

## Screening of aspartate dehydrogenase of bacteria

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Fifty-two strains of bacteria cultured under aerobic conditions and 12 strains of bacteria cultured under anaerobic conditions demonstrated high activity staining of aspartate dehydrogenase with  $\text{NAD}^+$ . Four strains of bacteria cultured under aerobic conditions and 7 strains of bacteria cultured under anaerobic conditions demonstrated high activity staining of aspartate dehydrogenase with  $\text{NADP}^+$ . Seven strains of bacteria cultured under aerobic conditions and 4 strains of bacteria cultured under anaerobic conditions demonstrated high specific activity of aspartate dehydrogenase with  $\text{NAD}^+$ . One strain of bacteria cultured under aerobic conditions and 5 strains of bacteria cultured under anaerobic conditions demonstrated high specific activity of aspartate dehydrogenase with  $\text{NADP}^+$ . A strain of *S. liquefaciens* IFO 12979 showed highest specific activity of aspartate dehydrogenase with  $\text{NAD}^+$  and  $\text{NADP}^+$ .

### Introduction

We have previously reported that the growth of *Klebsiella pneumoniae* IFO 13541 and the amount of vitamin  $\text{B}_{12}$  produced depended exclusively on the concentration of yeast extract added to the medium. The yeast extract components required were identified as aspartic acid and pyrroloquinoline quinone(PQQ). We have also reported the effects of aspartic acid and PQQ on the production of vitamin  $\text{B}_{12}$  by *K. pneumoniae* IFO 13541 and the metabolism of aspartic acid in the vitamin  $\text{B}_{12}$  production<sup>1)-3)</sup>.

On the other hand, a variety of L-amino acid dehydrogenases have been extensively studied, as reviewed by Ohshima and Soda<sup>4)</sup>, and used for the synthesis or measurement of L-amino acids<sup>4),5)</sup>. However, the specific enzyme for L-aspartic acid dehydrogenation has not yet been identified.

We reported a new reaction of aspartic acid dehydrogenation, novel  $\text{NAD}^+$  and  $\text{NADP}^+$  dependent aspartate dehydrogenase, in crude extract of vitamin  $\text{B}_{12}$  producing *K. pneumoniae* IFO 13541<sup>6)</sup>. However, there is no report about other bacteria. Therefore, we did a screenig of aspartate dehydrogenase of bacteria.

### Materials and Methods

#### Organisms

Sixty-eight strains of bacteria were used in this experiment.

#### Medium and culture conditions

The culture medium for bacteria consisted of 0.5% meat extract, 1.0% peptone, 0.5% NaCl and 0.5% aspartic acid(pH 7.0). Bacteria were grown in a test tube 16.5mm in diameter with 5ml of the medium at 28°C for 1day under aerobic conditions, or bacteria were grown in 300ml Erlenmyer flasks with 120ml of the medium at 28°C for 4days under anaerobic

conditions. After cultivation, the culture broth was centrifuged (4°C, 20min, 8000rpm) to collect the cells. The cells were washed twice with 0.85% NaCl and washed with 10mM Tris-HCl buffer (pH 7.0). The cells were stored at -20°C.

#### Preparation of cell free extract

The cells in 10mM Tris-HCl buffer (pH 7.0) were subjected to sonication with an ultrasonic oscillator (BRANSON, SONIFIER 250) 15sec × 2 times below 0~8°C. The undestroyed cells and debris were removed by centrifugation at 15,000rpm for 10min at 4°C. The supernatant solution obtained was used as the cell free extract.

#### Electrophoresis

Gel electrophoresis of the native enzyme was carried out with 7.5% polyacrylamide gel by the Davis<sup>7)</sup> method. Twenty  $\mu$ l of cell free extract containing 5 $\mu$ l of coloring liquid (0.1% bromphenol blue, 40% sucrose) were electrophoresized at 20mA per gel until the dye front reached the bottom of the gel. After electrophoresis, the gel was stained with enzymatic activity staining mixture containing 0.1M Tris-HCl buffer (pH 7.4), 15mM NAD<sup>+</sup>, or NADP<sup>+</sup>, 100mM aspartic acid, 25mM MgCl<sub>2</sub>, 6.5mM phenazine methosulfate and 12mM nitro blue tetrazolium.

#### Enzyme assay

The reaction mixture contained 160 $\mu$ l of 250mM Tris-HCl buffer (pH 7.5), 100 $\mu$ l of 100mM aspartic acid, 50 $\mu$ l of 12mM MgCl<sub>2</sub>, 50 $\mu$ l of crude extract, 50 $\mu$ l of 20 $\mu$ mol NAD<sup>+</sup> or NADP<sup>+</sup> and 190 $\mu$ l of water in a total volume of 600 $\mu$ l. The reaction mixture was replaced by water in a blank. Incubation was done at 30°C in a cuvette with a 1cm light path. The reaction was started by the addition of NAD<sup>+</sup> or NADP<sup>+</sup> and the reaction mixture was measured for absorbance at 340nm (time scan) with a HITACHI 150-20 double beam spectrophotometer. One unit of the enzyme was defined as the amount that catalyzed the formation of 1 $\mu$ mol of NADH or NADPH per mg of protein. Specific activity was expressed as

units per mg of protein. Protein was measured by the method of BIO-RAD Protein Assay with crystalline bovine serum albumin as the standard.

## Results and Discussion

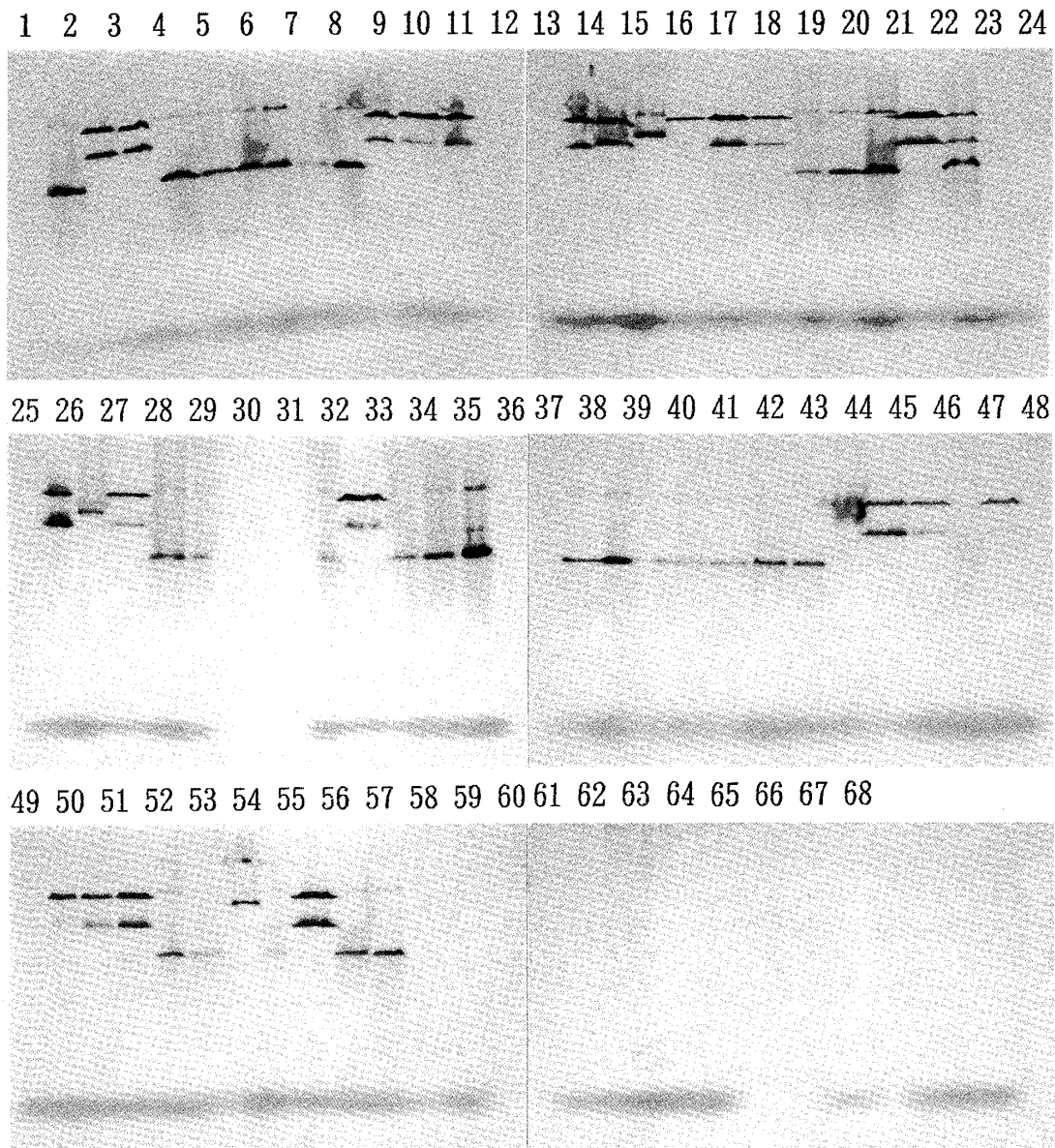
Polyacrylamide gel electrophoresis of the native enzyme followed by activity staining of aspartate dehydrogenase is shown in Fig. 1, 2, 3 and 4. The activity staining and specific activity of aspartate dehydrogenase of bacteria are summarized in Table 1. and 2.

Fifty-two (*Agrobacterium radiobacter* IAM 1526, *Agrobacterium tumefaciens* IAM B-26-1, *Alcaligenes faecalis* IAM B-141-1, *Alcaligenes polymorpha*, *Arthrobacter globiformis* IFO 12137, *Arthrobacter oxydans* IFO 12138, *Arthrobacter pascens* IFO 12139, *Arthrobacter ureafaciens* IFO 12140, *Bacillus brevis* IFO 3331, *Bacillus cereus* IFO 3001, *Bacillus megaterrium* NI 8100 NTH B12, *Bacillus pumilus* IFO3030, *Bacillus sphaericus* IFO 3525, *Bacillus subtilis* IFO 3007, *Bacterium cadaveris* IFO 3731, *Bacterium ketoglutamicus* SO(1) Sionogi Co., Ltd, *Bacterium orleanense* IFO 3259, *Brevibacterium sp.* P145 N. Kato, *Corynebacterium fascians* IAM 1079, *Corynebacterium glutamicum* No. 534 ACTT 13032, *Corynebacterium pseudodiphtheritium*, *Enterobacter cloacae* IAM1221, *Erwinia carotovora* IFO 3380, *Escherichia coli* K-12 IFO 3208, *Klebsiella pneumoniae* IFO 3317, *Klebsiella pneumoniae* IFO 12009, *Klebsiella pneumoniae* IFO 12932, *Klebsiella pneumoniae* IFO 13541, *Klebsiella pneumoniae* IFO 13703, *Micrococcus rubens* IFO 3768, *Proteus mirabilis* IFO 3849, *Proteus vulgaris* IFO 3988, *Pseudomonas fluorescens* IFO 3081, *Pseudomonas graveolens* IFO 3460, *Pseudomonas iodum* IFO 3558, *Pseudomonas solanacearum* IFO 3509, *Pseudomonas striafaciens* IFO 3309, *Sarcina aurantiaca* IFO 3064, *Serratia liquefaciens* IFO 12979, *Serratia plymuthica* IFO 3055, *Staphylococcus aureus* IFO 3060, *Staphylococcus epidermidis* IFO 3762) strains of bacteria cultured under aerobic

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conditions and 12 (*Alcaligenes faecalis* IAM B-141-1, *Bacillus sphaericus* IFO 3525, *Escherichia coli* K-12 IFO 3208, *Pseudomonas fluorescens* IFO 3081, *Pseudomonas graveolens* IFO 3460, *Pseudomonas iodium* IFO 3558, *Pseudomonas solanacearum* IFO 3509, *Pseudomonas striafaciens* IFO 3309, *Sarcina aurantiaca* IFO 3064, *Serratia plymuthica* IFO 3055, *Bifidobacterium adolescentis* M101-4, *Bifidobacterium bifidum*

A234-4) strains of bacteria cultured under anaerobic conditions demonstrated high activity staining of aspartate dehydrogenase with  $\text{NAD}^+$ . Four (*Alcaligenes faecalis* IAM B-141-1, *Bacillus brevis* IFO 3331, *Bacillus pumilus* IFO 3030, *Bacillus sphaericus* IFO 3525) strains of bacteria cultured under aerobic conditions and 7 (*Alcaligenes faecalis* IAM B-141-1, *Bacillus pumilus* IFO 3030, *Bacillus sphaericus* IFO



**Fig. 1.** Activity staining.

The activity staining of aspartate dehydrogenase with  $\text{NAD}^+$  was examined by the method described in materials and methods under aerobic conditions. Number 1~68 is the same as Table 1.

3525, *Serratia liquefaciens* IFO 12979, *Serratia marcescens* IFO 3054, *Bifidobacterium adolescentis* M101-4, *Bifidobacterium bifidum* A234-4) strains of bacteria cultured under anaerobic conditions demonstrated high activity staining of aspartate dehydrogenase with NADP<sup>+</sup>.

Seven (*Bacterium ketoglutamicus* SO(1), *Erwinia carotovora* IFO 3380, *Klebsiella pneumoniae* IFO 3317, *Proteus vulgaris* IFO 3988, *Pseudomonas*

*solanacearum* IFO 3509, *Serratia liquefaciens* IFO 12979, *Serratia plymuthmuthica* IFO 3055) strains of bacteria cultured under aerobic conditions and 4 (*Proteus vulgaris* IFO 3988, *Serratia liquefaciens* IFO 12979, *Bifidobacterium breve* I-53-8, *Lactobacillus clerbueckii* IFO 3202) strains of bacteria cultured under anaerobic conditions demonstrated high specific activity of aspartate dehydrogenase with NAD<sup>+</sup>. One (*Serratia li-*

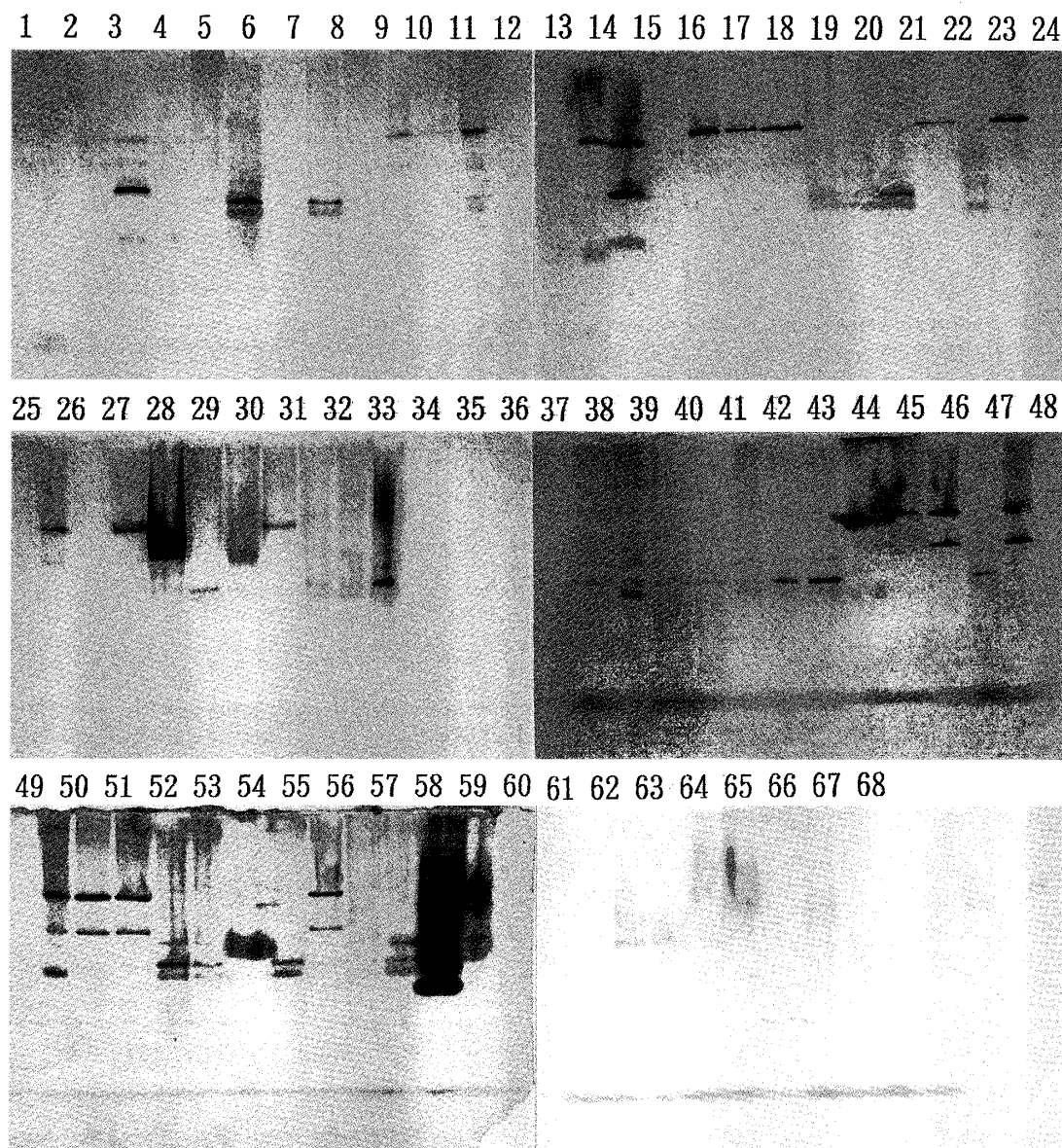


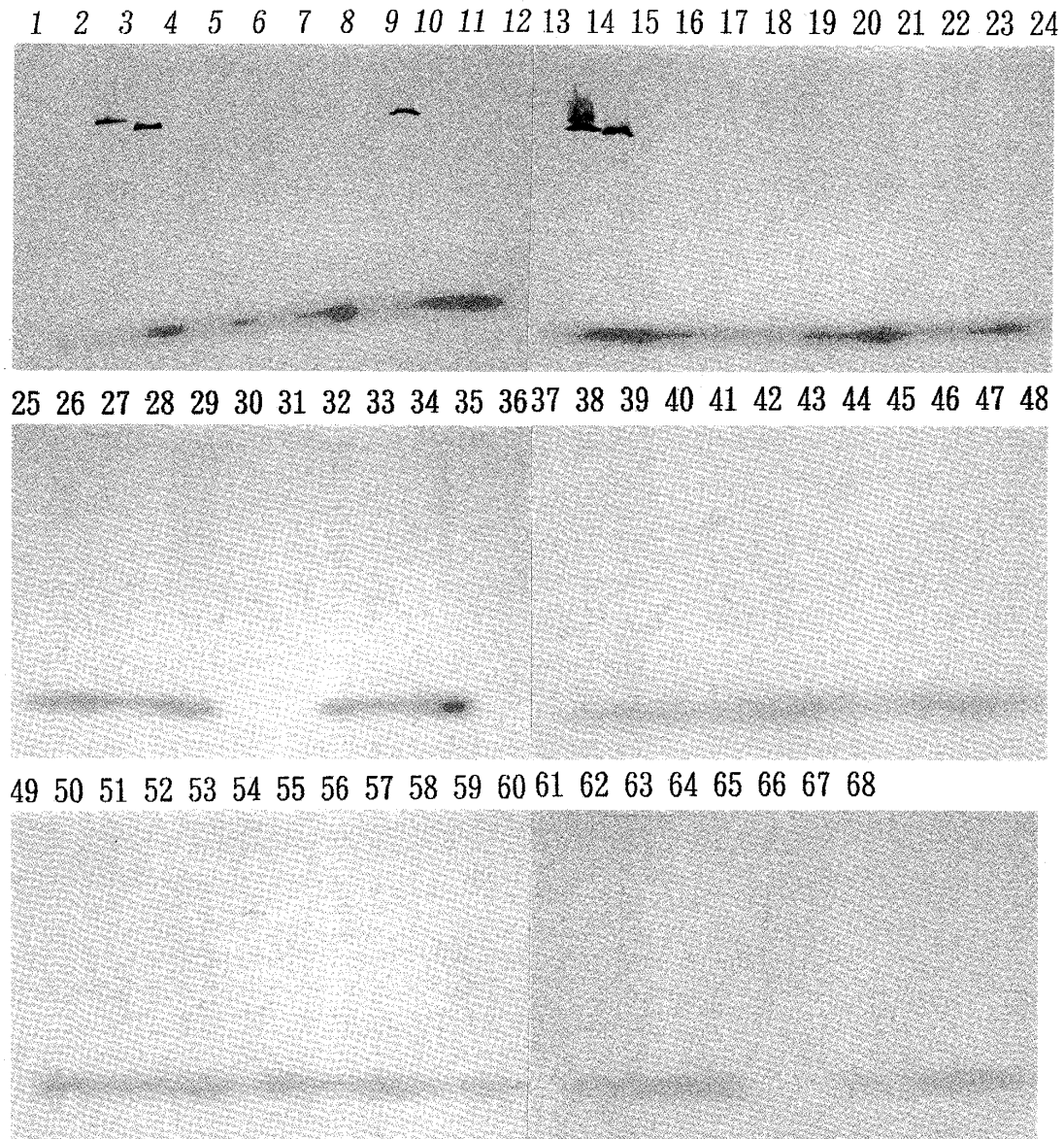
Fig. 2. Activity staining.

The activity staining of aspartate dehydrogenase with NAD<sup>+</sup> was examined by the method described in materials and methods under anaerobic conditions. Number 1~68 is the same as Table 1.

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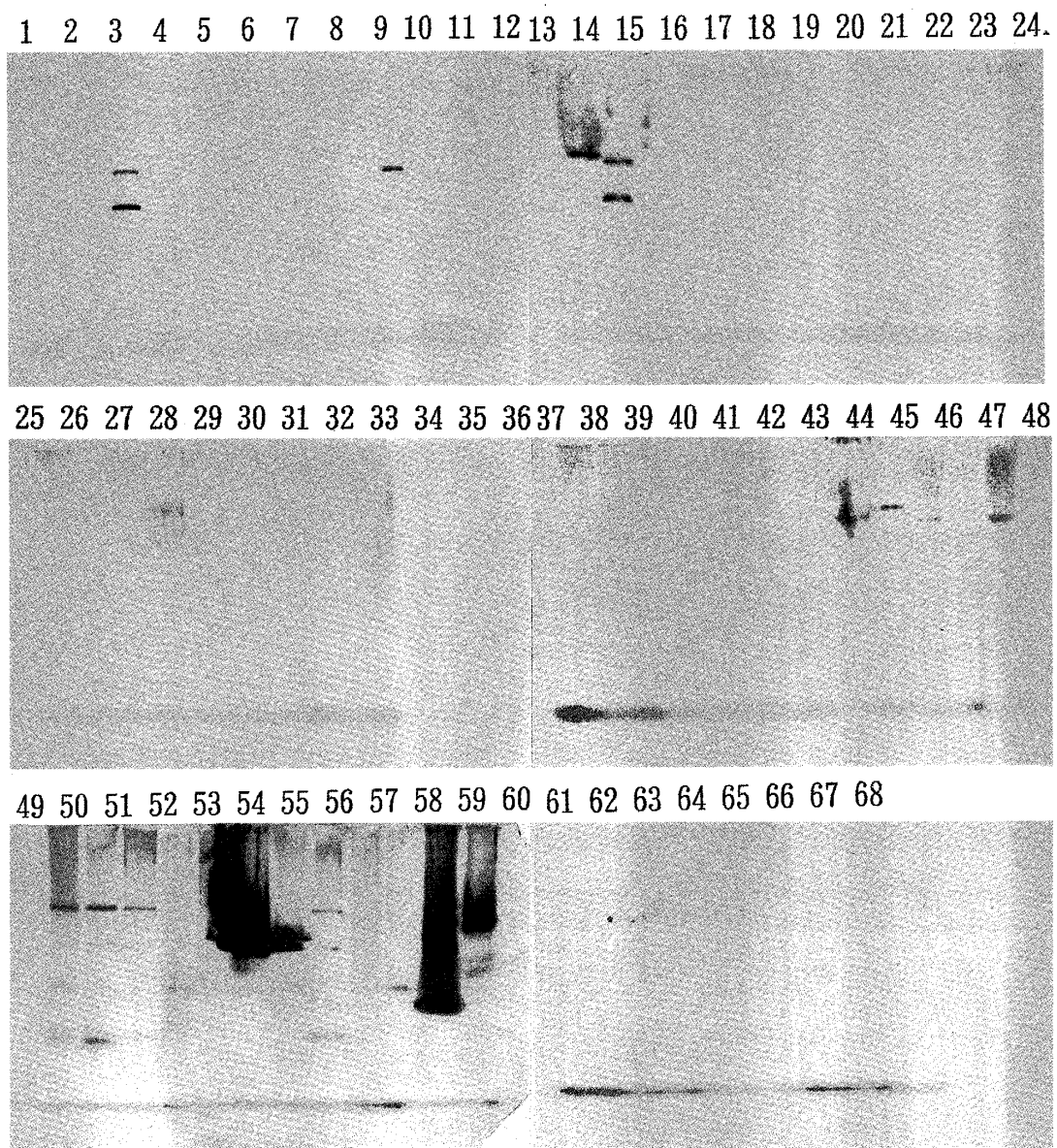
*quefaciens* IFO 12979) strain of bacteria cultured under aerobic conditions and 5 (*Alcaligenes faecalis* IAM B-141-1, *Bacillus sphaericus* IFO 3525, *Enterobacter aerogenes* IFO 3320, *Proteus vulgaris* IFO 3988, *Serratia liquefaciens* IFO 12979) strains of bacteria cultured under anaerobic conditions demonstrated high specific activity of aspartate dehydrogenase with NADP<sup>+</sup>. Judg-

ing from the above effects, *S. liquefaciens* IFO 12979 cultured under aerobic conditions and anaerobic conditions showed highest specific activity of aspartate dehydrogenase with NAD<sup>+</sup> and NADP<sup>+</sup>.



**Fig. 3.** Activity staining.

The activity staining of aspartate dehydrogenase with NADP<sup>+</sup> was examined by the method described in materials and methods under aerobic conditions. Number 1~68 is the same as Table 2.



**Fig. 4.** Activity staining.

The activity staining of aspartate dehydrogenase with  $\text{NADP}^+$  was examined by the method described in materials and methods under anaerobic conditions. Number 1~68 is the same as Table 2.

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Table 1. Activity staining and specific activity of aspartate dehydrogenase with NAD<sup>+</sup> of bacteria

Strain No.	Bacteria	Activity staining*		Specific activity** (Unit)	
		Aerobic	Anaerobic	Aerobic	Anaerobic
1	<i>Agrobacterium radiobacter</i> IAM 1526	++	+	0.000	0.000
2	<i>Agrobacterium tumefaciens</i> IAM B-26-1	++	+	0.000	0.000
3	<i>Alcaligenes faecalis</i> IAM B-141-1	++	++	0.000	0.000
4	<i>Alcaligenes polymorpha</i>	++	+	0.000	0.000
5	<i>Arthrobacter globiformis</i> IFO 12137	++	+	0.000	0.000
6	<i>Arthrobacter oxydans</i> IFO 12138	++	+	0.000	0.000
7	<i>Arthrobacter pascens</i> IFO 12139	++	-	0.000	0.000
8	<i>Arthrobacter simplex</i> IFO 3530	+	+	0.000	0.000
9	<i>Arthrobacter ureafaciens</i> IFO 12140	++	+	0.000	0.000
10	<i>Bacillus brevis</i> IFO 3331	++	+	0.024	0.000
11	<i>Bacillus cereus</i> IFO 3001	++	+	0.030	0.000
12	<i>Bacillus megaterrium</i> NI 8100 NTH B12	++	+	0.022	0.000
13	<i>Bacillus pumilus</i> IFO 3030	++	+	0.000	0.000
14	<i>Bacillus sphaericus</i> IFO 3525	++	++	0.000	0.000
15	<i>Bacillus brevis</i> IFO 3007	++	-	0.000	0.000
16	<i>Bacterium cadaveris</i> IFO 3731	++	+	0.039	0.017
17	<i>Bacterium ketoglutamicus</i> SO(1)Shionogi Co., Ltd	++	+	0.055	0.000
18	<i>Bacterium orleanense</i> IFO 3259	++	+	0.030	0.026
19	<i>Brevibacterium sp.</i> P145 Phage host	+	+	0.000	0.000
20	<i>Brevibacterium sp.</i> P145 N.Kato	++	+	0.000	0.000
21	<i>Corynebacterium fascians</i> IAM 1079	++	+	0.000	0.000
22	<i>Corynebacterium glutamicum</i> No.534 ATCC 13032	++	+	0.028	0.013
23	<i>Corynebacterium pseudodiphtheritium</i>	++	+	0.000	0.000
24	<i>Enterobacter aerogenes</i> IFO 3320	-	+	0.043	0.024
25	<i>Enterobacter cloacae</i> IAM 1221	++	+	0.041	0.000
26	<i>Erwinia arvideae</i> IFO 3830	+	-	0.004	0.000
27	<i>Erwinia carotovora</i> IFO3380	++	+	0.055	0.026
28	<i>Escherichia coli</i> K12 IFO 3208	++	++	0.000	0.000
29	<i>Escherichia freundii</i> S-96	+	+	0.000	0.000
30	<i>Escherichia intermedia</i> A-21	+	+	0.000	0.000
31	<i>Klebsiella pneumoniae</i> IFO 3317	++	+	0.059	0.019
32	<i>Klebsiella pneumoniae</i> IFO 12009	+	+	0.000	0.000
33	<i>Klebsiella pneumoniae</i> IFO 12019	++	+	0.000	0.000
34	<i>Klebsiella pneumoniae</i> IFO 12932	++	-	0.000	0.000
35	<i>Klebsiella pneumoniae</i> IFO 13541	++	+	0.000	0.000
36	<i>Klebsiella pneumoniae</i> IFO 13703	++	+	0.000	0.000
37	<i>Micrococcus luteus</i> IFO 3763	+	+	0.000	0.000
38	<i>Micrococcus lysodeikticus</i> Fleming IFO 3333	+	+	0.000	0.000
39	<i>Micrococcus roseus</i> IFO 3764	+	+	0.000	0.000
40	<i>Micrococcus rubens</i> IFO 3768	++	+	0.000	0.000
41	<i>Proteus mirabilis</i> IFO 3849	++	+	0.000	0.000
42	<i>Proteus morgani</i> IFO 3168	+	+	0.008	0.007
43	<i>Proteus vulgaris</i> IFO 3988	++	+	0.061	0.054
44	<i>Pseudomonas fluorescens</i> IFO 3081	++	++	0.043	0.013
45	<i>Pseudomonas fragi</i> IFO 3458	-	+	0.000	0.000
46	<i>Pseudomonas graveolens</i> IFO 3460	++	++	0.029	0.033
47	<i>Pseudomonas iodium</i> IFO 3558	++	++	0.039	0.018
48	<i>Pseudomonas solanacearum</i> IFO 3509	++	++	0.050	0.014
49	<i>Pseudomonas striafaciens</i> IFO 3309	++	++	0.029	0.000
50	<i>Sarcina aurantiaca</i> IFO 3064	++	++	0.000	0.000
51	<i>Sarcina lutea</i> IFO 1099	+	+	0.000	0.000
52	<i>Serratia liquefaciens</i> IFO 12979	++	+	0.111	0.222
53	<i>Serratia marcescens</i> IFO 3054	+	+	0.000	0.000
54	<i>Serratia plymuthica</i> IFO 3055	++	++	0.045	0.000
55	<i>Staphylococcus aureus</i> IFO 3060	++	+	0.000	0.000
56	<i>Staphylococcus epidermidis</i> IFO 3762	++	+	0.000	0.000
57	<i>Bifidobacterium adolescentis</i> M101-4	-	++	0.000	0.008
58	<i>Bifidobacterium bifidum</i> A234-4	-	++	0.000	0.004
59	<i>Bifidobacterium breve</i> I-53-8	-	-	0.000	0.129
60	<i>Bifidobacterium infantis</i> I-10-5	-	+	0.000	0.005
61	<i>Bifidobacterium longum</i> M101-2	-	+	0.000	0.014
62	<i>Lactobacillus bulgaricus</i> IFO 13953	-	+	0.000	0.000
63	<i>Lactobacillus clerbueckii</i> IFO 3202	-	+	0.000	0.039
64	<i>Lactobacillus paracasei</i> IFO 3953	-	-	0.000	0.000
65	<i>Lactococcus cremoris</i> IFO 3427	-	+	0.000	0.000
66	<i>Lactococcus lactis</i> IFO 12007	-	+	0.000	0.000
67	<i>Pediococcus acidilactici</i> IFO 3888	-	-	0.000	0.000
68	<i>Streptococcus thermophilus</i> IFO 13957	-	-	0.000	0.000

\*. Activity staining ++, higher; +, normal; -, no activity.

\*\*, Measurement by absorbance at 340nm.

Table 2. Activity staining and specific activity of aspartate dehydrogenase with NADP<sup>+</sup> of bacteria

Strain No.	Bacteria	Activity staining*		Specific activity** (Unit)	
		Aerobic	Anaerobic	Aerobic	Anaerobic
1	<i>Agrobacterium radiobacter</i> IAM 1526	—	—	0.000	0.000
2	<i>Agrobacterium tumefaciens</i> IAM B-26-1	—	—	0.021	0.000
3	<i>Alcaligenes faecalis</i> IAM B-141-1	++	++	0.049	0.037
4	<i>Alcaligenes polymorpha</i>	—	—	0.000	0.000
5	<i>Arthrobacter globiformis</i> IFO 12137	—	—	0.000	0.000
6	<i>Arthrobacter oxydans</i> IFO 12138	—	—	0.000	0.000
7	<i>Arthrobacter pascens</i> IFO 12139	—	—	0.000	0.000
8	<i>Arthrobacter simplex</i> IFO 3530	—	—	0.000	0.000
9	<i>Arthrobacter ureafaciens</i> IFO 12140	—	—	0.000	0.000
10	<i>Bacillus brevis</i> IFO 3331	++	+	0.000	0.000
11	<i>Bacillus cereus</i> IFO 3001	—	—	0.000	0.000
12	<i>Bacillus megaterrium</i> NI 8100 NTH B12	—	—	0.000	0.000
13	<i>Bacillus pumilus</i> IFO 3030	++	++	0.000	0.000
14	<i>Bacillus sphaericus</i> IFO 3525	++	++	0.041	0.012
15	<i>Bacillus brevis</i> IFO 3007	—	—	0.000	0.000
16	<i>Bacterium cadaveris</i> IFO 3731	+	+	0.000	0.000
17	<i>Bacterium ketoglutamicus</i> SO(1)Shionogi Co., Ltd	+	+	0.000	0.000
18	<i>Bacterium orleanense</i> IFO 3259	+	+	0.000	0.000
19	<i>Brevibacterium sp.</i> P145 Phage host	—	—	0.000	0.000
20	<i>Brevibacterium sp.</i> P145 N.Kato	—	—	0.000	0.000
21	<i>Corynebacterium fascians</i> IAM 1079	—	—	0.000	0.000
22	<i>Corynebacterium glutamicum</i> No.534 ATCC 13032	+	—	0.000	0.000
23	<i>Corynebacterium pseudodiphtheritium</i>	—	—	0.000	0.000
24	<i>Enterobacter aerogenes</i> IFO 3320	—	—	0.000	0.019
25	<i>Enterobacter cloacae</i> IAM 1221	+	+	0.000	0.000
26	<i>Erwinia arvideae</i> IFO 3830	—	—	0.007	0.000
27	<i>Erwinia carotovora</i> IFO3380	—	+	0.000	0.000
28	<i>Escherichia coli</i> K12 IFO 3208	—	+	0.000	0.000
29	<i>Escherichia freundii</i> S-96	—	—	0.000	0.000
30	<i>Escherichia intermedia</i> A-21	—	—	0.000	0.000
31	<i>Klebsiella pneumoniae</i> IFO 3317	—	+	0.000	0.000
32	<i>Klebsiella pneumoniae</i> IFO 12009	+	—	0.000	0.000
33	<i>Klebsiella pneumoniae</i> IFO 12019	—	—	0.000	0.000
34	<i>Klebsiella pneumoniae</i> IFO 12932	—	—	0.000	0.000
35	<i>Klebsiella pneumoniae</i> IFO 13541	—	+	0.000	0.000
36	<i>Klebsiella pneumoniae</i> IFO 13703	—	+	0.000	0.000
37	<i>Micrococcus luteus</i> IFO 3763	—	—	0.000	0.000
38	<i>Micrococcus lysodeikticus</i> Fleming IFO 3333	—	—	0.000	0.000
39	<i>Micrococcus roseus</i> IFO 3764	—	—	0.000	0.000
40	<i>Micrococcus rubens</i> IFO 3768	—	—	0.000	0.000
41	<i>Proteus mirabilis</i> IFO 3849	—	—	0.000	0.000
42	<i>Proteus morgani</i> IFO 3168	—	+	0.012	0.000
43	<i>Proteus vulgaris</i> IFO 3988	—	+	0.002	0.021
44	<i>Pseudomonas fluorescens</i> IFO 3081	—	+	0.002	0.000
45	<i>Pseudomonas fragi</i> IFO 3458	—	—	0.000	0.000
46	<i>Pseudomonas graveolens</i> IFO 3460	—	+	0.000	0.000
47	<i>Pseudomonas iodium</i> IFO 3558	+	+	0.000	0.000
48	<i>Pseudomonas solanacearum</i> IFO 3509	+	+	0.000	0.000
49	<i>Pseudomonas striafaciens</i> IFO 3309	+	+	0.000	0.000
50	<i>Sarcina aurantiaca</i> IFO 3064	—	+	0.000	0.000
51	<i>Sarcina lutea</i> IFO 1099	—	+	0.000	0.000
52	<i>Serratia liquefaciens</i> IFO 12979	+	++	0.476	0.072
53	<i>Serratia marcescens</i> IFO 3054	—	++	0.000	0.000
54	<i>Serratia plymuthica</i> IFO 3055	+	+	0.000	0.000
55	<i>Staphylococcus aureus</i> IFO 3060	—	+	0.000	0.000
56	<i>Staphylococcus epidermidis</i> IFO 3762	—	+	0.000	0.000
57	<i>Bifidobacterium adolescentis</i> M101-4	—	++	0.000	0.000
58	<i>Bifidobacterium bifidum</i> A234-4	—	++	0.000	0.000
59	<i>Bifidobacterium breve</i> I-53-8	—	—	0.000	0.000
60	<i>Bifidobacterium infantis</i> I-10-5	—	+	0.000	0.000
61	<i>Bifidobacterium longum</i> M101-2	—	+	0.000	0.000
62	<i>Lactobacillus bulgaricus</i> IFO 13953	—	+	0.000	0.000
63	<i>Lactobacillus clausenii</i> IFO 3202	—	+	0.000	0.000
64	<i>Lactobacillus paracasei</i> IFO 3953	—	—	0.000	0.000
65	<i>Lactococcus cremoris</i> IFO 3427	—	—	0.000	0.000
66	<i>Lactococcus lactis</i> IFO 12007	—	—	0.000	0.000
67	<i>Pediococcus acidilactici</i> IFO 3888	—	—	0.000	0.000
68	<i>Streptococcus thermophilus</i> IFO 13957	—	+	0.000	0.000

\*, Activity staining ++, higher; +, normal; —, no activity.

\*\*, Measurement by absorbance at 340nm.



### References

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