.10	0	48.7	48.7
0.20	0	36.0	36.0
SCS 0.05	19.4	22.7	42.1
0.10	23.8	22.4	49.2
0.20	0	48.3	48.3
0.40	0	44.8	44.8
SSS 0.05	48.7	0	48.7
0.10	47.0	0	47.0
0.20	45.4	0	45.4
0.40	42.0	0	42.0

Effect of surfactants. Table X shows the effect of surfactants. When the surfactants belonging to the anionic type, for example, SLS (sodium lauryl sulfate), SMS (sodium myristyl sulfate) or SCS (sodium cetyl sulfate)

were used, the accumulated NAD was almost leaked into the culture broth. SCS was especially suitable among the tested surfactants because the surfactant did not inhibit the accumulation of NAD, and the optimal concentration of SCS was found to be around 0.2% (Table XI).

Öbayashi¹⁵⁾ reported that the NAD of *S. cerevisiae* and *C. krusei* leaked by the action of cetyltrimethyl ammonium bromide (a cationic surfactant). In this case the leaked NAD was decomposed unless the yeast was treated at low temperature and at below pH 3.5. In the present investigation, the leaked NAD was not decomposed even at 37°C, at pH 5.0.

NAD accumulation under the optimal condition. A typical NAD accumulation is illustrated in Fig. 3. The experiment was

FIG. 3. Time Course of NAD Accumulation.

●—●: Growth, □—□: pH, ○—○: total NAD, ●—●: Extracellular NAD, △—△: Intracellular NAD.

conducted as follows: The yeast was cultured under the optimal conditions for 24 hr, and adenine and nicotinamide were added to make the final concentration of 0.3%, then cultivation was continued. At the period of 28-hr cultivation, SCS was added to make the final concentration of 0.2%. NAD increased line-

arly after the addition of adenine and nicotinamide. The accumulated NAD was leaked by adding SCS, which did not inhibit the growth of the cells. Finally, the accumulated NAD was completely leaked into the culture broth, and after a 72-hr culture period about 75 mg/100 ml of NAD was accumulated in the culture broth.

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REFERENCES

- 1) H. Holzer, S. Geidschmidt, W. Lamprecht and E. Heinrich, *Z. Physiol. Chem.*, **297**, 1 (1954).
- 2) I. Takebe and K. Kitahara, *J. Gen. Appl. Microbiol.*, **9**, 31 (1963).
- 3) K. Hayano, I. Takebe and K. Kitahara, *Nippon Nogeikagaku Kaishi*, **38**, 515 (1964).
- 4) K. Hayano, I. Takebe and K. Kitahara, *Amino acid and Nucleic acid*, **9**, 37 (1964).
- 5) J. London and M. Knight, *J. Gen. Microbiol.*, **44**, 241 (1966).
- 6) K. Nakayama, Z. Sato, H. Tanaka and S. Kinoshita, *Amino acid and Nucleic acid*, **18**, 25 (1968).
- 7) T. Sakai, T. Uchida and I. Chibata, *J. Chromatogr.*, **66**, 111 (1972).
- 8) M. M. Ciotti and N. O. Kaplan, "Methods in Enzymology," Vol. III, ed. by S. P. Colowick and N. O. Kaplan, Academic Press Inc., New York, N.Y., 1957, p. 890.
- 9) D. Miwa and H. Ueyama, *J. Fermt. Technol.*, **38**, 299 (1960).
- 10) D. Miwa and H. Ueyama, *ibid.*, **38**, 388 (1960).
- 11) R. J. P. Williams, F. L. Hoch and B. L. Vallee, *J. Biol. Chem.*, **232**, 465 (1958); *idem, ibid.*, **232**, 458 (1958).
- 12) B. L. Vallee, F. L. Hoch, S. J. Adelstein and W. E. C. Wacker, *J. Am. Chem. Soc.*, **78**, 5859 (1956).
- 13) B. L. Vallee and F. L. Hoch, *J. Biol. Chem.*, **225**, 185 (1957).
- 14) A. Nason, N. O. Kaplan and S. P. Colowick, *ibid.*, **188**, 397 (1951).
- 15) A. Öbayashi, *Nippon Nogeikagaku Kaishi*, **37**, 265 (1963).