Effect of Cultural Conditions on the Fumarate Metabolism in *Lactobacillus delbrueckii*

Chan Min JUNG*, Taku MIYAMOTO*, Kei KATAOKA*, Iichiroh OHHIRA*, and Takefumi YONEYA**

*Faculty of Agriculture, Okayama University, 1-1-1, Tsushimanaka, Okayama-shi 700
**Department of Food Science and Nutrition, Shizuoka Prefectural University, Hamamatsu College, 3-2-3, Nunohashi, Hamamatsu, Shizuoka 432

Organic acids such as fumaric, malic and lactic acids are produced by associative growth of fermentative microorganisms and contribute largely to flavor of fermented foods. In the present study, the effect of cultural conditions on the fumarate metabolism was tested for three strains of *Lactobacillus delbrueckii* possessing the ability in conversion of fumarate to L-malate, and an attempt was undertaken as preliminary studies for the flavor improvement of fumaric acid forming-mold starter foods in combination with lactic acid bacteria. These strains were incubated in the basal medium (glucose-yeast extract-peptone) containing 1% sodium fumarate at 34℃ under stand and shaking cultures and the cell growth and organic acid compositions during the incubation were tested. The effects of medium compositions and inorganic salts on the fumarate metabolism were investigated as well. Fumarate consumptions and the uptake of glucose by three strains were activated by the addition of fumarate to the basal medium. L-malate production decreased after 24 to 28 hours incubation under the stand culture, whereas the production showed a tendency to increase until 48 hours incubation under the shaking culture. However, the productive ratio of fumarate to L-malate was nearly same in both stand and shaking cultures. On the other hand, stimulatory effects on the cell growth of three strains were observed in fumarate-added basal medium supplemented with magnesium ions. Their fumarate-hydrolyzing abilities were also stimulated, although the abilities were inhibited in the presence of copper ions. (Received May 2, 1993)

Organic acids as products of microorganisms metabolism take an important role as ingredients of flavor or sourness of various kinds of fermented foods. However, the strength of sourness and quality of each organic acid differs respectively that due to its balance of volume, it might cause the lack of flavor. It is said that filamentous fungi of the genus *Rhizopus* (*1*) and fumaric acid produced by yeast of the genus *Candida* (*2*) in organic acids make products sour (*3*). On the other hand, with the activity of fumarate hydratase (EC4. 2. 1. 2., hereafter cited as, fumarase), L-malic acid converted from fumaric acid possesses poorer sour degree compared to fumaric acid but contains pleasant sour taste (*4*). Fumarase is distributed in mold (*5*), yeast (*6*), animals (*7*), the higher animals (*8*) and others. However, on fumarase of lactic acid bacteria, no report has been found other than that by Kitahara *et al*(*9*). It would be necessary to investigate basically on the prosperity and decline of organic acids dissolved by fumaric acid.

We selected three strains of *Lactobacillus delbrueckii* which showed high L-malic acid production ability in the result that the L-malic

acid production ability of fumaric acids from various lactic acid bacteria were investigated in aim to improve flavor of filamentous fungi fermented foods with the use of both filamentous fungi and lactic acid bacteria (*10*). Then, we will report the results here that we investigated changes of fumaric acid by time course that comes along with the growth of bacteria in the culture fumaric acid salt is added with three selected strains as basic experiment to investigate the metabolism formation of fumaric acid produced from glucose by filamentous fungi.

EXPERIMENTS

1. Sample strains

As reported in our previous study (*10*), we also sampled *L. delbrueckii* subsp. *delbrueckii* ATCC 9649 (hereafter cited as *L. delbrueckii*), *L. delbrueckii* subsp. *bulgaricus* 7235 (*11*) (hereafter cited as *L. bulgaricus*) and *L. delbrueckii* subsp. *lactis* 1135 (*11*) (hereafter cited as *L. lactis*) which showed high L-malic acid production ability from fumaric acid. These strains were transferred at 34℃ with GYP medium of pH6.8 (1% glucose, 0.5% yeast extract, 1% peptone, 0.1% Tween 80 and 0.01% L-Cystein).

2. Test Sample

We used GYP medium, FGYP medium (GYP medium supplemented with 1% fumaric acid disodium), FYP medium (glucose excluded from FYGP medium) and Na-FGYP medium (5% salt added to FGYP medium). Also, we investigated on the metal ion influence to metabolism of fumaric acid with the medium various kinds of inorganic salt to FGYP medium 0.5mM each. Yet, those inorganic salt we used are copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$), sulfuric acid magnesium ($MgSo_4 \cdot 7H_2O$), zinc sulfate ($ZnSO_4 \cdot 7H_2O$), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$), cobalt sulfate ($CoSO_4 \cdot 7H_2O$), ferrous sulfate ($FeSO_4 \cdot 7H_2O$), aluminium chloride ($AlCl_3 \cdot 6H_2O$) and ferric sulfate ($Fe_2(SO_4)_3$). Each medium was adjusted to pH6.8.

3. Culture condition

Testing the influence of culture condition to fumaric acid metabolism, we inoculated 0.1% culture medium cultured statically in GYP medium for 24 hours, we incubated at 34℃ under stand and shaking medium for prescribed hours. Shaking culture was done with 300mL Erlenmeyer flask in reciprocating shaking culture including 100mL FGYP.

4. Analysis

Measurement of organic acid in culture medium was done by high performance liquid chromatography in culture filtrate excluding fungi with Millipore filter (0.22 μm) (*12*). On the other hand, growth rate of bacteria was shown in optical density at 660nm.

RESULTS AND STUDY

1. Influence of culture media structure to fumaric acid metabolism

Table 1 shows the growth rate of three sample strains in each medium after 48-hour culture for stand culture and consumption.

The growth rate of bacteria in no glucose added FYP medium with fumaric acid as substrate and consumption of fumaric acid is generally low.

Table 1 Effect of medium composition on the fumaric acid metabolism by *Lactobacillus delbrueckii* strains

Medium[1)]	*L. delbrueckii* subsp. *bulgaricus* 7235		*L. delbrueckii* subsp. *lactis* 1135		*L. delbrueckii* subsp. *delbrueckii* ATCC 9649	
	Cell growth (OD 660 nm)	Fumarate utilized (%)	Cell growth (OD 660 nm)	Fumarate utilized (%)	Cell growth (OD 660 nm)	Fumarate utilized (%)
GYP	1.384	—	2.387	—	0.913	—
FYP	0.099	1.48	0.138	18.16	0.085	7.66
FGYP	1.843	93.77	2.158	97.64	1.536	96.82
Na-FGYP	0.064	0	0.056	0	0.051	0

1) Medium compositions are the following :
Composition of FGYP medium was 1% disodium fumarate, 1% glucose, 1% peptone, 0.5% yeast extract, 0.1% Tween 80 and 0.01% L-cysteine.
GYP medium and FYP medium were the same to FGYP medium but lacking disodium fumarate and glucose, respectively.
Na-FGYP medium was FGYP medium containing 5% sodium chloride.
All media were adjusted to pH 6.8.

The highest *L. lactis* was 18.16%. However, the growth rate at FGYP medium (1% glucose added to the previous medium was higher in *L. delbrueckii* and *L. bulgaricus* compared to GYP medium. In *L. lactis*, it was rather low value. And, fumaric acid was used mostly as 93.77 to 97.64% till 48th hour of cultivation in each strain. However, on investigating the growth rate of Na-FGYP medium 5% NaCl added to FGYP medium in consideration to the influence at salted foods and consumption of fumaric acid we could not find any growth of bacteria and consumption of fumaric acid.

Organic acid is believed to possess promoting effect in the growth of various kinds of lactic acid bacteria (*13,14*). Among these Harvey *et al.* (*15*) infer that it is because composing ability of cytoplasm structure ingredients formed with glucose as energy source would be strengthen by citric acid. Also, Branen *et al.* (*14*) report that citric acid, isocitric acid, α-ketoglutaric acid, succinic acid and L-malic acid promote the growth of *L. casei* 393 involving TCA circuit and fumaric acid inhibit the growth. On the contrary, our investigation showed growth promoting effect by fumaric acid. This growth promoting structure of fumaric acid is unknown. It is necessary to study in detail in the future.

On the other hand, Table 2 shows the results of influence of metal ions to the growth of three sample strains and fumaric acid metabolism. Fumaric acid consumption of each strain in FGYP medium differs to cultivation time (Fig.1). Table 2 shows the result of the 20th hour (Fig.1) where clear decrease of fumaric acid in *L. lactis* was observed.

In metal ions we used, it was Mg^{2+} on all three strains that promoted bacterial growth and fumaric acid consumption the most. On the other hand, when Cu^{2+} is added to culture medium, bacterial growth and fumaric acid consumption were inhibited. Similarly, it was inhibited in the medium with Zn^{2+} for *L. delbrueckii* and *L. lactis*. Bivalent positive ion was believed to be necessary for bacterial growth (*14*), organic acid metabolism (*16*) and ordinary cell division (*17*), Wright *et al.* (*18*) report that the growth of *L. bulgaricus* is promoted by adding Mg^{2+} to culture medium.

2. Influence of stand and shaking culture to fumaric acid metabolism

Since fumaric acid production from glucose by filamentous fungi is superior in shaking culture than stand culture (*19*), we investigated on influence of stand and shaking culture of three sample strains to fumaric acid assuming mix culture of filamentous fungi and lactic acid bacteria in this research.

Fig. 1 and Fig. 2 showed change with time of fumaric acid and each organic acid in stand and shaking culture. The bacterial growth rate of three sample strains in stand culture for 48 hours was almost 2-fold to that of shaking culture.
Also, fumaric acid contained around 74mM at the beginning of the culture started to decrease at 16th to 20th hour of cultivation, reaching in 48 hours to 4.89mM *L. bulgaricus*, 1.58mM *L. lactis* and 2.41mM *L. delbrueckii*. In contrast, the residue of fumaric acid around 48 hours in shaking culture was 14.23, 12.5 and 8.20mM respectively.

Table 2 Effect of inorganic salts on fumaric acid metabolism by *Lactobacillus delbrueckii* strains

Inorganic salt	*L. delbrueckii* subsp. *bulgaricus* 7235			*L. delbrueckii* subsp. *lactis* 1135			*L. delbrueckii* subsp. *delbrueckii* ATCC 9649		
	Cell growth (OD 660nm)	pH	Fumarate utilized (%)	Cell growth (OD 660nm)	pH	Fumarate utilized (%)	Cell growth (OD 660nm)	pH	Fumarate utilized (%)
None	1.826	4.69	39.88	2.034	4.47	75.59	0.624	5.22	27.53
$CuCl_2 \cdot 2H_2O$	1.732	4.76	32.59	1.134	4.96	46.72	0.513	5.33	13.67
$MgSO_4H_2O$	2.005	4.60	47.96	2.123	4.43	90.13	0.855	5.28	38.69
$ZnSO_4 \cdot 7H_2O$	1.731	4.66	41.48	1.440	4.78	45.97	0.412	5.39	16.67
$Na_2MoO_4 \cdot 2H_2O$	1.810	4.68	44.53	1.958	4.50	72.70	0.590	5.26	23.80
$CoSO_4 \cdot 7H_2O$	1.354	4.78	33.36	1.783	4.76	69.48	0.574	5.53	22.75
$FeSO_4 \cdot 7H_2O$	1.673	4.65	42.29	1.912	4.41	83.34	0.630	5.16	31.22
$AlCl_3 \cdot 6H_2O$	1.755	4.66	42.32	1.859	4.48	85.06	0.577	5.22	23.74
$Fe_2(SO_4)_3$	1.711	4.65	40.66	1.879	4.35	85.09	0.559	5.18	25.49

Each strain was incubated at 34℃ for 20 hours in the FGYP medium supplemented with 0.5mM inorganic salt.

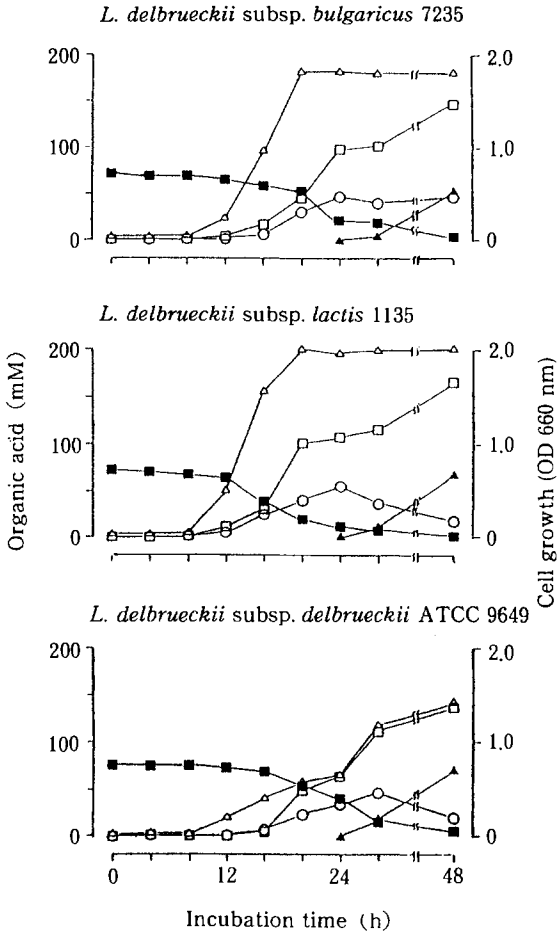

Fig. 1 Metabolism of fumarate in the FGYP medium incubated with *Lactobacillus delbrueckii* at 34℃ under stand culture

■, fumaric acid ; ○, L-malic acid ; □, lactic acid ; ▲, acetic acid ; △, cell growth.

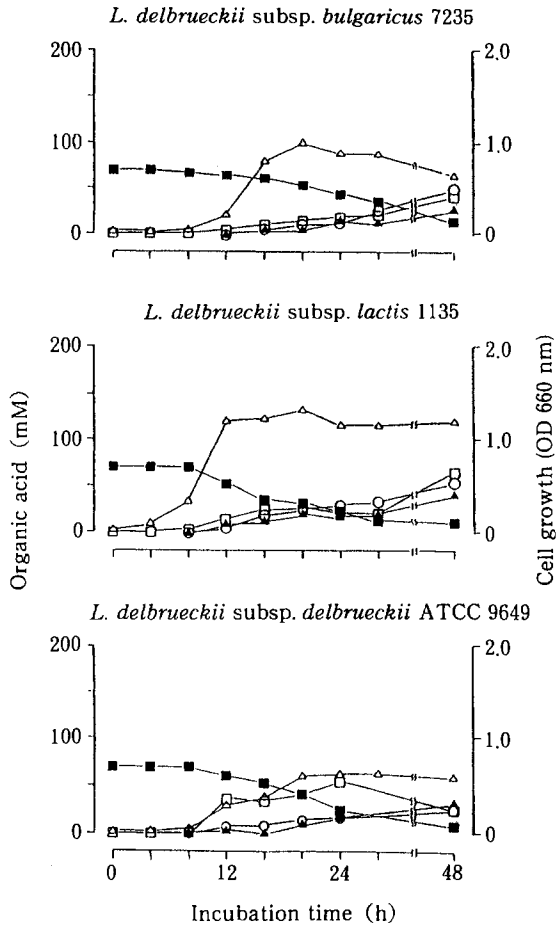

Fig. 2 Metabolism of fumarate in the FGYP medium incubated with *Lactobacillus delbrueckii* at 34℃ under shaking culture Symbols were shown in Fig. 1.

While L-malic acid produced from fumaric acid, *L. bulgaricus* and *L. lactis* in stand culture at the 24th hour after cultivation, L.delbrueckii at the 28th hour of cultivation, became the maximum (49.71mM, 55.37mM and 47.80mM, respectively) and started declining from then on. However, in shaking culture, all strain show increasing tendency to the 48th hour and *L. bulgaricus* showed 50.72mM, *L. lactis* showed 52.34mM and *L. delbrueckii*showed 34.51mM. That time, the ratio of fumaric acid and L-malic acid in stand cultrue is 1:2.1 to 4.3, and 1:36 to 4.2 in shaking culture. From this, L-malic produced by fumarase activity from fumaric acid, they were almost the same value in their ratio under stand and shaking culture. Kitahara *et al.* (*20*) report that on the conversion of L-malic acid from fumaric acid by fumarase of lactic aicd bacteria, their ratio was 1 : 3.3 (23% : 77%). We also had a same result in this research.

On the other hand, when we confirmed optical rotation of productive lactic acid with enzyme method (*21*), productive lactic acid was almost D-lactic acid and L-lactic acid was a small volume (data not shown). Radler (*22*) says that malic acid dihydrate enzyme, malic acid dissolution enzyme and malo-lactic enzyme are involving to the metabolism of L-malic acid. And carbon dioxide and L-lactic acid are produced as the last products.

Also, Hara (*23*) determines that the activity of these enzyme differ to their pH or the kinds of lactic acid bacteria, the more pH become neutral,

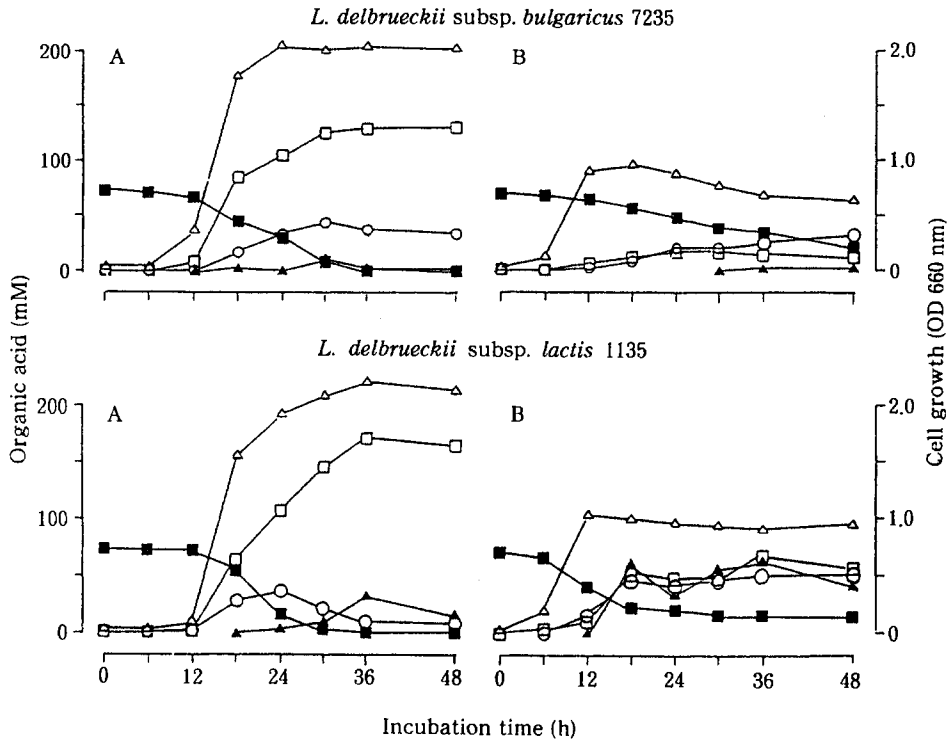

Fig. 3 Metabolism of fumarate in the FGYP medium containing 0.5 mM magnesium ion incubated with *Lactobacillus delbrueckii* at 34℃ under stand and shaking culture conditions

A, stand culture ; B, shaking culture.
Symbols were shown in Fig. 1.

the more easily happen malic acid dissolving enzyme system, and then produced also acetic acid. Moreover, sample strains produce D-lactic acid from glucose (*24*). Then, it is considered that lactic acid produced is mostly originated in glucose.

On the studying results that is shown in Table 2, we investigated change with time of organic acid content in stand and shaking culture with FGYP medium containing Mg^{2+} since supplementing Mg^{2+} is the most effective in fumaric acid metabolism of sample strains. Fig. 3 showed the results of *L. bulgaricus* and *L. lactis*.

Consumption of fumaric acid in stand culture starts from the 12th hour in culture on both strains, *L. bulgaricus* was 28.99mM until the 24th hour of cultivation and *L. lactis* decreased to 19.86mM. While in shaking culture, *L. bulgaricus* decreased fumaric acid consumption, *L. lactis* decreased 41.93 mM at the 12th hour of cultivation. Conversion of L-malic acid from fumaric acid is admitted in a short time compared to stand culture. On the other hand, L-malic acid produced from fumaric acid showed the maximum (*L. bulgaricus* was 42.13mM and *L. lactis* was 38.51mM) in 24 to 30 hours after cultivation in stand culture, showing decrease afterward. Then, we increased the cultivation time to 48 hours in shaking culture, 33.23 and 52.75mM respectively.

Therefore, fumaric acid described in this research was produced from glucose by filamentous fungi and make tempeh (produced from wheat and cereals) sour (*3*). However, L-malic acid converted from fumaric acid contain pleasant sourness. In this investigation, L-malic acid from fumaric acid by three sample strains is produced with the bacterial growth, its production ratio did not influence in stand and shaking culture. Also, in case of *L. lactis*, when Mg^{2+} is applied to FGYP medium, fumaric acid was metabolized to L-malic acid in a short time. From this, conversion of L-malic acid is determined by mix culture of filamentous fungi with fumaric acid productive ability and fumarase producing lactic acid bacteria and estimated of adding pleasant flavor to filamentous fungi fermented foods.

SUMMARY

We gained the following results on investigating cultivation condition to fumaric acid metabolism of three strains of *Lactobacilus delbrueckii.*

(1) By applying fumaric acid to the medium at stand cultivation, bacterial growth was promoted.

(2) By investigating metal ion influence to fumaric acid metabolism activity, Mg^{2+} added medium promoted fumaric acid metabolism activity but Cu^{2+} added medium inhibited fumaric acid metabolism activity.

(3) On stand culture, the production volume of L-malic acid from fumaric acid showed the maximum at the 24th hour of cultivation for *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis* and the 28th hour of cultivation for *L. delbrueckii* subsp. *delbrueckii.* While in shaking culture, all three strains showed increasing tendency untill the 48th hour of cultivation. However, in any culture, L-malic acid production ratio from fumaric acid was almost the same value.

(4) Under the existence of Mg^{2+}, by having *L. delbrueckii* subsp. *lactis* in shaking culture, fumaric acid was converted to L-malic acid in a short period.

REFERENCES

1) Yoneya, T. and Sato, T.: *J. of Jpn. Society for Bioscience, Biotechnology and Agrochemistry,* **53**, 363 (1979)

2) Yamada, K., Furukawa, T. and Nakahara, T.: *Agr. Biol. Chem.,* **34**, 670 (1970)

3) Wang, H. L. and Hesseltine, C.W.: *Cereal Chem.,* **43**, 563 (1966)

4) Shiraishi, T.: *New Food Industry,* **29**, No.5, 51 (1987)

5) Jacobson, K. P.: *Biochem. Z.,* **234**, 401 (1931)

6) Favelukes, G. and Stoppani, A.O.M.: *Biochemi. Biophysi. Acta,* **28**, 654(1958)

7) Kanarek, L. and Hill, R. L.: *J. Biol. Chem.,* **239**, 4202 (1964)

8) Pierpoint, W. S.: *Biochem. J.,* **75**, 511 (1960)

9) Kitahara, K., Fukui, S. and Misawa, M.: *J. of Jpn. Society for Bioscience, Biotechnology and Agrochemistry,* **33**, 528 (1959)

10) Jung, C.M., Miyamoto, T., Kataoka, K., Ohhira, I. and Yoneya, T.: *Nippon Shokuhin Kogyo Gakkaishi (J.of Jpn. Society for Food and Technology),* **40**, 316 (1993)

11.)Reddy, N. S., Miyamoto, T., Kataoka, K. and Nakae, T: *Jpn. J. of Dairy and Food Science,* **35**, A-5 (1986)

12) Tsukioka, H., Hiroi, T. and Suzuki, T.: *J. of Jpn. Society for Bioscience, Biotechnology and Agrochemistry,* **62**, 971 (1988)

13) Glenn, W.E. and Prouty, C. C.: *Appl. Microbiol.,* **3**, 317 (1955)

14) Branen, A. L. and Keenan, T. W.: *J. Dairy Sci.,* **53**, 593 (1970)

15) Harvey, R. J. and Collins, E.B.: *J. Bacteriol.,* **86**, 1301 (1963)

16) Kaneko, T., Suzuki, H. and Takahashi,T.: *Agric. Biol. Chem.,* **51**, 2315(1987)

17) Ito, S., Yoshizuka, N., Kobayashi, T., Saitoh, M. and Morichi, T.: *Agric. Biol. Chem.,* **49**, 231 (1985)

18) Wright, C.T. and Klaenhammer, T.R.: *Appl. Environ. Microbiol.,* **46**, 785 (1983)

19) Steinkraus, K.H.: Jpn. J. of *Food Industry,* **32**, No.12, 20 (1989)

20) Kitahara, K. and Katagiri, T.: *J. of Jpn. Society for Bioscience, Biotechnology and Agrochemistry,* **14**, 752 (1938)

21) Noll, F.: *Methods of Enzymatic Analysis,* (Bergmeyer, H.U.ed.)3rd., vol.VI (Verlag Chemie, Weinheim, Deerfiled Beach/Florida, Basel) p.582 (1984)

22) Radler, F.: *Lactic Acid Bacteria in Beverages and Food,* Carr, J.G. Cutting, C.V. and Whiting, G.C. ed (Academic press, London, New York and San Francisco) p.17 (1975)

23) Hara, M.: *Maturation of Food,* supervised by Sato., S. (Kourin, Tokyo) p.96 (1984)

24) Sneath, P. H. A., Mair, N. S., Sharpe, M. E. and Holt, J. G.: *Bergey's Manual of Systematic Bacteriology, Vol.2*(Williams and Wilkins, Baltimore), p.1223 (1986)

Received May 12, 1993