Screening for Lactic Acid Bacteria Transforming Fumarate to L-Malate and Some Properties of Selected Strains

Chang 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 they contribute largely to flavor of fermented foods. In the present study, an attempt was made to select lactic acid bacteria producing strong fumarate hydratase (EC 4.2.1.2, known as fumarase) to transform fumarate to L-malate. Forty-nine strains of lactic acid bacteria were assayed for the ability exhibiting the conversion of fumarate to L-malate in cell suspensions incorporated with disodium fumarate and toluene. The ability was determined by an enzymatic method and high performance liquid chromatography. Most strains of *Lactobacillus* species showed high ability to transform fumarate to L-malate, although *Streptococcus, Enteroccocus, Lactococcus* and *Leuconostoc* species had weak L-malate producing ability. Among the tested strains, *Lactobacillus delbrueckii* subsp. *bulgaricus* strain 7235 exhibited the highest ability of L-malate production in the reaction mixture without sodium chloride, whereas *Lactobacillus delbrueckii* subsp. *lactis* strain 1135 was the highest in the reaction mixture supplemented with 5% sodium chloride. The optimal pH of L-malate producing ability in strain 7235 and strain 1135 were 6 and 7, respectively, and higher L-malate production by both strains was observed in the range from 30℃ to 45℃. L-malate production by strain 7235 and strain 1135 was maximal at the concentration of 3% and 10% sodium chloride, respectively. (Received Jun.25,1992)

The role of microorganisms in fermented foods is extremely important in forming flavor components (*1,2*). Among them, organic acids as microorganisms metabolism producer takes an important role as flavor and acid ingredients for various kinds of fermented foods as *miso* or soybean paste, *shoyu* or soy sauce and yogurt; fruits juice or soft drinks. This shows that some kinds of organic acids are used as sour seasoning ingredients (*3*). However, strength and quality of sourness of each organic acid differ respectively and due to the balance of these in volume, insufficiency of flavor could be brought about.

L-malate which is utilized mainly in soft drink, ice cream or sorbet as sour ingredients is transformed from fumarate with the activity of fumarate hydratease (EC4.2.1.2, hereafter cited as fumarase). Fumarate producing fungi are known as filamentous fungi, the genus *Rhizopus* (*4*), for L-malate producing, *Asperigillus flavus* (*5,6*), *Asp. parasiticus* (*5,6*), *Asp. oryzae* (*5,6*) and *Schizophyllum commune* (*7*). In contrast, Kitahara *et al.* (*8-10*)

report that *Lactobacillus brevis* have strong fumarase activity. But no report has been seen after that about L-malate production from fumarate by fumarase of lactic acid bacteria. Therefore, along with screening L-fumarate producing activity from fumarate of lactic acid bacteria of 49 strains 6 genera kept in our laboratory, we investigated on active properties of lactic acid bacteria strain containing high L-fumarate producing activity.

METHODS

1. Sample strains

We tested 49 strains 6 genera of lactic acid bacteria kept in our laboratory. Table 1 shows the details: the genus *Streptococcus* (3), *Enterococcus* (5), *Lactococcus* (8), *Leuconostoc* (3), *Pediococcus* (6) and *Lactobacillus* (24). These strain were precultured for 24 hours earlier at appropriate temperature for each strains (30℃, 34℃ or 37℃) with GYP medium (pH 6.8) (of 1% glucose, 0.5% yeast extract, 1% peptone, 0.01% L-cysteine and 0.1% Tween 80) we sampled in the following L-malate producing activity test from fumarate.

2. Determination method of L-malate

We measured L-malate producing activity from fumarate according to Kitahara's (*8*) method. That is, after inoculating 0.1% preculture solution to GYP medium, we suspended the microbes (250 to 350mg dried microbes) prepared by centrifuging by washing from culture solution cultured statically for 24 hours in 10mL 0.2M phosphoric acid buffer (pH7.4). With the reaction mixture the above is added to 0.4mL toluen or 10mL 0.2M fumarate-sodium solution (pH7.4) as substrate, after the prescribed hours, the volume of L-malate produced from fumarate was measured by enzyme method. Also, in order to research the salt influence on this reaction we investigated L-malate producing activity on the reaction 5% NaCl added. Then we used high performance liquid chromatography to research the time change from fumarate to each organic acid by strains with high L-malate activity.

(1) Determination of L-malate by enzyme method

After 2-days reaction at 30℃, fungus bodies were eliminated with Millipore filter (0.22 μ m), we measured the volume of L-malate acid in filtrate. For measuring the volume of L-malate, we used F-kit (Boehringer Mannheim-Yamanouchi Pharmaceutical Co., Ltd.) to measure NADH or dihydronicotinamide adenine dinucleotide its optical density at 340nm which is produced by MDH or malate dehydrogenase under NAD or nicotinamide adenine dinucleotide. The result was shown in μg for the volume of L-malate produced from fumarate by 1mg microbe. And, on the strain high in L-malate producing activity, we investigated temperature, pH and salt influence from fumarate to L-malate producing activity.

(2) Determination of fumarate and L-malate by high performance liquid chromatography

For determination of fumarate and L-malate, we used high performance liquid chromatography. That is after 0, 1, 2, 3, 4, 5, 7 and 15-day reaction at 30℃, we eliminated fungus bodies with Millipore filter (0.22 μ m), we determined organic acid in the buffer with the following conditions:

apparatus: Shimadzu LC-6A
column: SCR-101 H (Shimadzu), 7.9mm ϕ × 30cm
migration phase: phosphoric acid (pH2.2)
flow speed: 0.8mL/min
detector: Shimadzu SPD-6A (UV, 210nm)
volume of sample added: 10 μL
recorder: Shimadzu CR 6-5 A

Also, using standard sample which each organic acid is diluted to distilled water as contrast, we identified the peak of organic acid in each sample from retention time detected from chromatogram of organic acid standard solution. Also, the measuring line was processed from peak area value and concentration (mg/mL) of chromatogram of organic acid standard solution.

Table 1 Malic acid production in the reaction mixture with and

Strains[1]		L-Malic acid (μg/mg)[2]	
		0% NaCl	5% NaCl
Streptococcus salivarius			
subsp. *thermophilus*	NIAI 510	0.08	0.02
	16230	0	0.02
	18235	0	0.02
Enterococcus faecalis	RIMD 3116001	1.29	0.01
	5 C 3	0.06	0.01
	3311	0	0.01
	8301	0.01	0.02
E. faecium	IFO 3826	0.04	0.05
Lactococcus lactis			
subsp. *cremoris*	NIAI H-61	0.01	0.01
	525	0.14	0.02
	5 B 9	0.02	0.03
	5 M 1	0.01	0.01
subsp. *lactis*	NIAI N-7	0.02	0.02
	NIAI 527	0.03	0.02
	KM	1.66	0.01
	10302	0	0.01
Leuconostoc mesenteroides			
subsp. *cremoris*	IAM 1087	0.14	0.07
subsp. *dextranicum*	IAM 1122	0.37	2.29
subsp. *mesenteroides*	IAM 1046	0.03	0
Pediococcus acidilactici	NCDO 1859	5.58	4.89
	2-5	2.90	5.51
P. halophilus	NCDO 1635	8.33	8.99
P. pentosaceus	RW 2	3.86	3.99
	IAM 12296	26.00	17.11
P. urinaeequi	NCDO 1636	0.13	0.01

1) The strain were held in the reaction mixture for 2 days.
2) The malic acid was expressed as the amount per 1 mg of dry cell weight.

RESULTS AND STUDY

1. Production of L-malate from fumarate by lactic acid bacteria

Table 1 shows the results of determination of L-malate production from fumarate by 49 strains of sampled lactic acid bacteria by enzyme method on the second day. As a result, strains of the genera *Streptococcus, Enterococcus, Lactococcus* and *Leuconostoc* showed weak L-malate production while most of the strains did not show transformation of L-malate from fumarate. However, in the genus *Lactobacillus* all strains we sampled were assumed to contain fumarase. The strain showed especially high L-malate production was *Lactobacillus delbrueckii,* among them it was *L. delbrueckii* subsp. *bulgaricus* 7235. Also some strains of the genus *Pediococcus* contained high L-malate productivity.

On the other hand, in consideration of influence of salt added foods, we investigated L-malic acid production from fumaric acid 5% NaCl added. *L. delbrueckii* subsp. *bulgaricus* 7235, *L. delbrueckii* subsp. *delbrueckii* ATCC9649 and *L. plantarum* IFO 3070 showing high productivity of L-malate in 0% NaCl, showed similar or poorer productivity to that in 0% NaCl even under the condition supplemented with 5% NaCl. *L. delbrueckii* subsp. lactis 1135, *L. fermentum* 9235 and *L. curvatus* 921 showed more production volume L-malate than under 0% NaCl. And, in the genus *Pediococcus, P. pentosaceus* IAM 12296 showed more volume of L-malate in 0% NaCl than 5% NaCl. *P. halophilus* known as halotolerance lactic acid bacteria was not

without sodium chloride by lactic acid bacteria

Strains[1]		L-Malic acid (μg/mg)[2]	
		0% NaCl	5% NaCl
Lactobacillus acidophilus	NIAI L-54	3.45	4.50
	IID 893	2.51	6.84
	AHU 1042	0.42	3.95
L. brevis	IFO 3345	3.65	2.79
L. delbrueckii			
subsp. *bulgaricus*	NIAI B-5 b	4.22	18.55
	7235	30.07	27.43
subsp. *delbrueckii*	ATCC 9649	22.39	14.28
subsp. *lactis*	1135	24.67	30.48
L. casei subsp. *casei*	NIAI 34143	9.00	7.93
	ATCC 393	2.99	1.32
	NIAI L-14	5.29	5.72
subsp. *pseudoplantarum*	ATCC 25598	2.11	5.23
subsp. *tolerans*	ATCC 25599	2.18	1.60
L. curvatus	921	17.24	23.50
L. fermentum	9235	17.85	29.97
L. helveticus	NIAI B-1	0.30	8.95
	AHU 1687	2.47	4.56
	4811	12.93	1.85
"L. japonicus"	IAM 10068	22.33	20.85
L. plantarum	IFO 3070	27.16	25.27
	6201	16.98	19.32
	12111	13.08	8.63
	6214	8.78	8.76
L. rhamnosus	ATCC 7649	7.22	5.43

influenced by NaCl added but it showed almost the same value of L-malic acid volume produced with both reaction mixture.

2. Production of L-malic acid from fumaric acid with *Lactobacillus delbrueckii*

On *L. delbrueckii* subsp. *bulgaricus* 7235 and *L. delbrueckii* subsp. *lactis* 1135 that showed the highest L-malate productivity in both 0% and 5% NaCl reaction mixture, we investigated the changes by time passing in production of L-malic acid than fumaric acid with high performance liquid chromatography.

Table 2 shows that *L. delbrueckii* subsp. *bulgaricus* 7235 in 0% NaCl and *L. delbrueckii* subsp. *lactis* 1135 in 5% NaCl had most of fumaric acid transformed to L-malic acid up to the second day. The transformation from then on became slow and the volume of L-malic acid produced at the 15th day was almost the same in both strains. But the transformation of L-malic acid produced to other organic acid was not admitted.

Fig. 1 shows the result of influence of toluen application to reaction mixture to investigate on the conversion of L-malic acid to other organic acid with *L. delbrueckii* subsp. *bulgaricus* 7235. In case of no toluene added, not only L-malic but also oxalacetic acid and pyruvic acid were produced. By the way, enzyme involving in the metabolism of L-malic acid to lactic acid are malo-lactic enzyme (*12*), malic acid dissolving enzyme (*12*) and malic acid dehydronase (*12*). In this strain, no malo-lactic enzyme exists. It is assumed that malic acid dehydronase was involving in metabolism of L-malic acid.

Table 2 Conversion of fumaric acid to malic acid in the reaction mixture by lactic acid bacteria

Strains	Reaction time (days)	Fumaric acid (μg/mg)[1]	L-Malic acid (μg/mg)[1]
Lactobacillus delbrueckii subsp. *bulgaricus* 7235	0	56.85	0
	1	13.83	40.61
	2	6.40	50.93
	3	7.08	50.89
	4	6.83	51.35
	5	6.37	51.85
	7	6.87	51.92
	15	5.25	52.89
Lactobacillus delbrueckii subsp. *lactis* 1135[2]	0	58.59	0
	1	16.94	38.49
	2	4.74	47.99
	3	5.56	51.07
	4	5.47	52.62
	5	4.81	49.46
	7	5.27	50.44
	15	5.50	51.64

1) The organic acids were expressed as the amount per 1 mg of dry cell weight.
2) Strain 1135 was investigated in the reaction mixture supplemented with 5% sodium chloride.

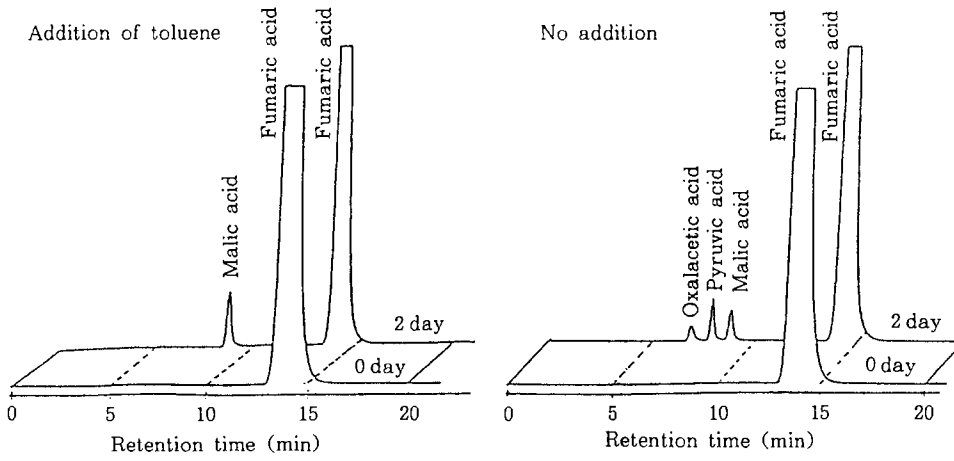

Fig. 1 Conversion of fumaric acid to organic acids in the reaction mixture with and without toluene

3. Influence of temperature, pH and salt in production of L-malic acid from fumaric acid by *Lactobacillus delbrueckii*

In 0% NaCl and 5% NaCl, we investigated the influence of temperature and pH in production of L-malic acid from fumaric acid by *L. delbrueckii* subsp. *bulgaricus* 7235 with high productivity of L-malic acid in 0% and 5% NaCl and *L. delbrueckii* subsp. *lactis* 1135. (Fig. 2 and 3)

The appropriate temperature of two strains we sampled to L-malic acid production was in the range between 30℃ to 45℃. *L. delbrueckii* subsp. *lactis* 1135 showed high productivity even at 50℃. However, *L. delbrueckii* subsp. *bulgaricus* 7235 had L-malic acid productivity decreased from 20℃and from 50℃, so did *L. delbrueckii* subsp. *lactis* 1135 from 25℃ and from 55℃. Both strains had L-malic acid production extremely lowered at 10℃ and 60℃. On the contrary, the optimal pH of L-malate producing ability in *L. delbrueckii* subsp. *lactis* 1135 and *L. delbrueckii* subsp. *bulgaricus* 7235 were 7 and 6, respectively, and higher L-malate production was observed in the range from pH 4 to 9. Kitahara *et al.* (*10*) reveal that the optimal condition for fumarase activity *L. brevis* contains is pH6.7 at 37℃ showing activity even at 0℃. Also, Massey V. (*13*) announces that fumarase purified from pig's heart showed high activity as around pH7 and relatively stable activity in neutral zone.

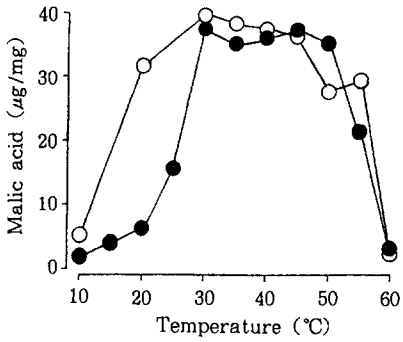

Fig. 2 Effect of temperature on the conversion of fumaric acid to L-malic acid in the reaction mixture (pH 7.4) held for 48 hours

● : *Lactobacillus delbrueckii* subsp. *lactis* 1135

○ : *Lactobacillus delbrueckii* subsp. *bulgaricus* 7235

The reaction mixture was composed of 0.2 M disodium fumarate 10 ml, 0.2 M phosphate buffer 10 ml and toluene 0.4 ml. Strain 1135 was investigated in the reaction mixture supplemented with 5% sodium chloride.

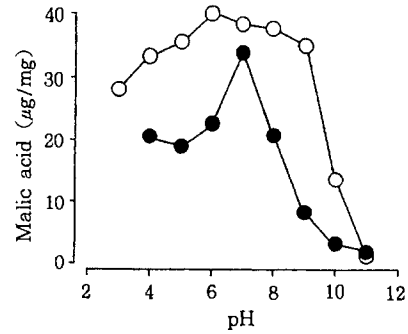

Fig. 3 Effect of pH on the conversion of fumaric acid to L-malic acid in the reaction mixture held for 48 hours at 30°C

Composition of the reaction mixture and symbols were shown in Fig. 2.
Strain 1135 was investigated in the reaction mixture supplemented with 5% sodium chloride.

Next, on investigating on these two strains the salt influence to L-malic acid production from fumaric acid, *L. delbrueckii* subsp. *bulgaricus* 7235 in 3% NaCl and *L. delbrueckii* subsp. *lactis* 1135 in 10% NaCl showed the highest L-malic acid production (Fig. 4).

On studying L-malic acid production from fumaric acid by lactic acid bacteria as above, *L. delbrueckii* subsp. *bulgaricus* 7235 and *L. delbrueckii* subsp. *lactis* 1135 showed high L-malic acid production. In the future we need to study fumarase these strains produce with the properties on enzymology. Fumaric acid is produced in volume by strains of the genus *Rhizopus* (*3*).

Hesseltein (*14*) says that on tempeh production, a traditional fermented soybean foods in Indonesia, a big volume of fumaric and lactic acid production by tempeh filamentous fungi is observed. Also, when tempeh produced from wheat or cereals is supplemented by *Rhizopus oryzae*, glucose formed by amylase becomes fumaric acid by fermentation and it is said that it becomes sour products. Application of in flavor improvement of filamenteous fungi fermentation together with lactic acid bacteria strain possessing high L-malic acid production obtained in determination of this study is therefore anticipated.

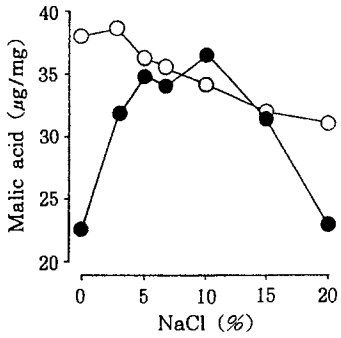

Fig. 4 Effect of NaCl on the conversion of fumaric acid to L-malic acid in the reaction mixture (pH 7.4) held for 48 hours at 30°C

Composition of the reaction mixture and symbols were shown in Fig. 2.

SUMMARY

We obtained the following results of 6 genera 49 strains of lactic acid bacteria comparing L-malic acid production from fumaric acid.

(1) The strains of the genera *Streptococcus, Enteroccocus, Lactococcus* and *Leuconostoc* species had weak L-malate producing ability and conversion from fumalic acid to L-malic acid was scarcely admitted.

(2) In the genus *Pediococcus,* although *P. pentosaceus* IAM 12296 in 0% NaCl reaction mixture showed high ability of production, in other strains, the ability of production in both reaction mixture was almost similar.

(3) The genus *Lactobacillus* possessed strains showing high ability of production. Among them, *L. delbrueckii* subsp. *bulgaricus* 7235 in 0% NaCl and *L. delbrueckii* subsp. *lactis* 1135 in 5% showed the highest L-malic acid producing activity.

(4) In above two strains showing high producing activity, most of fumaric acid were convereted to L-malic acid till the second day of reaction.

(5) The optimal pH of L-malate producing ability in *L. delbrueckii* subsp. *lactis* 1135 and *L. delbrueckii* subsp. *bulgaricus* 7235 were 6 and 7, respectively, and higher L-malate production by both strains was observed in the range from 30℃ to 45℃. L-malate production by strain 7235 and strain 1135 was maximal at the concentration of 3% and 10% NaCl respectively.

REFERENCES

1) James, M. J.: *Modern food microbiology, 3rd ed.* (Van Nostrand Reinhold company, New York), p.326 (1986)

2) Brian, J. B. W.: *Microbiology of Fermented Foods, Vol.1* (Elsevier Applied Science Publishers, London and New York), p.167, p.263 (1985)

3) Iwamoto, M.: *Nipppon Shokuhin Kogyo Gakkaishi* (*J. of Jpn. Society for Food and Technology*) **27**, 464 (1980)

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

5) Abe, S., Furuya, A., Saito, K. and Takayama, K.: *J. of Jpn. Society for Bioscience, Biotechnology, and Agrochemistry,* **33**, 571 (1959)

6) Abe, S., Saito, K. and Takayama, K.: *J. of Jpn. Society for Bioscience, Biotechnology, and Agrochemistry,* **34**, 66 (1960)

7) Tachibana, S. and Shiode, J.: *XIIIth Intern. Congr. Microbiol., Abstrs.,* (Montreal Canada) p. 67 (1962)

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

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

10) Kitahara, K., Hukui, S. and Misawa, M.: *J. Gen. Appl. Microbiol.,* **6** 108(1960)

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

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

13) Massey, V.: *Biochem. J.,* **53**, 67 (1953)

14) Hesselstein C. W.: *Nipppon Shokuhin Kogyo Gakkaishi (J. of Jpn. Society for Food and Technology)* **32**, No.12, 34 (1989)

(Received Jun.25,1992)