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Factors Affecting the Lactate Dehydrogenase Activity of a Spore-forming Lactic Acid Bacteria

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胞子形成 乳酸菌의 Lactate Dehydrogenase 力價에 미치는 諸要因

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ABSTRACT

Several strains of spore-forming lactic acid bacteria were isolated from natural sources such as soils, cereals, and foods. The general morphological and physiological characteristics of the strain 6-4 were investigated and compared with some other industrial strains.

The effects of fructose-1,6-diphosphate (FDP), adenosine triphosphate (ATP), and *p*H on the lactate dehydrogenase (LDH) activity of the strain were studied, and the changes in LDH activity and spore formation under various cultural conditions were researched.

The results were as follows.

1. This strain was identified to *Bacillus coagulans* Hammer and distributed widely in natural sources.

2. The strain strongly converted various fermentation substrates into L(+)-lactic acid in anaerobic conditions, and many spores that were of great advantages to the industrial application were formed easily in the aerobic condition.

3. The LDH activity of this strain was activated by FDP and inhibited by ATP. The optimal *p*H for the enzyme activity was 6.0-6.5.

4. In the anaerobic culture condifion, the large amount of glucose added in the medium increased the LDH activity, but the cells were not committed to sporulate.

5. When none or a very small amount of glucose (less than 0.5%) was added to culture medium in the aerobic condition, the LDH activity was decreased and many spore were produced with final *p*H higher than 8.5.

6. The addition of large amount of glucose (more than 2.0%) in aerobic culture increased the LDH activity and inhibited strongly the spore formation with final *p*H lower than 6.0.

INTRODUCTION

It had been reported that some strains of spore-forming bacilli possessed the capability to produce the lactic acid as a fermentation product (Hammer, 1915; Anderson *et al.*, 1938; Smith *et al.*, 1946). A group of spore-forming lactic acid bacte-

ria have been described by Nakayama (1960), Kitahara *et al.*(1963), Nakayama *et al.* (1967) and Misawa *et al.* (1972). Wolin (1964) demonstrated that lactate dehydrogenases (LDH) from several members of the genus *Streptococcus* were activated specifically by fructose-1, 6-diphosphate(FDP). Wittenberger *et al.*(1970, 1972) confirmed and extended these results, and biochemcally characterized the interaction of FDP with the Streptococcal LDHs. LDHs from *Bifidobacterium*(Vries *et al.*, 1968; Kandler *et al.*, 1974) and *Acholeplasma laidlawii* (Neimark *et al.*, 1973) also were activated by FDP. Among the *Lactobacilli*, four species—*L. plantarum* (Mizushima *et al.*, 1974), *L. casei* (Vries *et al.*, 1970), *L. xylosus* (Buchanan *et al.*, 1674), *L. curvatus* (Steeter *et al.*, 1973)-possessed LDHs that required FDP and Mn^{++} ion. Carlsson and Griffith (1974) caused an almost complete suppression of lactate procuction by substrate limitation. Yamada *et al.* (1975) reported that the control of lactate production could be achieved by any alteration of the intracellular pool of FDP.

In the present work, several strains of spore-forming lactic acid bacteria were isolated from natural sources, and among them the strain, 6-4, which has more powerful acitivity in lactate production and spore formation, was investigated on the morphological and physiological properties. The effects of FDP, ATP and *p*H on the LDH activity of the strain were compared with those of *Lactobacillus* and *Streptococcus*, and the interaction of glucose added to medium with both LDH activity and spore formation were studied in aerobic and anaerobic conditions.

MATERIALS AND METHODS

1. Isolation of strains

Small amounts of samples from 30 kinds of matural sources were dipped in test tubes with medium A in table 1, and incubated at 45°C for 48 hours. The cultures without gas and pellicle were diluted with sterilized distilled water and plated on medium B. Chalk dissolving colonies were picked up and inoculated on medium C slant. The slants were incubated at 45°C for 48 hours, and examined microscopically. The spore-forming rods were transferred to medium E broth, and cultured in shaken incubator. The strains with powerful ability to sporulate were inoculated in medium D broth. After 7 days' anaerobic cultivation the residual glucose, dissolved calcium and volatile acids were estimated. Thus the active strains in the lactate fermentation and spore formation were screened.

2. Bacteriological properties of the isolate

General morphological and physiological properties were examined according to Mannual of Microbiological Methods

Table 1. Composition of culture med'a

Medium for		Composition(%) Glucose	Yeast ext.	Peptone	$CaCO_3$	Agar	*p*H
Enrichment	(A)	0.5	0.5	0.5			5.5
Plating	(B)	0.5	0.5	0.5	0.5	1.5	6.0
Slant	(C)	0.2	0.5	0.25		1.5	6.8
Fermentation	(D)	10.0	0.5	0.25	7.0		6.8
Sporulation	(E)		1.0	0.5			5.0

(Conn *et al.*, 1957).

3. Cultivation and enumeration of spores

General cultivation was carried out stationally in 45°C incubator and aerobic culture was run at 45°C on the reciprocal shaker with 130-140 stroke per minute. Viable cells were counted on the medium B plate in table 1. The heat resistant spores were enumerated on the same plate after heat treatment at 85°C for 30 minutes.

4. Analysis of fermentation

The Residual glucose was estimated by means of Fehling-Lehmann-Schrool method, the dissolved calcium by the titration with EDTA, and the volatile acids by steam distilation of acidified culture filtrates. The volatile acids were calculated as acetic acid, nonvolatile acids as lactic acid, and the lactic acid was confirmed by means of paper chromatography.

5. Optical properties of the lactic acid

The dissolved calcium lactate of the filtrate was extracted with ether and concentrated with zink carbonate into zink lactate. The solution of 5% zink lactate was examined in the configuration tube with natorium lamp (Zeis). In this condition the *laevo* lactate indicated positive value, *dextro* lactate negative value under (α) d=±7-8′.

6. Estimation of LDH activities

1) Preparation of crude cell-free extracts : The cells were harvested by centrifugation, washed with 0.05M phosphate buffer (pH 7.0) three times, and broken by grinding with alumina. The broken cells were centrifuged at 15,000× g for 20 min, and the clear supernatants were used for the assay of protein and LDH.

2) Protein assay: The protein was determined according to Lowry *et al.*(1951) with crystalline bovine serum albumin as a standard.

3) LDH assay : The reaction mixture was consisted of the following components in total volume of 3.15ml; phosphate buffer, pH 7.0 48mM; sodium pyruvate, 0.6mM; NADH, 0.18mM; FDP, 0.8mM. Reactions were initiated by the addition of 0.1ml enzyme extracts, and LDH activity was measured by the decrease rate in the absorption at 340nm resulting from the pyruvate-dependent oxidation of NADH. One unit is the amount of enzyme required to catalyze the oxidation of 1μ mole of NADH per min., and specific activity is expressed as units per milligram of protein. The reaction was run with Beckman QV 50 spectrophotometer, and sodium pyruvate, β-NADH, FDP$(CHA)_4 \cdot 10H_2O$ were perchased from the Sigma Chemical Co.

RESULTS AND DISCUSSION

1. Isolation of strains and general properties

35 strains of spore-forming latic acid bacteria were isolated from 30 samples of natural sources such as soils, cereals and foods. The strains were grouped by the fermentation rates and lactate yields. And the number of isolates belonging to each group A,B,C, and D are shown in Table 2. 12 strains in A group were more than 90% in the fermentation rate and the yield of lactic acid. One strain, 6-4, was selected and investigated on its general bacteriological properties. This is Gram-positive, rod-shaped with rounded ends in anaerobic culture enrichmented with the sufficient fermentation substrates, and

bears oval spores terminally or subterminally in the aerobic culture with the protein hydrolysates such as pepton or yeast extracts. Optimal temperature for the growth is 40°-45°C and optimal *p*H 6.0-7.0. The another morphological and physiological characteristics are listed in Table 3. Nakayama (1967) grouped the spore-forming lactic acid bacteria isolated by himself into 5 kinds of type according to their catalase activity and optimal properties of lactic acid produced by them. The strain 6-4 is presumed to belong to *Bacillus coagulans* as compared to Naka-

Table 2. Results of isolation

Sources	No. of materials for isolation	No. of isolates belonging to each group of fermentation activity*				
		A	B	C	D	Total
Soil	11	3	8	2	3	16
Cereal	10	4	2	2	3	11
Bread	3	2				2
Milk	6	3	3			6
Total	30	12	13	4	6	35

	A	B	C	D
*Degree of fermentation %	>90	>80	>50	>50
Yield of lactic acid %	>90	>80	>50	>50

Table 3. Description of strain 6—4

Cell size (μ)		H_2S production	−
Vegetative	0.6−1.0×5−7	Indole production	−
Spore	0.9−1.0×1.0−1.2	VP test	+
Spore situation	Terminal or subterminal	Urea production	−
Growth temperature	35-55C	Utilization of carbon compounds	
Growth *p*H	5.0−8.5	glucose, sucrose, lactose, xylose, soluble starch maltose, mannitol	+
Type of lactic acid	L(+)		
Gram stain	+		
Growth in 7% NaCl	−	arabinose, sorbitol, inositol	−
Catalase activity	+		
Nitrate reduction	+		

Table 4. Fermentation test by various sugars with strain 6-4

Sugars	Initial sugar (%)	Residual sugar (%)	Degree of fermentation (%)	Volatile acid* (%)	Nonvolatile acid** (%)	Yield of lactic acid (%)
Glucose	10.00	0.65	93.54	0.078	8.79	93.97
Sucrose	10.00	0.73	92.66	0.096	8.84	95.37
Lactose	10.00	1.07	89.27	0.120	8.25	92.34
Xylose	10.00	1.29	87.12	0.210	5.55	63.67
Starch	10.00	4.56	54.44	0.126	5.10	79.16

*as acetic acid **as lactic acid

Table 5. Fermentation test by various strains with glucose

Strains	Initial sugar (%)	Residual sugar (%)	Degree of fermentation (%)	Volatile acid* (%)	Nonvolatile acid ** (%)	Yield of lactic acid(%)
strain 6-4	10.00	0.65	93.54	0.078	8.79	93.97
Nakayama strain	10.00	1.03	89.69	0.126	8.83	98.47
Shiroda strain	10.00	1.13	88.66	0.084	6.98	78.64
Lactobacillus bulgaricus	10.00	1.72	82.76	0.120	8.00	96.37

*as acetic acid **as lactic acid

yama's key and Bergy's manual (Buchanan *et al.*, 1974).

Glucose, sucrose and lactose of carbohydrates as fermentation substrate appeared to be more usable than xylose and starch (Table 4). The fermentative ability of the strain 6-4 with glucose was compared to that of Nakayama strain, a spore-forming lactic acid bacterium applicated widely to the medical use in Japan, Shiroda strain and *L. bulgaricus*, nonspore-forming lactic acid bacilli being used generally in the dairy products (Table 5). By these results, the strain 6-4 could be expected much of the application to the medical drug, food, dairy technology and lactate production because of its high activity in the lactate fermentation and spore formation.

2. Factors affecting the LDH activity

The lactate dehydrogenases of several species of the genus *Streptococcus* have been shown to be markedly activated by the glycolytic intermediate, fructose-1, 6-diphosphate (FDP) (Anders *et al.*, 1970), however, among *Lactobacillus* a few species only have been proved to have the requirement of FDP and Mn^{++} ion for the LDH activity (de Vries *et al*, 1970; Stetter *et al.*, 1973). The LDH activity of the strain 6-4 was highly activated by FDP, but not by Mn^{++} ion(Fig. 1). The concentration of FDP required to yield the maximal activity of LDH was influenced by the *p*H at which the assay was carried out. As the *p*H of the assay mixture was increased, so was the concentration of FDP for the maximal enzyme activity. The maximal enzyme activity was appeared with 0.8mM of FDP at *p*H 6.0, 1.2 mM of FDP at *p*H 7.0 and 1.8mM of FDP at *p*H 8.0 respectively. However, the maximal activity of LDH was decreased with the increase of the *p*H. In the case of the absence of FDP, all the level of LDH activity in the all range of the *p*H

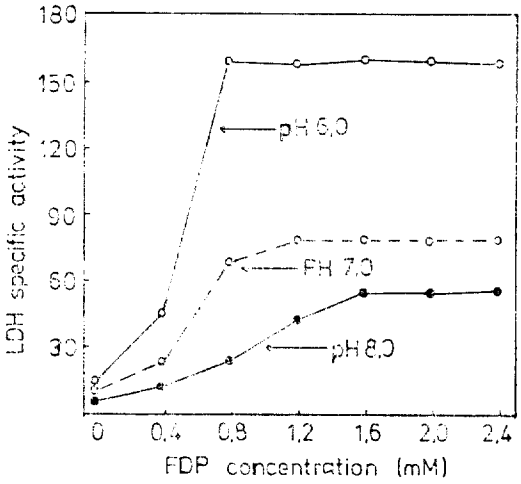

Fig. 1. Dependence of LDH activity on Fructose-1, 6-diphosphate at *p*H 6.0, 7.0 and 8.0 The final reaction mixture(3.5ml) contained phosphate (buffer) 48mM, sod. pyruvate 0.6 mM, NADM 0.18mM, enzyme extract 0.1ml, and FDP was varied as shown.

tested was very low. This result was different from that of *Streptococcus cremoris* in which the *p*H optimum of the LDH was 8.0 in the absence of FDP and changed to a broad plateau between *p*H 5.0 and *p*H 7.0 in the presence of FDP

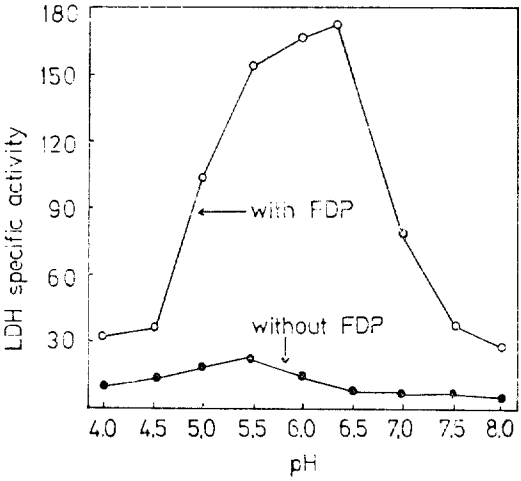

Fig. 2. LDH activites at various *p*H with (0.8 mM) or without FDP. The reaction mixture was same as described before except that *p*H was varied as shown.

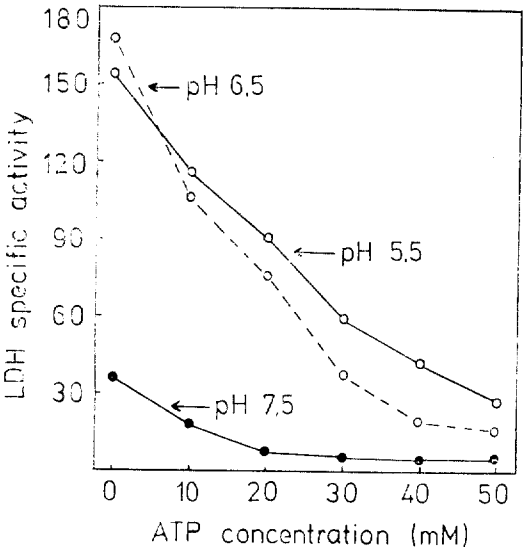

Fig. 3. Inhibition of LDH activity by ATP at *p*H 5.5, 6.5 and 7.5. The reaction mixture was same as described before except that ATP concentration was varied as shown.

(Jonas *et al.*, 1972). But it was agreeable with the results from *Lactobacillus casei* (Mizushima *et al.*, 1962).

The results from several studies have shown that the LDHs from many lactic acid bacteria were inhibited by adenosine triphosphate (ATP) (Wittenberger, 1968; Tarmy *et al.*, 1968; Brown *et al.*, 1972). The activity of the LDH from the strain 6-4 was also inhibited strongly by ATP in proportion to its concentrations (Fig. 3).

This inhibition was sensitive especially in the *p*H 5.5-6.5 which is the optimal *p*H for the enzyme activity. The properties of the LDH of the spore forming lactic acid bacterium isolated resemble that of *Lactobacillus casei* in respect of the activation by FDP, the inhibition by ATP and its optimal *p*H.

The LDH activities of the cells grown at various temperatures is shown in Fig. 4. The enzyme activities of the cells grown at 40°-45°C, the optimal temperature for growth, were very high and at 30° or 55°C very low. Mou *et al.* (1972) reported that the increase in the temperature of growth from 25° to 40°C decreased markdely the levels of D-LDH activity present in the cells in contrast to the levels of L-LDH which remained fairly constant. The result in this work was agreeable with Mou's as regarding that the maximal activity appeared at the optimal temperature.

As the strain was grown at the various glucose concentrations aerobically or anaerobically, the LDH activity and sporulation rate of the cells are revealed in Fig. 5. In anaerobic condition, the LDH activity reached an almost constant level in the glucose concentration more than

1.5%, while in aerobic condition the glucose concentration for the maximal enzyme activity was more than 2.0%. In all anaerobic culture the cells were not committed to sporulate, and in aerobic culture, the ratio of sporulation was decreased markedly by the addition of glucose in the medium. It was reported by Carlsson *et al.* (1975) that glucose limitation in culture medium decreased the intracellular level of FDP and the production of lactic acid, but didn't effect on the LDH activity. In the present work, when glucose was not added or added with the small amount less than 0.5%, the LDH activity was very low. The presence of an excess of glucose increased the LDH activity and inhibited the spore formation even in aerobic condition.

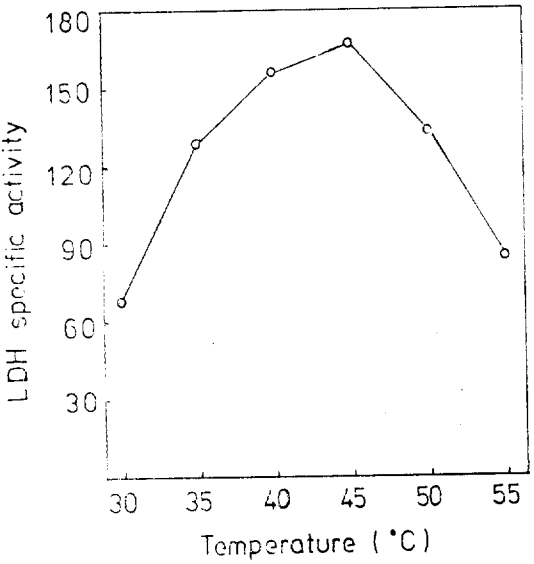

Fig. 4. Effects of cultural temperatures on LDH activity. The cultures were grown stationarily for 30 hrs. with medium D in table 1.

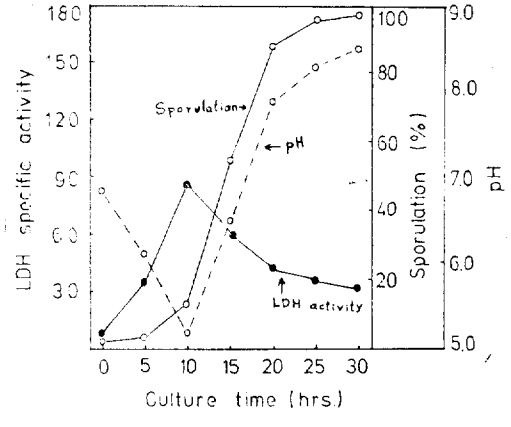

Fig. 5. Effects of glucose added in culture media on LDH activity and sporulation with or without aeration after 30 hr cultivation. In cultures without aeration, spores were not detected.

These facts have assumed that the addition of an access of glucose in aerobic condition has induced the intracellular LDH and activated the enzyme activity owing to the accumulation of intracellular FDP by glycolysis, and inhibited the spore formation of the cells owing to the depression of TCA cycle enzymes (Ohme *et al.*, 1974) and other metabolites serving for sporulation by the catabolite repression (Schaeffer *et al.*, 1965).

In case of the addition of less glucose or not, the intracellular level of FDP is insufficient for the catalytic activity of LDH and the excess ATP obtained by aerobic repiration decreases the LDH

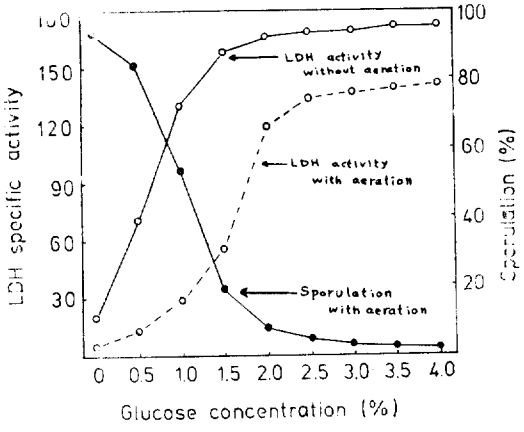

Fig. 6. Changes of LDH activity, *p*H and sporulation of strain 6-4 in aerobic culture contained 0.5% glucose.

activity. Thus the lactate fermentation must be inhibited and sporulation can be occurred.

When the cuture was carried out in aerobic condition with 0.5% glucose, the changes of LDH activity, the *p*H and the sporulation degree are shown in Fig. 6. The LDH activity was appeared to be in contrast to the *p*H changes. After 10 hour-culture, the *p*H moved from the initial *p*H 6.8 toward the *p*H 5.5, while the LDH activity reached its maximal level on this time. After then the *p*H continued to increrase to the final *p*H more than 8.5, and LDH activity decreased slowly, and the sporulation rate increased with the *p*H movement from neutrality toward alkalinity, after 20 hours it exceeded the level of 90%.

The general lactic acid bacteria such as *Lactobacillus* and *Streptococcus* don't possess an aerobic respiration system. In the facultatives which have two energy yielding patterns, lactate fermentation and aerobic respiration, the effects of the fermentation substrates such as glucose on the regulation for lactate fermentation, the aerobic respiration, the intracellular physiological control of the metabolic pathways, and the expression mechanisms of the genes governing the lactate fermentation, aerobic respiration and bacterial sporulation are much worth researching extensively.

摘 要

토양, 곡물, 우유등 자연시료로부터 胞子形成乳酸菌을 數株 분리하여 한 분리주(Strain 6-4)에 대하여 일반 형태 및 生理學的 성질을 조사하였으며 몇가지 산업균주와 비교하였다.

또한 本菌의 조효소액의 LDH 역가에 미치는 FDP, ATP 및 *p*H의 작용에 대하여 조사하였으며 몇가지 배양조건이 LDH 역가 및 spore 형성에 미치는 영향을 검토하여 다음과 같은 결과를 얻었다.

1) 분리한 포자형성유산균은 *Bacillus coagulans*로 생각되고 토양·곡물등 자연에 널리 존재하며 본 분리방법에 의하여 쉽게 분리할 수 있었다.
2) 다른 산업균주에 비하여 당해분해율 및 유산생성력이 우수하며 강한 내성을 갖는 포자를 얻을 수 있어 여러 방면의 산업적 이용이 기대된다.
3) 본균의 LDH 역가는 FDP에 의하여 촉진되고 ATP에 의해 저해를 받으며 *p*H optimum은 6.0~6.5이였다.
4) 염기성배양에서는 LDH 역가가 높으며 발효기질의 90%이상을 L(+)-lactic acid로 만들며 spore는 전혀 형성되지 않았다.
5) 호기성배양에서는 LDH 역가가 저하되고 glucose가 첨가되지 않거나 소량(0.5% 이하) 첨가되면 final *p*H는 8.5이상으로 되며 spore를 형성하였다.
6) 호기성배양에서 glucose가 다량(2.0%이상) 첨가되면 LDH 역가는 높아지고 final *p*H는 6.0이하였으며 spore 형성은 심한 저해를 받았다.

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