Characterization of Lactic Acid Bacteria Isolated from Sauce-type *Kimchi*

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Abstract

This study was carried out to investigate the isolation and characterization of lactic acid bacteria (LAB) from naturally fermented sauce-type *kimchi*. Sauce-type *kimchi* was prepared with fresh, chopped ingredients (Korean cabbage, radish, garlic, ginger, green onion, and red pepper). The two isolated bacteria from sauce-type *kimchi* were identified as *Pediococcus pentosaceus* and *Lactobacillus brevis* by 16S rDNA sequencing and tentatively named *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2, respectively. *Pediococcus* sp. IJ-K1 was isolated from the early and middle fermentation stages of sauce-type *kimchi* whereas *Lactobacillus* sp. IJ-K2 was isolated from the late fermentation stage. The resistance of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 to artificial gastric and bile acids led to bacterial survival rates that were 100% and 84.21%, respectively.

Key words: sauce-type *kimchi*, lactic acid bacteria, *Pediococcus* sp., *Lactobacillus* sp.

INTRODUCTION

Kimchis are fermented using vegetables mixed with spicy seasoning as traditional Korean fermented food and categorized by seasonings, areas, and ingredients (1). Cabbage *kimchi*, which is a fermented cabbage seasoned with radish, garlic, ginger, green onion, red pepper, and red pepper powder, is commonly prepared in Korean homes (2). The quality of *kimchi* produced in each household, however, differs because of fermenting microorganisms and various environmental conditions such as temperature, density of salt, pH, oxygen, species of cabbage, and side ingredients (3).

Kimchi has anti-cancer and anti-oxidative functional properties and is now prepared by industrial process in response to market factors that include nuclear family and women working outside (4-7). Recently, functional properties have contributed to the globalization of *kimchi*.

In Han's study, multi-purpose sauce was developed by using *kimchi* (8). Sauce-type *kimchi* is different from *mul-kimch*, which is one type of *kimchi* enriched in seasoned water (9). In *kimchi*, selection of a proper starter is crucial because it should be fermentable under harsh conditions, such as high concentrations of seasoning. Many studies have been focused on the fermenting lactic acid bacteria (LAB) (4,10-14). Generally, *Leuconostoc mesenteroides* is found at an early fermentation stage and *Lactobacillus plantarum* generates over-ripeness and

acidification after maturity of *kimchi*.

In this study, LAB was isolated from naturally fermented sauce-type *kimchi*. The isolated LAB were identified and analyzed for their physicochemical properties. Quality-controlled sauce-type *kimchi* was also produced with the isolated LAB as a starter and its properties were characterized.

MATERIALS AND METHODS

Preparation of sauce-type *kimchi*

To prepare sauce-type *kimchi*, fresh ingredients (Table 1) including Korean cabbage, radish, garlic, ginger, green

Table 1. Recipes of sauce-type *kimchi*

Ingredients	w/o starter culture	w/ starter culture
Korean cabbage	20.0[1)]	20.0
Radish	5.0	5.0
Garlic	5.4	5.4
Ginger	0.1	0.1
Green onion	1.5	1.5
Red pepper	1.2	1.2
Red pepper powder	2.3	2.3
Salt	0.9	0.9
Sugar	1.4	1.4
Starter	0.0	20.0
dH$_2$O	82.2	62.2
Total (%)	100.0	100.0

[1)]w/w %

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onion, and red pepper, were purchased from a grocery store in An-seong, Korea, 2009. All ingredients were washed, chopped, mixed together, and fermented with and without a starter at room temperature with shaking (150 rpm) for 60 hr.

Isolation and identification of LAB

To obtain LAB from the fermented sauce-type *kimchi*, the samples were independently fermented without a starter at room temperature for 20, 40, and 60 hr. The *kimchi* samples diluted with 0.85% NaCl solution were spread on MRS agar (Difco, Detroit, MI, USA) and incubated in a 2.5 L jar with an anaerobic pack at 37°C for 24 hr.

The isolated LAB was identified by analysis of the 16S rDNA sequence. The chromosomes of LAB were extracted using a G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea) with Mutanolysin (Sigma-Aldrich, St. Louis, MO, USA) and used as PCR templates. PCR was carried out over 30 cycles (initial denaturation at 97°C for 5 min, denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and polymerization at 72°C for 1.5 min) with a final 4-min polymerization step at 72°C with forward primer 5′-AGA GTT TGA TCM TGG CTC AG-3′ and reverse primer 5′-GG YTA CCT TGT TAC GAC TT-3′ using PCR System 2700 (Applied Biosystems, Foster City, CA, USA) (15). PCR products were purified with a gel extraction kit (AtmanBio, Uiwang, Korea) and sequenced at Macrogen (Seoul, Korea). The sequences of the 16S rDNA gene were analyzed with the EzTaxon server (16). A phylogenetic tree was constructed by using the neighbor-joining method that produced a unique final tree under the principle of minimum evolution and the MEGA4 program (17,18).

Physicochemical properties of the isolated LAB

Isolated LAB was analyzed for morphological properties, gram staining, and catalase activity according to Bergey's Manual (19). The growth patterns and pH changes of isolated LAB were analyzed with MRS agar plate (Difco™, Franklin Lakes, NJ, USA) at 37°C for 48 hr and a pH meter (Orion 420A, Orion Research, Boston, MA, USA). The initial inocula of IJ-K1 and IJ-K2 were 5.6×10^2 and 9.4×10^2 CFU/mL, respectively. Salt resistances were analyzed with 2%, 4%, and 6% NaCl in MRS broth (20). Carbonate fermentation properties, fermentation efficacy of sugars, and enzyme activities were analyzed with an API 50 CH Kit, API 50 CHL System Kit, and API ZYM Kit, respectively, according to the procedures described by the manufacturer (BioMérieux, Lyon, France).

Production of sauce-type *kimchi* concentrate using the isolated LAB

The sauce-type *kimchi* concentrate was produced with the isolated LAB *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 as starters. Starter ($\sim 1 \times 10^8$ cells/mL) was inoculated with supplementary nutrients and fermented for 60 hr at room temperature. Various ratios of each starter strain were applied to the different batches of sauce-type *kimchi* concentrates, such as 5:0, 0:5, 2.5:2.5, 3.5:1.5, and 1.5:3.5 of *Pediocuccus* sp. IJ-K1 to *Lactobacillus* sp. IJ-K2. Population of LAB, pH, and acidity of sauce-type *kimchi* concentrate were measured during fermentation (21,22).

Sensory evaluation

A panel of eight participants performed a sensory evaluation, assessing taste (bitterness, sourness, and savouriness), odor, and overall acceptability using the interval scale method (23). A score of 10 indicated that the feature assessed was very strong or very good, and a score of 1 indicated very weak or very poor.

Statistical analysis

All values of experimental data were obtained in triplicate and analyzed using the SPSS software package (SPSS, Chicago, IL, USA). Multiple comparisons were performed between all the data using Duncan's multiple range test at $p \leq 0.05$.

RESULTS AND DISCUSSION

Isolation and identification of LAB from sauce-type *kimchi*

To isolate LAB from naturally fermented *kimchi*, sauce-type *kimchi* was produced without adding any starter culture. During fermentation, the pH values changed from 5.8 to 3.9, and total acidity reached 0.86% (Fig. 1). LAB were isolated in the initial, middle, and late stages of fermentation. Analysis of the 16S rDNA se-
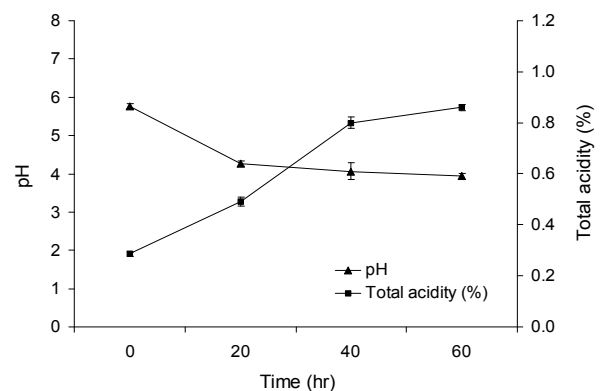

Fig. 1. Changes in pH and acidity of sauce-type *kimchi* during fermentation.

quences (>1,400 bps) revealed that the LAB isolated from the initial and middle stages displayed 99.86% homology with *Pediococcus pentosaceus* ATCC 33316[T]. In LAB isolated from the late stage of fermentation, sequences were determined to share 99.93% homology with *Lactobacillus brevis* ATCC 14869[T]. Each strain was tentatively named *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 (Fig. 2), which were predominant at each stage. The 16S rDNA gene sequences IJ-K1 and IJ-K2

were deposited in GenBank under accession number JX444059 and JX444060, respectively. Generally, *Leuconostoc* sp. is the dominant strain of traditional *kimchi* (24); however, this strain was not isolated from sauce-type *kimchi*. Besides *Pediococcus* sp. and *Lactobacillus* sp., *Bacillus* spp. were developed in the initial stage of fermentation. Microbial flora were influenced by various conditions such as composition of ingredients and culturing methods. In this study, *Pediococcus* IJ-K1 was pre-
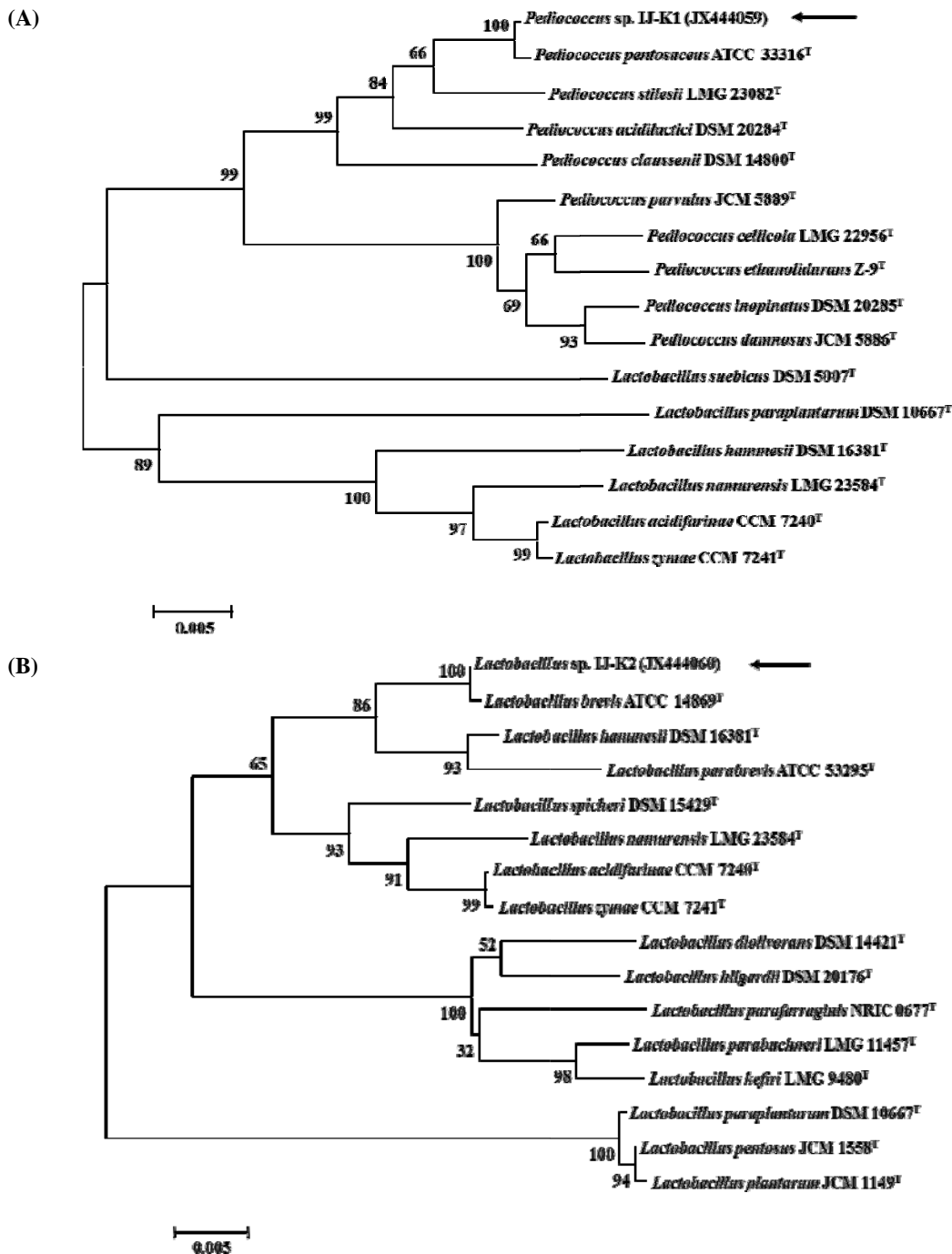

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rDNA gene sequence. A, *Pediococcus* sp. IJ-K1; B, *Lactobacillus* sp. IJ-K2. Numbers of node are levels of bootstrap support (%) from 1000 resample dataset. Bar, 0.1, nt substitution per position.
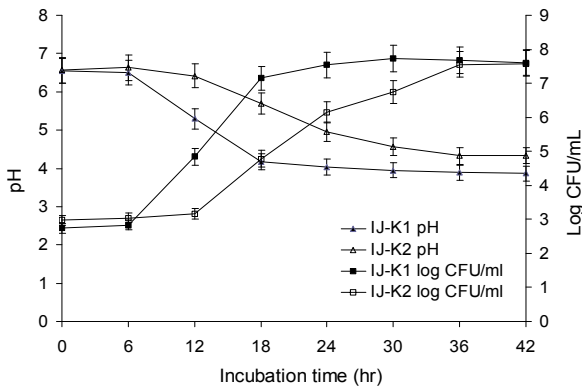
Fig. 3. Growth patterns and pH changes of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2. Squared symbol, growth pattern; triangle symbol, pH change.
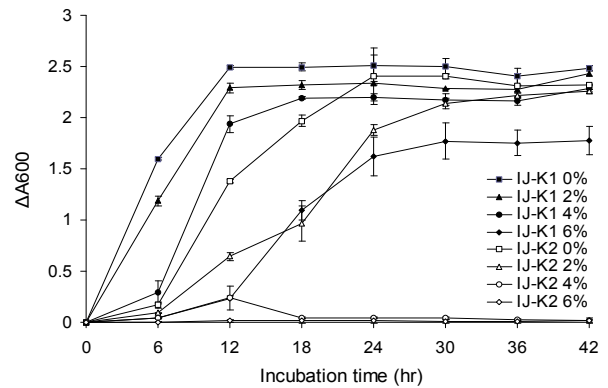

Fig. 4. Halo-tolerance analyses of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2. Closed symbol, growth pattern; open symbol, pH change. The data represent the means of three independent experiments. The error bar represents standard deviations.

dominant because of its halo-tolerance in the high salt condition of sauce-type *kimchi* (Fig. 4).

Physicochemical properties of the isolated LAB

Round-shaped *Pediococcus* sp. IJ-K1 and rod-shaped *Lactobacillus* sp. IJ-K2 cells were gram positive and catalase negative. In the growth patterns of the isolated LAB at 37°C, the duration of lag phase of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 was 6 hr and 12 hr and the stationary phase was reached after 18 hr and 36 hr of incubation, respectively. After incubation at 37 °C for 30 hr, the pH values of bacteria culture of IJ-K1

and IJ-K2 were 4.03 and 4.95, respectively (Fig. 3). In addition, the halo-tolerance of *Pediococcus* sp. IJ-K1 was higher than that of *Lactobacillus* sp. IJ-K2 (Fig. 4). The stationary phase of *Pediococcus* sp. IJ-K1 was reached in 6% NaCl after 30 hr of incubation at 37°C. However, *Lactobacillus* sp. IJ-K2 was completely inhibited from the start of lag phase at the same culture condition.

Utilization of carbohydrates with the API 50 CHL Kit is summarized in Table 2. l-Arabinose, Ribose, d-Galac-

Table 2. Carbohydrate fermentation patterns analysis of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 by API 50 CHL KIT[1)]

Carbon source	IJ-K1	IJ-K2	Carbon source	IJ-K1	IJ-K2
Control	−	−	Esculine	+	−
Glycerol	−	−	Salicin	+	−
Erythritol	−	−	Cellobiose	+	−
d-Arabinose	−	−	Maltose	+	+
l-Arabinose	+	+	Lactose	+	−
Ribose	+	+	Melibiose	+	−
d-Xylose	−	+	Sucrose	+	−
l-Xylose	−	−	Trehalose	−	−
Adonitol	−	−	Inulin	−	−
β-Methy-xyloside	−	−	Melezitose	−	−
d-Galactose	+	+	d-Raffinose	+	−
d-Glucose	+	+	Starch	−	−
d-Fructose	+	+	Glycogen	−	−
d-Mannose	+	−	Xylitol	−	−
l-Sorbose	−	−	Gentiobiose	+	−
Rhamnose	−	−	d-Turanose	−	−
Dulcitol	−	−	d-Lyxose	−	−
Inositol	−	−	d-Tagatose	+	−
Mannitol	−	+	d-Fucose	−	−
Sorbitol	−	−	l-Fucose	−	−
α-Methyl-d-mannoside	−	−	d-Arabitol	−	−
α-Methyl-d-glucoside	−	+	l-Arabitol	−	−
N-Acetylglucosamine	+	+	Gluconate	−	+
Amygdaline	+	−	2-Ketogluconate	−	+
Arbutine	+	−	5-Ketogluconate	−	−

[1)]+, positive; −, negative.

tose, d-Glucose, d-Fructose, d-Mannose, N-Acetylglucosamine, Amygdaline, Arbutine, Esculine, Salicin, Cellobiose, Maltose, Lactose, Melibiose, Sucrose, d-Raffinose, Gentiobiose, and d-Tagatose were utilized by *Pediococcus* sp. IJ-K1. *Lactobacillus* sp. IJ-K2 showed higher utilization rates with l-Arabinose, Ribose, d-Xylose, d-Galactose, d-Glucose, d-Fructose, Mannitol, α-Methyl-d-glucoside, N-Acetylglucosamine, Maltose, Gluconate, and 2-Ketogluconate. In contrast to *Pediococcus* sp. IJ-K1, *Lactobacillus* sp. IJ-K2 did not utilize lactose. Although *Lactobacillus* sp. IJ-K2 is one of many lactic acid bacteria, all LAB do not necessarily utilize lactose (25). Comparison with the API database (https://apiweb. biomerieux.com) (26) revealed 99.2% homology of IJ-K1 with *P. pentosaceus*, and 99.9% homology of IJ-K2 with *L. brevis*. In the API ZYM enzyme assay kit, *Pediococcus* sp. IJ-K1 exhibited the enzymatic activities of esterase (C4), esterase lipase (C8), leucine arylasmidase, valine arylamidase, β-galactosidase, α-glucosidase, and N-acetyl-β-glucosaminidase. In *Lactobacillus* sp. IJ-K2, the enzymatic activities of leucine arylasmidase, α-galactosidase and β-galactosidase were confirmed (Table 3). β-Glucuronidase is a carcinogenic enzyme (27), but its activity was not detected in either isolated strain.

Production of sauce-type *kimchi* concentrate using the isolated LAB

Sauce-type *kimchi* concentrate was produced with the

Table 3. Enzyme activities of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 as determined by API ZYM KIT[1]

Enzyme	IJ-K1	IJ-K2
Control	ND[2]	ND
Alkaline phosphatase	ND	ND
Esterase (C$_4$)	1	ND
Esterase lipase (C$_8$)	1	ND
Lipase (C$_{14}$)	ND	ND
Leucine arylamidase	5	2
Valine arylamidase	2	ND
Crystine arylamidase	ND	ND
Trypsin	ND	ND
α-Chymotrypsin	ND	ND
Acid phosphatase	ND	ND
Naphtol-AS-BI-phosphohydrolase	5	ND
α-Galactosidase	ND	2
β-Galactosidase	4	2
β-Glucuronidase	ND	ND
α-Glucosidase	2	ND
β-Glucosidase	ND	ND
N-Acetyl-β-glucosamidase	5	ND
α-Mannosidase	ND	ND
α-Fucosidase	ND	ND

[1]Enzyme activity was determined by using color-strength values: 0, negative reaction; 1, 5 nmol; 2, 10 nmol; 3, 20 nmol; 4, 30 nmol; 5, $\geq$40 nmol. ND, not detected.
[2]ND, not detected.

Table 4. Sensory quality of sauce-type *kimchi* produced with the isolated lactic acid bacteria

Starter ratio (IJ-K1 : IJ-K2)[1]	Final acidity (%)	Final pH
Control[2]	0.65	3.67
5:0	0.82	3.77
0:5	1.0	3.42
2.5:2.5	0.93	3.51
3.5:1.5	0.79	3.64
1.5:3.5	0.97	3.56

[1]IJ-K1: *Pediococcus* sp. IJ-K1, IJ-K2: *Lactobacillus* sp. IJ-K2.
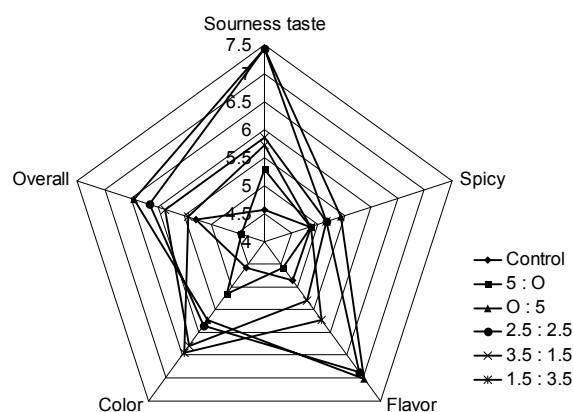[2]Naturally fermented *kimchi* without adding any starter culture.

Fig. 5. Sensory evaluation of sauce-type *kimchi* produced with various ratios of starter cultures, *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2. Various ratios of each starter strain were applied to the different batches of sauce-type *kimchi* concentrates, such as 5:0, 0:5, 2.5:2.5, 3.5:1.5 and 1.5:3.5 of *Pediocuccus* sp. IJ-K1 to *Lactobacillus* sp. IJ-K2.

isolated LAB as starter culture. The pH values of non-starter-treated sauce-type *kimchi* as control was 3.67 and starter-treated sauce-type *kimchies* were 3.42 to 3.77. Final acidities of starter-treated sauce-type *kimchi* samples were increased under the range from 0.79% to 1.0%, whereas final acidity in control was 0.65% (Table 4). Final pH was the lowest one (pH 3.42) and total acidity was the highest one (1.0%) at the inocula ratio of 0:5 (*Pediococcus* sp. IJ-K1 to *Lactobacillus* sp. IJ-K2). This sauce-type *kimchi* gave a high sourness taste during the sensory evaluation test (Fig. 5). Lee et al. (12) reported that *L. brevis* is very tolerant to acid and plays a role in the later stage of fermentation. These results suggested that the isolated *Lactobacillus* sp. IJ-K2 as starter was proper to produce sauce-type *kimchi* concentrate and its product gave better value concerning sourness taste, flavor, and overall evaluation.

CONCLUSION

The objective of this study was to isolate and identify LAB from the naturally fermented sauce-type *kimchi* and to characterize their physicochemical properties. During

the 60 hr fermentation, naturally fermented sauce-type *kimchi* was investigated for pH, acidity and dominant microbial strains. The pH values of sauce-type *kimchi* were changed from 5.8 to 3.9 and the acidity reached 0.86%. The dominant LAB were isolated and identified as *Pediococcus* sp. and *Lactobacillus* sp. by the 16S rDNA sequencing and tentatively named *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2, respectively. The pH values of *Pediococcus* sp. IJ-K1 and *Lactobacillus* sp. IJ-K2 cultures after 24 hr incubation at 37°C were 4.03 and 4.95, respectively. *Pediococcus* sp. IJ-K1 was more resistant to NaCl than *Lactobacillus* sp. IJ-K2. Sauce-type *kimchi* was produced with the isolated LAB and analyzed. An inoculum ratio of 0:5 (*Pediococcus* sp. IJ-K1 to *Lactobacillus* sp. IJ-K2) got the highest overall evaluation score, 6.5. The isolated LAB are judged to be suitable for the manufacture of sauce-type *kimchi* concentrate, the type favored to prepare various dishes as well as convenience of storage and distribution.

ACKNOWLEDGMENTS

REFERENCES

1. Cheigh HS, Park KY. 1994. Biochemical, microbiological, and nutritional aspects of *kimchi* (Korean fermented vegetable products). *Crit Rev Food Sci Nutr* 34: 175-203.
2. Park WS, Lee IS, Han YS, Koo YJ. 1944. Kimchi preparation with combined Chinese cabbage and seasoning mixture stored separately. *Korean J Soc Food Sci Technol* 26: 231-238.
3. Lim CR, Park HK, Han HU. 1989. Re-evaluation of isolation and identification of gram-positive bacteria in kimchi. *Korean J Microbiol* 27: 404-414.
4. Park KY. 1995. The nutritional evaluation, and antimutagenic and anticancer effects of Kimchi. *J Korean Soc Food Nutr* 24: 169-182.
5. Lee YO, Park KY, Cheigh HS. 1996. Antioxidative effect of Kimchi with various fermentation periods on the lipid oxidation of cooked ground meat. *J Korean Soc Food Nutr* 25: 261-266.
6. Choi WY, Park KY. 1999. Anticaner effects of organic Chinese cabbage *Kimchi. J Food Sci Nur* 4: 113-116.
7. Park JY, Rhee SH, Park KY. 2000. Enhancement of anticancer activities of *Kimchi* by manipulating ingredients. *J Food Sci Nur* 5: 126-130.
8. Han GJ, Shin DS, Cho YS, Lee SY. 2007. Development of a multi-purpose sauce using *Kimchi. Korean J Food Cookery Sci* 23: 281-287.
9. Lee SK, Ji GE, Park YH. 1999. The viability of bifidobacteria introduced into kimchi. *Lett Appl Microbiol* 28: 153-156.
10. So MH, Shin MY, Kim YB. 1996. Effects of psychrotrophic lactic acid bacterial starter on Kimchi fermentation. *Korean J Soc Food Sci Technol* 28: 806-813.
11. Mheen TI, Kwon TW. 1984. Effect of temperature and salt concentration on Kimchi fermentation. *Korean J Food Sci Technol* 16: 443-450.
12. Lee CW, Ko CY, Ha DM. 1992. Microloral changes of the lactic acid bacteria during Kimchi fermentation and identification of the isolates. *Korean J Appl Microbiol Biotechnol* 20: 102-109.
13. Lee HJ, Baek JH, Yang M, Han HU, Ko YD, Kim HJ. 1993. Characteristics of lactic acid bacterial community during kimchi fermentation by temperature downshift. *Korean J Microbiol* 31: 346-353.
14. Park JA, Heo GY, Lee JS, Oh YJ, Kim BY, Mheen TI, Kim CK, Ahn JS. 2003. Change of microbial communities in Kimchi fermentation at low temperature. *Korean J Microbiol* 39: 45-50.
15. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 173: 697-703.
16. Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW. 2007. EzTaxon: A web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequence. *Int J Syst Evol Microbiol* 57: 2259-2261.
17. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406-425.
18. Tamura K, Dudley J, Nei M, Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24: 1596-1599.
19. Peter HA, Nicholas SM. 1983. *Bergey's Manual of Systematic Bacteriology*. 2nd eds. Williams/Wilkins, Baltimore MD, USA. p 1208.
20. So MH, Lee YS, Kim HS, Cho EJ, Yea MJ. 1996. An influence of salt concentrations on growth rates of lactic acid bacteria isolated from Kimchi. *Korean J Food Nutr* 9: 341-347.
21. Shin EH. 2002. Studies on growth characteristics of *Lactobacillus brevis* isolated from Kimchi. *Korean J Food Nutr* 15: 215-219.
22. Choi IK, Jung SH, Kim BJ, Park SY, Kim JH, Han HU. 2003. Novel *Leuconostoc citreum* starter culture system for the fermentation of Kimchi, a fermented cabbage product. *Antonie Van Leeuwenhoek* 84: 247-253.
23. Herbert A, Jeol LS. 2004. *Sensory evaluation practices*. 3rd eds. Academic Press, San Diego, CA, USA. p 79.
24. Kim BJ, Lee HJ, Park SY, Kim JH, Han HU. 2000. Identification and characterization of *Leuconostoc gelidum*, isolated from Kimchi, a fermented cabbage product. *J Microbiol* 38: 132-136.
25. Akcelik M. 2001. Identification of a lactose utilization and copper resistance plasmid in *Lactococcus lactis* sub sp. *lactis* MCL64. *Turk J Vet Anim Sci* 25: 783-787.
26. Cox RP, Thomsen JK. 1990. Computer-aided identification of lactic acid bacteria using the API 50 CHL system. *Lett Appl Microbiol* 10: 257-259.
27. Cole CB, Fuller R, Carter SM. 1989. Effect of probiotic supplements of *Lactobacillus acidophilus* and *Bifidobacterium adolescentis* 2204 on β-glucosidase and β-glucuronidase activity in the lower gut of rats associated with a human fecal flora. *Microbiol Ecol Health Dis* 2: 223-225.