Effects of Sodium Chloride, Ethanol, Glucose Content and pH on the Growth of Lactic Acid Bacteria Isolated from Salted "Daikon" (*Raphanus sativus*)

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The effects of salt (6.3~8.3%), ethanol (0~2%), glucose (0.3~2.3%) and pH (4.0~5.0) on the growth of 6 species of lactic acid bacteria (*Lactobacillus plantarum, L. brevis, L. bavaricus, L. coryniformis* subsp. *torquens, L. coryniformis* subsp. *coryniformis,* and *Lactobacillus* sp. 1), which were isolated from salted "Daikon", were studied by using fractional factorial experimental design for factors at three levels. *L. plantarum* and *L. brevis* grew in the presence of 6.3~8.3% sodium chloride, while no growth of the other 4 species was observed in the medium containing over 7.3% salt. The growth of *L. plantarum* and *L. brevis* was inhibited when the concentration of sodium chloride was increased and when the medium pH was decreased. Ethanol had an inhibitory effect at increased concentrations on the growth of *L. brevis,* but not on the growth of *L. plantarum.* Inhibition of growth of *L. plantarum* and *L. brevis* was most marked by pH, followed by sodium chloride and ethanol. Effects of glucose on the growth of these lactic acid bacteria (6 species) were not clearcut.

During the storage of salted "Daikon" (*Raphanus sativus*) in 5~6% NaCl (low salt concentration) with the newly developed nitrogen gas packing system, the growth of *Debaryomyces hansenii* was inhibited and *Saccharomyces cervazzii* grew dominantly in the cover brine. After growth of *Sacch. servazzii* it has been found that the cover brine shows the increased pH of 4.3 and contains 2% ethanol and 0% reducing suger. By using the nitrogen gas packing system, it is possible to maintain these conditions for extended period of time, which inhibits growth of harmful microorganisms. The salted "Daikon" may be kept in good condition for a long period of storage by the nitrogen gas packing system[1]. However, when the ambient temperature is over 20°C, putrefaction may occur due to acidification as the result of accumulation of excess amounts of lactic acid owing to growth of lactic acid bacteria[2].

In general, the growth of lactic acid bacteria may be inhibited by decreasing the pH, increasing the salt and ethanol content, and decreasing the reducing sugar content. However, the relationship between the growth of lactic acid bacteria and the above-mentioned 4 factors have not been reported.

We have studied the effects of 4 factors ; pH, NaCl, ethanol and reducing sugar on the growth of lactic acid bacteria isolated from salted "Daikon".

1. Materials and method

Bacterial strains : The lactic acid bacteria used in this study were *Lactobacillus plantarum, L. brevis, L. bavaricus, L. coryniformis* subsp. *torquens, L. coryniformis* subsp. *coryniformis, Lactobacillus* sp. 1. All the strains were isolated from salted "Daikon" and stored at −80°C.

Experimental design : Fractional factorial experimental design for factors at three levels was used. The factors (pH, NaCl, ethanol, and reducing sugar content) were assigned to the orthogonal table ($L_{27}(3^{13})$) as described in Table 2. The levels, which were the quantitative conditions of the factors were three as follows : pH ; 4.0, 4.5, 5.0, NaCl ; 6.3%, 7.3%, 8.3%, ethanol ; 0%, 1.0%, 2.0% , reducing sugar (glucose) ; 0.3%, 0.8%, 2.3% (Table 1). Appropriate levels were determined on the basis of changes in constituents in cover brine observed during the storage of salted "Daikon"[1].

Cultivation medium : GAM broth (Nissui Tokyo) was used as the basal medium. It was prepared to get various combinations of pH,

Table 1 Influences of salt, suger, ethanol, and pH on the growth of six species of lactic acid bacteria in GAM broth media

Exp. No.	(Factors) NaCl (%)	Sugar (%)	pH	Ethanol (%)	Increment of optical density at 660 nm in the media after cultivation for 10 days L. pla	L. bre	L. bav	L. cor tor	L. cor cor	L. sp. 1
1	6.3	2.3	5.0	0.0	+++	+++	+++	++	+++	++
2	6.3	2.3	4.5	1.0	+++	+++				
3	6.3	2.3	4.0	2.0						
4	6.3	0.8	5.0	1.0	+++	+++	+++	+++	+++	+++
5	6.3	0.8	4.5	2.0	++	++				
6	6.3	0.8	4.0	0.0						
7	6.3	0.3	5.0	2.0	+++	++				
8	6.3	0.3	4.5	0.0	+++	+++				
9	6.3	0.3	4.0	1.0						
10	7.3	2.3	5.0	1.0	+++	++				
11	7.3	2.3	4.5	2.0		+				
12	7.3	2.3	4.0	0.0						
13	7.3	0.8	5.0	2.0	+++	++				
14	7.3	0.8	4.5	0.0	+++	+++				
15	7.3	0.8	4.0	1.0						
16	7.3	0.3	5.0	0.0	+++	+++				
17	7.3	0.3	4.5	1.0		++				
18	7.3	0.3	4.0	2.0						
19	8.3	2.3	5.0	2.0						
20	8.3	2.3	4.5	0.0		+				
21	8.3	2.3	4.0	1.0						
22	8.3	0.8	5.0	0.0	++	++				
23	8.3	0.8	4.5	1.0		+				
24	8.3	0.8	4.0	2.0						
25	8.3	0.3	5.0	1.0	++	++				
26	8.3	0.3	4.5	2.0						
27	8.3	0.3	4.0	0.0						

The abbreviations used are as follows: L. pla, *Lactobacillus plantarum*; L. bre, *Lactobacillus brevis*; L. bav, *Lactobacillus bavaricus*; L. cor. tor, *Lactobacillus coryniformis* subsp. *torquens*; L. cor. cor; *Lactobacillus coryniformis* subsp. *coryniformis*; L. sp. 1; *Lactobacillus* sp. 1

Symbols: +++, grow actively (Increment of O.D. was more than 0.40); ++, grow (Increment of O.D. was between 0.20 and 0.39); +, grow weakly (Increment of O.D. was between 0.10 and 0.19). Bacterial growth was measured as increment of optical density at 660 nm. GAM (Nissui) broth media were prepared under various conditions in each experiment.

NaCl, ethanol and reducing sugar at different levels (Table 1), as follows : first, salt and sugar was added to GAM broth and dissolved. After it had been adjusted to get the required pH, the broth was autoclaved for 15 min, at 15 pounds pressure (121°C), and ethanol was added to it under aseptic conditions. After the various broths were left for 2~3 days, 4 m*l* of the upper part of the broths were transferred into test tubes with plugs (10 m*l* with 14 mm i.d.).

Fermentation test : Actively growing cultures were suspended into approx. 7 m*l* of physiological saline solution (0.85% sodium chloride) in 10 m*l* plugged test tubes (14 mm i.d.). The transmittance of the suspension was adjusted to 60% at 660 nm by adding sterilized physiological saline. The suspension (0.2 m*l*) was transferred into 4 m*l* of the cultivation medium in the plugged tubes.

Each strain in the medium was cultivated under non-agitated atmospheric conditions at 35°C for 10 days.

Measurement of cellular growth : Cellular growth was judged by the increment of optical density at 660 nm after 10 days cultivation. Its O.D. was measured with a Shimazu spectronic 70 spectrometer (Shimazu, Kyoto).

2. Result and discussion

The effects of increasing the salt level from 6.3% to 8.3% and the ethanol content from 0.0 % to 2.0%, as well as decreasing the pH value from 5.0 to 4.0 and the reducing sugar (glucose) content from 2.3% to 0.3% on the growth of 6 species lactic acid bacteria (*L. plantarum, L. brevis L. bavaricus, L. coryniformis* subsp. *torquens, L. coryniformis* subsp. *coryniformis,* and *Lactobacillus* sp. 1) in GAM broth media are shown Table 1.

L. plantarum and *L. brevis* grew under the conditions of salt concentration ranging from 6.3 to 8.3%. However, the 4 other species did

Table 2 Analysis of variance table for the effects of salt, sugar, ethanol and pH on the growth of *Lactobacillus plantarum*

Array No.	Factors	Level means			D.F.	S.S.	F	Ratio
		1	2	3				
1	A	0.17	0.01	−0.18	2	0.57	4.69*	22.65
2	B	−0.03	0.02	0.01	2	0.01	0.10	0.48
5	C	0.25	−0.00	−0.25	2	1.10	9.05**	43.67
9	D	0.08	0.02	−0.10	2	0.14	1.17	5.65
3	A B	0.02	−0.03	0.01	4	0.07	0.30	2.93
4		0.05	−0.07	0.02				
8	B C	−0.02	0.00	0.02	4	0.01	0.05	0.48
11		−0.01	−0.01	0.02				
6	e	−0.03	0.14	−0.11	10	0.61		24.13
7		0.05	0.01	−0.06				
10		0.01	0.04	−0.05				
12		−0.06	0.06	−0.01				
13		−0.04	−0.07	0.11				

Symbols : A, NaCl ; B, sugar ; C, pH ; D, ethanol ; e, error ; D.F. degrees of freedom ; S.S, sum of squares of devitions ; F, F value

Single and double asterisks indicate significance at the 5% level (F : 4.10) and 1% level (F : 7.56), respectively.

Table 3 Analysis of variance table for the effects of salt, sugar, ethanol and pH on the growth of *Lactobacillus brevis*

Array No.	Factors	Level means			D.F.	S.S.	F	Ratio
		1	2	3				
1	A	0.07	0.03	−0.10	2	0.15	8.43**	18.68
2	B	−0.01	0.00	0.01	2	0.00	0.11	0.23
5	C	0.11	0.06	−0.17	2	0.41	23.63**	52.36
9	D	0.06	0.01	−0.08	2	0.09	5.03*	11.14
3	A B	0.05	−0.04	0.00	4	0.05	1.36	6.01
4		0.03	−0.01	−0.02				
8	B C	0.00	−0.01	0.01	4	0.00	0.11	0.49
11		0.01	−0.01	−0.01				
6		−0.00	0.03	−0.03				
7		0.05	−0.01	−0.04				
10	e	0.01	0.01	−0.02	10	0.09		11.08
12		−0.00	0.02	−0.02				
13		0.01	−0.04	0.03				

The symbols are the same as in Table 2.

not grow in media containing more than 7.3% salt. It seemed that *L. plantarum* and *L. brevis* were more tolerant to salt than the 4 other species.

The effects of salt and pH on the growth of *L. plantarum* and *L. brevis* were significant at the 5% level. The growth of *L. plantarum* and *L. brevis* were inhibited when salt level was increased and the pH was decreased. The effect of ethanol on *L. brevis* was significant at the 5% level but that on *L. plantarum* was not significant (Tables 2 and 3). The growth of *L. brevis* was inhibited by increasing the ethanol content but that of *L. plantarum* was not inhibited. *L. plantarum* was more tolerant to ethanol than *L. brevis*. pH (5.0~4.0) was the most inhibitory factor to the growth of *L.* plantarum and *L. brevis*, followed by salt (6.3 ~8.3%) and then ethanol (0~2%). The effect s of glucose (0.3~2.3%) on the growth of these 6 species of lactic acid bacteria were not significant at the 5% level.

The growth of the 6 species was inhibited completely by the conditions consisting of combinations of 2 or 3 of the following factors : with 6.3% salt and pH≦4.0 ; with 7.3 % salt, pH≦4.5 and ethanol≧2.0% ; with 8.3 % salt, pH≦5.0 and ethanol≧2.0% (Table 1).

In conclusion, it seems that the growth of *L. bavaricus, L. coryniformis* subsp. *torquens, L. coryniformis* subsp. *coryniformis*, and *Lactobacillus* sp. 1 are inhibited by the conditions of cover brine with approx. 6% salt, pH 4.3, and ethaonl 2.0% in the nitrogen gas packing system for the storage of salted "Daikon", whereas the growth of *L. plantarum* and *L. brevis* is not inhibited.

We consider that it is necessary to maintain the cover brine under the above mentioned conditions, that is, with 6.3% salt, pH≦4.0 ; with 7.3% salt, pH≦4.5 and ethanol≧2.0% ; with 8.3% salt, pH≦5.0 and ethanol≧2.0 in order to prevent the putrefaction with accumulation of excess amounts of lactic acid.

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塩漬大根より分離した乳酸菌の生育に及ぼす食塩，エタノール，グルコース濃度及び pH の影響

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塩漬大根（食塩 5～6%）貯蔵中から分離した 6 種類の乳酸菌（*Lactobacillus plantarum*, L. brevis, *L. bavaricus, L. coryniformis* subsp. *coryniformis, L. coryniformis* subsp. *torquens, Lactobacillus* sp. 1）の生育に与える食塩（6.3～8.3%），エタノール（0～2%），グルコース（0.3～2.3%）濃度及び pH（4.0～5.0）の影響について多因子実験により検討した．*L. plantarum, L. brevis* は，食塩 6.3～8.3% の条件下で生育したが，他の 4 種の乳酸菌は，7.3% 以上では生育しなかった．*L. plantarum, L. brevis* の生育は，食塩濃度の増加，および *pH* の低下によって抑制された．*L. brevis* の生育は，エタノール濃度の増加によって抑制されたが，*L. plantarum* では，抑制効果は認められなかった．*L. plantarum, L. brevis* の生育抑制効果は，pH，食塩，エタノールの順に大きかった．これら 6 種類の乳酸菌の生育に対するグルコース濃度の影響は，明らかではなかった．