Identification and Growth Characteristics of Alkalophilic *Corynebacterium* sp. Which Produces NAD(P)-Dependent Maltose Dehydrogenase and Glucose Dehydrogenase

Yoshinori KOBAYASHI* and Koki HORIKOSHI

*The Institute of Physical and Chemical Research, Wako,
Saitama 351, Japan*

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An alkalophilic bacterium, strain No. 93-1, was isolated from soil. On the basis of the morphological and biochemical characteristics, this strain was identified to belong to the genus *Corynebacterium*. A crude extract of this strain showed NAD-dependent dehydrogenase activity on maltose (NAD-MalDH) and glucose (GlcDH). The ratio between these two dehydrogenase activities varied by cultural conditions. And NADP was also a suitable co-factor for dehydrogenase activity on maltose (NADP-MalDH). There were remarkable differences in an optimal temperature and pH stability among these three activities. According to these experimental results, we expected that, at least, three kinds of NAD (P)-dependent dehydrogenase acted on maltose and glucose in the crude extract of this microorganism.

In the last decade, many alkalophilic microorganisms have been isolated and characteristics of extracellular enzymes produced by them have been reported.^{1~7)} Also, physiological properties of these bacteria have been studied on amino acids transport,⁸⁾ protein synthesis,⁹⁾ and RNA synthesis.¹⁰⁾ But up to now, we have little knowledge about the reason why such high pH is necessary for the growth. In order to recognize this unique property of these bacteria, it is very important to get much information of intracellular enzymes in main metabolic pathways.

Many species of microorganisms which possessed the oxidation activity of glucose and other aldoses have been reported. And one of the responsible enzymes, glucose dehydrogenase was reviewed by Hauge and Sadoff.¹¹⁾ In Bacillaceae, NAD(P)-dependent glucose dehydrogenase (GlcDH; EC 1.1.1.47) has been studied in a view point of spore-specific enzyme.^{12~15)} As regards dehydro-

genase which acts on disaccharides, we have few reports. Nishizuka and Hayaishi reported on a partially purified lactose dehydrogenase of *Pseudomonas graveolens*.¹⁶⁾ Westermarck and Eriksson described about cellobiose dehydrogenase of *Polyporus versicolore* and *Sporotrichum pulverulentum*.¹⁷⁾

We isolated an alkalophilic *Corynebacterium* strain which produced three kinds of NAD(P)-dependent dehydrogenase which acted on carbohydrates. The first was a NAD-dependent dehydrogenase which exhibited high activity on maltose, "NAD-dependent maltose dehydrogenase (NAD-MalDH)." The second was "NADP-dependent maltose dehydrogenase (NADP-MalDH)." And the third was a NAD-dependent glucose dehydrogenase (GlcDH). This paper deals with the isolation and identification of this bacterium, some cultural conditions for enzyme production, and properties of the crude extract.

MATERIALS AND METHODS

Isolation of alkalophilic microorganisms. Alkalophilic microorganisms were isolated from soils in Japan²⁾ using Basal Medium II; 1% soluble starch, 0.5% peptone (polypepton), 0.5% yeast extract, 0.1%

* On leave from Meito Sangyo Co., Ltd.

Abbreviations; NAD-MalDH, NAD-dependent maltose dehydrogenase; NADP-MalDH, NADP-dependent maltose dehydrogenase; GlcDH, NAD-dependent glucose dehydrogenase.

K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, 2% agar, 1% Na_2CO_3 (separately sterilized).

Culture.

(a) *Screening.* Ten ml of the liquid Basal Medium II in a test tube (24×200 mm) was autoclaved at 120°C for 15 min. One loopful of isolated microorganisms was inoculated and cultured aerobically for 2 days at 37°C on a reciprocal shaker.

(b) *Examination of cultural conditions for enzyme production.* Effect of initial pH of the medium, carbon sources and nitrogen sources were examined as follows; one loopful of the bacteria was inoculated to 50 ml of the liquid medium in a 300-ml Erlenmeyer flask and cultured at 30°C for 24 hr on a rotary shaker. Time course examination of enzyme production was examined in 600 ml of the liquid medium in a 2-L Erlenmeyer flask at 30°C on a rotary shaker.

Preparation of the crude extract. Cells were harvested and washed with 50 mM phosphate buffer, pH 7.5, by centrifugation. The same buffer was added to cells and subjected to sonic oscillation at 20 KHz for 3 to 5 min under cooling with ice. The supernatant fluid was obtained as the crude extract by centrifugation at $20,000 \times g$ for 20 min.

Enzymes assay. NAD(P)-MalDH and GlcDH activities were assayed spectrophotometrically by the measurement of the increase of O.D. 340 nm with an Hitachi spectrophotometer model 200-10 at 30°C. Assay system contained 0.5 ml of 100 mM glycine-NaOH buffer, pH 10.0, 0.1 ml of 30 mM NAD(P) solution, 0.1 ml of 1 M maltose or glucose solution, the crude extract and water to make up a total volume of 1 ml in a cuvette with a light path of 1 cm. One unit of these enzyme activities was defined as the amount of enzyme which catalyzed the reduction of 1 μ mol of NAD(P) per min at 30°C. NAD(P)H oxidase activities were also assayed spectrophotometrically by the measurement of the decrease of O.D. 340 nm with the same equipment at 30°C. Assay mixture contained 0.5 ml of 100 mM glycine-NaOH buffer, pH 10.0, 0.1 ml of 1 mM NAD(P)H solution, the crude extract and water to make up a total volume of 1 ml in a cuvette with a light path of 1 cm.

Characterization and identification of the isolated bacteria. The isolated bacterium was characterized and identified according to Komagata's reports¹⁸⁻²²⁾ and "Bergey's Manual of Determinative Bacteriology 8th ed."

Analytical methods. The bacterial growth was measured by the measurement of O.D. 610 nm of the culture broth. Protein was determined from the absorbancies of O.D. at 260 and 280 nm.

Chemicals. NAD, NADP, NADH and NADPH were purchased from Kyowa Hakko Kogyo Co. Ltd. Maltose was obtained from Nakarai Chemical Co., Ltd. Glucose was purchased from Wako Pure Chemical Co. Ltd.

RESULTS

Characterization and identification of the isolated microorganism.

About 150 soils were examined and approximately 600 colonies of bacteria, a few colonies of molds and actinomycetes were isolated. In these colonies, one yellow colored bacterium, strain No. 93-1, possessed strong activity of NAD-dependent dehydrogenase acted on maltose (NAD-MalDH). And also, this crude extract contained two or three times higher activity of NAD-dependent glucose dehydrogenase (GlcDH) than NAD-MalDH. The isolated alkalophilic microorganism, strain No. 93-1, was an aerobic gram positive, non-spore forming, motile, rodshaped bacterium and had peritrichous flagella. The morphological, cultural and biochemical characteristics are summarized in Table I. This bacterium showed good growth at the range of pH 7 to 11 in Basal Medium II. In nutrient broth, the alkaline condition (pH 10) was more suitable for the growth than neutral condition (pH 7). On the basis of classification reported by Komagata *et al.*,²²⁾ this bacterium had both properties of genus *Corynebacterium* and *Brevibacterium*. In "Bergey's Manual of Determinative Bacteriology 8th ed.," genus *Brevibacterium* is omitted. Therefore, this bacterium should belong to *Corynebacterium*. Although, there has been no report on a *Corynebacterium* sp. which is able to grow on such alkaline medium, the strain is designated as *Corynebacterium* sp. No. 93-1 in this study.

Cultural conditions for enzyme production

(a) *Effect of initial pH on enzyme production.* The effect of the initial pH of cultural medium on enzyme production was investigated using modified Basal Medium

TABLE I. MORPHOLOGICAL, CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF STRAIN No. 93-1

Morphological characteristics		
Form	Rods	
Size (μ)	0.8-1.0 $\times$ 2.0-3.0	
Motility	Positive	
Flagellation	Peritrichous flagella	
Spore formation	Negative	
Gram stain	Positive	
Acid fastness	Negative	
Metachromatic granule	Negative	
Type of cell division	Snapping	
Pleomorphism	Not distinct	
Cultural characteristics		
	pH 7	pH 10
Nutrient broth	±	+
Nutrient agar slant	±	+
Nutrient glucose broth	+	+
Nutrient glucose agar slant	+	++
Basal Medium I*	++	++
Basal Medium I agar slant	++	++
Basal Medium II	++	++
Basal Medium II agar slant	++	++
Growth in NaCl		
5% in Basal Medium I	+	+
7%	+	+
10%	+	+
Liquefaction of gelatine	+	+
Litmus milk	-	peptonized
Biochemical characteristics		
Obligate aerobic		
Temperature for growth (Basal Medium II, pH 10); 20°C to 40°C		
pH for growth (Basal Medium II); 7 to 11		
Main amino acid of cell wall; DL-DAP		
GC content (%); 65.8		
	pH 7	pH 10
Denitrification	+	+
Reduction of nitrate to nitrite	N.G.**	+
Methylred test	N.G.	N.T.***
V-P test	N.G.	-
Indole production	-	-
H ₂ S production	-	-
O-F test	Oxidation (no gas)	Oxidation (no gas)
Urease test	+	+
Oxidase test	+	+
Catalase test	+	+
Hydrolysis of starch	+	+
Hydrolysis of casein	+	+
Hydrolysis of cellulose	-	-
Utilization of citric acid		
Koser medium	-	-
Christensen medium	+	+
Utilization of ammonium salts	-	-
Water soluble pigment production	-	-
Acid production from sugars (pH 10)		
Positive (without gas); glucose, fructose, xylose, sorbose, lactose, maltose, cellobiose, dextrin, starch.		
Negative; arabinose, rhamnose, mannose, galactose, sucrose, trehalose, raffinose, inulin, glycerol, erythritol, adonitol, mannitol, dulcitol, sorbitol, inositol, arbutin, salicin, α -methyl glucoside.		

* Basal Medium I; 1% glucose, 0.5% peptone (polypepton), 0.5% yeast extract, 0.1% K₂HPO₄, 0.02% MgSO₄·7H₂O, 1% Na₂CO₃ (pH 10).

** N.G.; No growth.

*** N.T.; Not tested.

TABLE II. EFFECT OF INITIAL pH ON ENZYME PRODUCTION

Na ₂ CO ₃ conc. (%)	Initial pH	Final pH	Bacterial growth (O.D. 610)	Protein (mg)	NAD-MalDH (total units)	GlcDH (total units)
0	7.18	8.00	1.08	7.41	0.471	1.32
0.05	8.00	8.43	1.20	8.23	0.508	1.49
0.1	8.89	8.43	1.30	8.92	0.557	2.01
0.2	9.60	8.90	1.32	9.06	0.726	2.98
0.3	9.90	9.12	0.72	4.94	0.690	2.90
0.5	10.20	9.55	0.58	3.98	0.448	1.82
1.0	10.50	10.20	0.35	2.40	0.289	1.16

TABLE III. EFFECT OF CARBON SOURCES ON ENZYME PRODUCTION

Carbon source (1%)	Final pH	Bacterial growth (O.D. 610)	Protein (mg)	NAD-MalDH (total units)	GlcDH (total units)
D-Glucose	8.20	1.82	9.80	0.049	0.067
D-Mannose	8.68	1.91	11.0	0.138	0.420
D-Galactose	8.85	1.92	10.3	0.672	2.40
D-Fructose	8.58	1.98	9.24	0.029	0.082
D-Xylose	8.82	1.97	11.7	0.696	2.52
D-Ribose	8.50	1.38	12.1	0.402	1.00
D-Arabinose	9.23	0.07	0.53	0	0
Maltose	8.73	1.58	10.7	0.540	2.82
Lactose	8.80	1.81	12.6	0.504	1.56
Cellobiose	8.53	2.48	13.4	0.116	0.696
Melibiose	8.72	2.42	11.0	0.570	2.23
Sucrose	8.38	2.44	8.93	0	0.050
D-Mannitol	8.48	2.00	9.45	0.064	0.192
D-Xylitol	9.10	0.83	3.15	0.348	1.44
D-Sorbitol	8.98	1.72	5.04	0.462	1.83
D-Galactitol	9.02	0.96	6.83	0.504	1.39
Inositol	8.78	1.55	4.20	0.346	1.50
Soluble starch	9.02	1.07	7.35	0.660	2.40
Glycerol	8.80	1.28	4.20	0.024	0.044
None	9.03	0.95	5.25	0.372	1.38

II(yeast extract 0.1%). The initial pH of the medium was adjusted by the addition of Na₂CO₃. Table II shows that the optimal concentration of Na₂CO₃ for enzyme production was 0.2%. In the following experiments, this concentration was used.

(b) *Effect of carbon sources on enzyme production.* Various carbohydrates were tested as a carbon source in the modified Basal Medium II (soluble starch was omitted) for enzyme production. As shown in Table III, several kinds of carbohydrates were effective for the bacterial growth and enzyme production. Maltose and glucose were not essential for enzyme production. Therefore, it

was clear that these enzymes were not an inducible type. We chose maltose as a carbon source and the optimal concentration of maltose for enzyme production was 1%.

(c) *Effect of nitrogen sources on enzyme production.* The effect of various nitrogen sources was investigated in the modified Basal Medium II (without peptone (polypepton), containing 1% maltose). Table IV shows that organic nitrogen sources such as soya-bean casein etc. were essential for the growth. But inorganic nitrogen sources such as NH₄NO₃, NH₄Cl, NaNO₃, KNO₃, NH₄H₂PO₄ and (NH₄)₂HPO₄ were not effective. The optimal concentraion of soya-bean

TABLE IV. EFFECT OF NITROGEN SOURCES ON ENZYME PRODUCTION

Nitrogen source (0.5%)	Final pH	Bacterial growth (O.D. 610)	Protein (mg)	NAD-MalDH (total units)	GlcDH (total units)
Peptone (polypepton)	8.90	0.98	4.40	0.419	1.35
Soya-bean casein	8.19	10.6	45.5	3.27	9.68
Yeast extract	8.90	0.97	3.85	0.183	0.637
Beef extract	8.91	0.95	3.05	0.194	0.766
Corn steep liqure	8.70	3.41	21.5	1.29	3.01
Casamino acids	9.02	0.85	3.63	0.254	0.790
Urea	9.71	0.21	0.225	0	0
Ammonium sulfate	8.88	0.48	0.310	0.011	0

casein for enzyme production was 1%. Therefore, for enzyme production, following composition of medium was used; 1% maltose, 1% soya-bean casein, 0.1% yeast extract, 0.1% K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$ and 0.2% Na_2CO_3 .

(d) *Time course examination of enzyme production.* Time course of the bacterial growth and enzyme production were investigated. As shown in Fig. 1, after the decrease of pH, rapid bacterial growth appeared and the enzyme activities reached maximum at 24 hr-culture.

FIG. 1. Time Course of Bacterial Growth and Enzyme Production.

Experimental conditions are described in MATERIALS AND METHODS. $\square$, bacterial growth; $\bullet$, pH; $\circ$, NAD-MalDH activity; $\triangle$, GlcDH activity.

Properties of the curde extract

(a) *Co-factor requirement.* When maltose and glucose were used as the substrate, the requirement of NAD and NADP was examined. As shown in Fig. 2, NADP was slightly effective than NAD on dehydrogen-

FIG. 2. Co-factor Requirement.

Examination was carried out in accordance with the assay method described in MATERIALS AND METHODS. a, maltose+NAD; b, maltose+NADP; c, glucose+NAD; d, glucose+NADP; e, NAD; f, NADP.

ation of maltose and NAD was significantly effective than NADP on dehydrogenation of glucose. And no NAD(P)H oxidase activity was detected during the assay process.

(b) *Effect of pH.* Using NAD and NADP as a co-factor, optimal pH of dehydrogenase activity on maltose was examined. Fig. 3 shows that optimal pH of NAD-MalDH

FIG. 3. Optimal pH of Enzyme Activity.

pH 7-8, phosphate buffer; pH 9-11, glycine-NaOH buffer. $\circ$, NAD-MalDH; $\bullet$, NADP-MalDH; $\triangle$, GlcDH.

FIG. 4. pH Stability of Enzyme Activity.

After the treatment of the crude extract in each buffer for 30 min at 30°C, the residual activity was measured. pH 4.5~5, succinate buffer; pH 6~8.5, phosphate buffer; pH 9~11, glycine-NaOH buffer. ○, NAD-MalDH; ●, NADP-MalDH; △, GlcDH,

was around 10.5 and that of NADP-MalDH was around 10.0. As shown in Fig. 2, NADP-dependent glucose dehydrogenase activity was very low. Therefore, only optimal pH of NAD-dependent glucose dehydrogenase activity was investigated. Fig. 3 shows that optimal pH of GlcDH was around 10.0 to 10.2. Fig. 4 shows pH stability of these three dehydrogenase activities. NAD-MalDH was stable at the pH range of 6.5 to 9.0. NADP-MalDH was stable at the pH range of 7.0 to 8.0. The stable pH range of GlcDH was 6.0 to 8.0.

(c) *Effect of temperature.* Fig. 5 shows the optimal temperature of these activities. Optimal temperature of NAD-MalDH was 50°C and that of NADP-MalDH was 40 to 45°C. As regards GlcDH, 35°C was optimal. Thermal stability was shown in

FIG. 5. Optimal Temperature of Enzyme Activity.
○, NAD-MalDH; ●, NADP-MalDH; △, GlcDH.

FIG. 6. Thermal Stability of Enzyme Activity.

After the treatment of the crude extract with each temperature for 10 min in 50 mM phosphate buffer pH 7.0, the residual activity was measured. ○, NAD-MalDH; ●, NADP-MalDH; △, GlcDH.

Fig. 6. NAD-MalDH was stable up to 40°C. NADP-MalDH and GlcDH were stable up to 35°C and lost these activities in the similar degree at the above temperature.

DISCUSSION

Glucose dehydrogenase has been known in various microorganisms and reviewed by Hauge and Sadoff.¹¹⁾ The enzyme was classified into two types. One was a particle-bound type and existed mainly in *Pseudomonas* and *Acetobacter* species. The enzyme in *Pseudomonas* sp. could use oxygen, 2,6-dichlorophenolindophenol and phenazine methosulfate as an electron acceptor, but could not use NAD and NADP. This enzyme acted on several kinds of aldoses, but it showed low activity on maltose and lactose. However, when it was solubilized, it acted on these disaccharides in the same degree on glucose. The other was a soluble type and found in *Bacillaceae*. This soluble type glucose dehydrogenase has been studied in a view point of spore-specific enzyme. This enzyme could use NAD and NADP as electron acceptors.

In regards to microorganisms which could oxidize disaccharides, e.g., lactose, maltose and cellobiose to corresponding aldobionic acids, *Pseudomonas* sp. has been well known. Bently *et al.* reported about the oxidation of monosaccharides and disaccharides to cor-

responding aldonic acids and aldobionic acids by the extract of *Pseudomonas* sp.²³⁾ Nishizuka and Hayaishi reported on the partially purified lactose dehydrogenase of *Pseudomonas graveolens*.¹⁶⁾ This enzyme could use artificial electron acceptor such as 2,6-dichlorophenolindophenol, methylen blue and ferri-cyanide, but could not use oxygen, pyridine nucleotides and heart muscle cytochrome C. In spite of their attempt of further purification, the enzyme was not purified completely. Westermarck and Eriksson reported on cellobiose:quinone oxidoreductase (cellobiose dehydrogenase) of white-rot fungi, *Polyporus versicolore* and *Sporotrichum pulverulentum*, in wood degradation. This enzyme showed high specificity on cellobiose and did not act on maltose and glucose.

We searched for NAD-dependent dehydrogenase which showed high activity on maltose in alkalophilic microorganisms. An isolated strain was identified as *Corynebacterium* sp. As shown in Table II, this strain showed good growth at the range of pH 7 to 10 and recognized as facultative alkalophilic bacteria. The cell-free extract of this strain showed high NAD-dependent dehydrogenase activity on maltose and glucose. One of the main point of the examination of cultural conditions for enzyme production was to make clear whether these two activities were separate entities or not. In the results shown in Tables II to IV, the ratio of the activity of GlcDH/NAD-MalDH varied from about 2.3 to 6. In the examination of properties of the crude extract, we found that NADP was also a suitable co-factor for dehydrogenase activity on maltose. Therefore, we compared the properties of dehydrogenases. As shown in Figs. 3 and 6, there were slight differences in optimal pH and thermal stability among these three activities. In pH stability and optimal temperature, there were remarkable differences. In addition, these three activities were located in soluble fraction obtained by the centrifugation at $100,000\times g$. On the basis of these experimental results, we expected

that, at least, three kinds of NAD or NADP-dependent dehydrogenase acted on maltose and glucose existed in the crude extract and these NAD-MalDH and NADP-MalDH were new type enzymes. Therefore, works on the purification of these enzymes are in progress.

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