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# Isolation and Characterization of a Novel Lactic Acid Bacterium for the Production of Lactic Acid

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**Abstract** We isolated a novel lactic acid bacterium from a Korean traditional fermented food, soybean paste. The newly isolated strain, dubbed RKY2, grew well on glucose, sucrose, galactose, and fructose, but it could not utilize xylose, starch, or glycerol. When the partially amplified 16S rDNA sequence (772 bp) of the strain RKY2 was compared with 10 reference strains, it was found to be most similar to *Lactobacillus pentosus* JCM 1588$^T$, with 99.74% similarity. Therefore, the strain RKY2 was renamed *Lactobacillus* sp. RKY2, which has been deposited in the Korean Collection for Type Cultures as KCTC 10353BP. *Lactobacillus* sp. RKY2 was found to be a homofermentative lactic acid bacterium, because its end-product from glucose metabolism was found to be mainly lactic acid. It could produce more than 90 g/L of lactic acid from MRS medium supplemented with 100 g/L of glucose, with 5.2 g $L^{-1}$ $h^{-1}$ of productivity and 0.95 g/g of lactic acid yield.

*Keywords*: characterization, homofermentation, isolation, lactic acid, *Lactobacillus*

## INTRODUCTION

Lactic acid ($CH_3CHOHCOOH$) is a naturally occurring, chiral, hydroxycarboxylic acid, with a long history of usage in the food industry [1]. It can be used for the production of principal chemicals, such as propylene glycol, acetaldehyde, acrylic acid, 2,3-pentanedione, and propanoic acid, and for the polymerization of biodegradable polylactic acid (PLA) [2]. Lactic acid exhibits a low volatility, and self-esterification also often occurs in lactic acid solution, due to its hydroxyl and carboxyl groups [3,4]. Although lactic acid can be produced either by biotechnological fermentation or chemical synthesis [4], the biotechnological production of lactic acid has been gradually gaining support from industry, due to the depletion of petroleum resources and environmental pollution problems [5,6]. Lactic acid is considered to be a relatively mature fine chemical in that only its use in new applications, as, for example, a monomer in plastics, or as an intermediate in the synthesis of high-volume oxygenated chemicals, would cause a significant increase in anticipated demand [2,3,7]. Recently, the scale of lactic acid production has been considerably rising, in order to provide raw materials for the production of PLA, principally at the Cargill-Dow LLC NatureWorks$^{TM}$ plant, in Blair, Nebraska, USA [8].

Lactic acid bacteria (LAB) consist of the gram-positive genera; *Lactobacillus* (*Lb.*), *Lactococcus* (*Lc.*), *Enterococcus*, *Leuconostoc*, *Carnobacterium*, *Oenococcus*, *Pediococcus*, and *Streptococcus* [1,10]. Strains of *Lb. debrueckii* are often used for the commercial production of lactic acid [3]. Currently, the strains used in the lactic acid industry are all but proprietary. However, it is believed that most of the microorganisms used belong to the genus *Lactobacillus* [11-13]. Lactic acid bacteria may be generally classified into two groups, based on their carbohydrate metabolism. Those groups are 1) the homofermentative LAB, which convert glucose almost quantitatively into lactic acid, and 2) the heterofermentative LAB, which ferment glucose into ethanol and $CO_2$, as well as lactic acid. However, only the homofermentative LAB is really adequate for industrial lactic acid production [5,13]. The homofermentative LAB catabolize glucose through glycolysis. As glycolysis results in only lactic acid as an end-product of glucose metabolism, two lactic acid molecules are produced from each molecule of glucose, typically with a yield of more than 0.9 g/g [13,14].

In this paper, we attempt to isolate and characterize a novel lactic acid bacterium from Korean traditional fermented foods, in order to efficiently produce lactic acid. In particular, we investigated the homofermentative characteristics of the isolated strain, using high glucose concentrations.

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## MATERIALS AND METHODS

### Isolation of a Novel Lactic Acid Bacterium

The isolating contents were taken from a Korean traditional soybean paste, and used as an inoculum for the enrichment medium, which was Lactobacilli MRS broth (Difco, Detroit, MI, USA). The broth was composed of the following components (per liter); 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g glucose, 1 g polysorbate 80, 5 g ammonium citrate, 5 g sodium acetate, 0.1 g $MgSO_4$, 0.1 g $MnSO_4$, and 2 g $K_2HPO_4$. The culture was serially diluted to a concentration of $10^{-10}$ in sterilized distilled water containing 8.5 g/L of NaCl. The diluted cultures (0.025 mL) were spread onto agar plates containing MRS broth, 20 g/L of agar, and 10 g/L of $CaCO_3$, and then incubated at 38°C. After 48 h of incubation, single-isolated colonies forming relatively large clear zones were removed with sterilized platinum wire and suspended in 20 mL vials, containing 15 mL enrichment medium. These primary isolates were incubated on a shaking incubator (KMC-8480SF, Vision Scientific Co., Daejon, Korea) at 38°C for 48 h, and final products were analyzed by high-performance liquid chromatography (HPLC) in order to screen for a homofermentative lactic acid bacterium.

### Growth and Fermentation Conditions

To evaluate the cultural characteristics of the isolated strain, the vial-type cultures were performed in 50 mL vials, with 40 mL of working volume. The inoculated vials were incubated at 38°C on a shaking incubator (Vision Scientific Co.) at 200 rpm.

In order to investigate the lactic acid production capacity and the homofermentative characteristics of the isolated strain, batch fermentation, using a jar-type fermentor, was performed on a KF 2.5-L fermentor (Kobiotech Co., Incheon, Korea) with 1 L of working volume. This fermentation was carried out at 38°C and 200 rpm, and the culture pH was maintained at 7.0 by automatic addition of 10 N NaOH. The samples were withdrawn at desired intervals, and were frozen at -20°C for further analysis.

Unless stated otherwise, the medium for vial-type and fermentor-type cultures was similar to Lactobacilli MRS broth.

### Partial 16S rDNA Sequencing

Genomic DNA was isolated from the screened bacterium, using a modified Rochelle method [15]. The 16S rDNA coding region was amplified by polymerase chain reaction (GenAMP™ PCR System 9700, Perkin-Elmer, Boston, MA, USA) using two oligonucleotide primers, 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-AAGGAGG T GWTCCARCC-3' [16]. The PCR product was purified using a Wizard PCR Preps DNA Purification System (Promega Co., Madison, WI, USA), and direct sequencing was performed on an ABI PRISM™ 310 Genetic Analyzer (Perkin-Elmer) and BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer).

### Similarity Index Analysis

The partial 16S rDNA sequence of the isolated strain was compared with those of several reference strains for determination of similarity index. The accession numbers for the sequences used as reference strains (type strains) were as follows; *Lactobacillus pentosus* JCM 1588$^T$ (D79 211), *Lactobacillus plantarum* JCM 1149$^T$ (D79210), *Lactobacillus sakei* subsp. *sakei* ATCC 15521$^T$ (M58829), *Lactobacillus collinoides* JCM 1123$^T$ (AB005893), *Lactobacillus brevis* ATCC 14869$^T$ (M58810), *Lactobacillus brevis* NCDO 1749$^T$ (X61134), *Lactobacillus alimentarius* ATCC 29643$^T$ (M58804), *Lactobacillus rhamnosus* ATCC 7469$^T$ (D16552), *Lactobacillus hilgardii* ATCC 8290$^T$ (M58821), and *Lactobacillus rhamnosus* DSM 20021$^T$ (M58815).

### Scanning Electron Microscopy

To determine the morphology of the isolated strain, the samples were fixed in 2% (w/v) glutaraldehyde, buffered with 0.1 M sodium cacodylate buffer solution (pH 7.2) for 2 h, postfixed in 0.5% (w/v) osmium tetroxide using the same buffer for 1 h, dehydrated in graded ethanol (50, 60, 70, 80, 90, and 100%) for 10 min, and then dried at the critical point, in $CO_2$ using a critical point dryer. The samples were finally sputtered with gold in a sputter coater (JFC-1100, Jeol Co., Tokyo, Japan), and observed using a scanning electron microscope (JSM-5400, Jeol Co.) under 20 kV.

### Analytical Methods

Lactic acid concentration was quantified by a Waters HPLC system (Millipore Co., Milford, MA, USA) equipped with a Waters 486 tunable absorbance detector, set at 210 nm. An ion-exclusion column (300×7.8 mm, Aminex HPX-87H, Bio-Rad, Hercules, CA, USA) was eluted with 5 mM $H_2SO_4$ as mobile phase at a flow rate of 0.6 mL/min, while the column temperature was maintained at 35°C. Glucose concentration was enzymatically measured by the glucose oxidase-peroxidase (GOD-POD) method using a Glucose-E kit (YD Diagnostics, Seoul, Korea), and cell growth was measured as optical density by a UV-160A spectrophotometer (Shimadzu Co., Tokyo, Japan) set at 660 nm.

## RESULTS AND DISCUSSION

### Morphological and Cultural Characteristics

Among the several strains isolated from soybean paste, we selected one strain based on the degree of formation of a relatively large clear zone. This strain was initially called RKY2. In terms of morphology, the strain RKY2 was rod-shaped, occurring singly, in pairs, or short

**Fig. 1.** Scanning electron micrograph of the strain RKY2.

**Table 1.** Morphological and cultural characteristics of the strain RKY2

| Traits | | Characteristics |
|---|---|---|
| Cell shape | | Rods |
| Gram staining | | + |
| Catalase | | - |
| Oxygen response | | Facultative anaerobe |
| | Aerobic | + |
| | Anaerobic | + |
| Growth at | | |
| | 10°C | + |
| | 45°C | + |
| | 50°C | - |
| | pH 3.0 | + |
| | pH 6.0 | + |
| | pH 8.0 | + |
| | pH 9.0 | - |
| | pH 10.0 | - |
| Growth with | | |
| | 6.5% NaCl | + |
| | 10.0% NaCl | - |
| Utilization of | | |
| | Glucose | + |
| | Fructose | + |
| | Galactose | + |
| | Maltose | + |
| | Sucrose | + |
| | Lactose | + |
| | Xylose | - |
| | Mannitol | - |
| | Glycerol | + |
| | Starch | - |
| | Pyruvic acid | - |
| | Malic acid | - |
| | Acetic acid | - |
| | Lactic acid | - |

+: Positive, -: Negative

5'-CGAACTCTGGTATTGATTGGTGCTTGCATCATGATTTACATTTGAGTGAG
TGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCATAAGCGGGGGATA
ACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTC
CGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGTCCCGCGGCGT
ATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCG
ACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGG
AGCAACGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGT
TAAAGAAGAACATATCTTGAGAGTAACTGTTCAGGTATTGACGGTATTTA
ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGG
TGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTT
TTTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAA
ACTGGGAAACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGG
TGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTG
GTCTGTAACTGACGCTGAGGCTCGAAAGTATGGGTAGCAAACAGGATTAG
ATACCCTGGTAGTCCATACCGT-3'

**Fig. 2.** Nucleotide sequence of the partially amplified 16S rDNA gene from the strain RKY2.

chains (Fig. 1). The strain RKY2 was around 1.2 μm in length, and 0.5 μm in width. Colonies had a chalky white appearance. The strain RKY2 grew rapidly on MRS broth. Table 1 summarizes the morphological and cultural characteristics of the strain RKY2. It was a gram-positive rod-cell-type bacterium, and grew well at 30-38°C, but not at all at 50°C. The strain RKY2 was a facultative anaerobe, and was thus able to grow both aerobically and anaerobically. It could grow on 6.5% (w/v) NaCl, but not on 10% (w/v) NaCl. The strain RKY2 could grow at a broad range of initial pH between 3.0 and 8.0, but did not grow at all at pH 9.0. As shown in Table 1, the strain RKY2 could utilize several carbon sources, such as glucose, fructose, galactose, lactose, sucrose, maltose, *etc.*

## Similarity Index

The 16S rDNA partial sequence (772 bp) was determined for the strain RKY2 (Fig. 2), and this sequence was compared with several reference strains in the database. From these sequence comparisons, the strain RKY2 clearly belonged to the genus *Lactobacillus*. Table 2 shows the similarity indexes, based on the above comparisons with 10 reference strains. The strain RKY2 was closely related to *Lactobacillus pentosus* JCM $1588^T$ (99.74% similarity) and *Lactobacillus platarum* JCM $1149^T$ (99.61% similarity). From these results, it could be concluded that the strain RKY2 should be included in the genus *Lactobacillus*. Therefore, the strain RKY2 has been dubbed *Lactobacillus* sp. RKY2, and deposited in the Korean Collection for Type Cultures as KCTC 10353BP.

## Homofermentative Characteristics

To verify the lactic acid production capacity and homofermentative characteristics of *Lactobacillus* sp. RKY2, lactic acid fermentation, using MRS broth supplemented with 100 g/L of glucose was experimented. The results of this experiment are shown in Fig. 3. *Lactobacillus* sp.

**Table 2.** Similarity index of 16S rDNA sequence between the strain RKY2 and related taxa

| Strains | Accession number | Similarity (%) |
|---|---|---|
| *Lactobacillus pentosus* JCM 1588$^T$ | D79211 | 99.74 |
| *Lactobacillus plantarum* JCM 1149$^T$ | D79210 | 99.61 |
| *Lactobacillus sakei* supsp. *sakei* ATCC 15521$^T$ | M58829 | 91.98 |
| *Lactobacillus collinoides* JCM 1123$^T$ | AB005893 | 91.92 |
| *Lactobacillus brevis* ATCC 14869$^T$ | M58810 | 91.62 |
| *Lactobacillus brevis* NCDO 1749$^T$ | X61134 | 91.31 |
| *Lactobacillus alimentarius* ATCC 29643$^T$ | M58804 | 91.21 |
| *Lactobacillus rhamnosus* ATCC 7469$^T$ | D16552 | 90.74 |
| *Lactobacillus hilgardii* ATCC 8290$^T$ | M58821 | 90.74 |
| *Lactobacillus rhamnosus* DSM 20021$^T$ | M58815 | 90.62 |

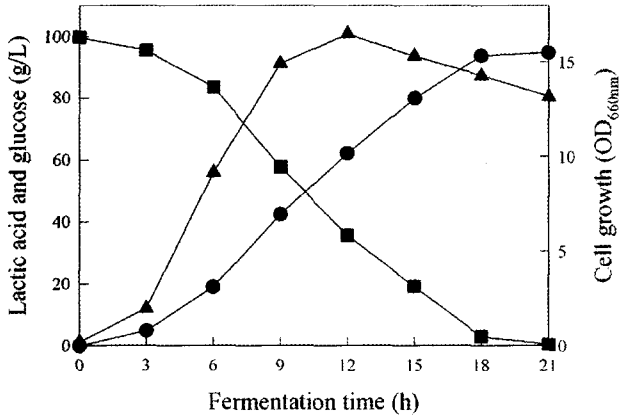


**Fig. 3.** Profiles of lactic acid fermentation from Lactobacilli MRS broth supplemented with 100 g/L of glucose by batch culture of *Lactobacillus* sp. RKY2. Symbols are represent lactic acid (●), glucose (■), and cell growth (▲), respectively.

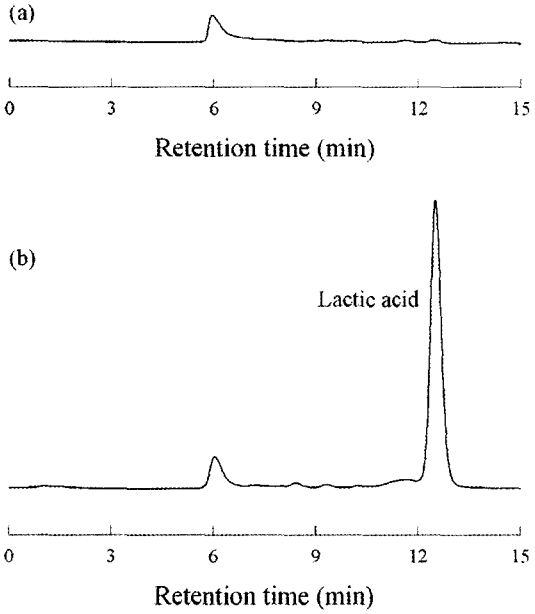


**Fig. 4.** HPLC chromatograms of metabolite from glucose fermentation by batch culture of *Lactobacillus* sp. RKY2. a, 0 h; b, after 18 h of fermentation.

RKY2 completely metabolized 100 g/L of glucose after 21 h of fermentation, and lactic acid obtained was more than 90 g/L. Under this experimental condition, lactic acid productivity and yield were 5.2 g $L^{-1}$ $h^{-1}$ and 0.95 g/g, respectively. Furthermore, cell growth increased up to 16.5 optical density units at 12 h of fermentation. These results suggest that *Lactobacillus* sp. RKY2 is a homofermentative lactic acid bacterium.

Fig. 4a and Fig. 4b show the HPLC chromatograms of fermentation broth after 0 and 18 h of fermentation, respectively. From HPLC chromatograms shown in Fig. 4, we deduced that *Lactobacillus* sp. RKY2 converted glucose into lactic acid via glycolysis without byproducts. In other words, byproducts other than lactic acid were rarely observed. While the heterofermentative LAB uptake carbohydrates through group translocation and produce ethanol and $CO_2$, as well as lactic acid, *via* pentose-phosphate pathway, the homofermentative LAB uptake carbohydrates through the sugar-phosphotransferase system, and produce lactic acid *via* glycolysis [17,18]. Finally, we conclude that *Lactobacillus* sp. RKY2 metabolizes glucose into lactic acid through the homofermentative pathway.

Table 3 shows the results from this work and from several literatures previously reported about final lactic acid concentration, lactic acid yield, and productivity in batch fermentation of glucose into lactic acid. Among the previously published results, Hujanen & Linko [19] gave the highest lactic acid productivity, through batch fermentation of *Lb. casei*. *Lactobacillus* sp. RKY2 used in this study was able to produce 94.8 g/L of lactic acid from 100 g/L of glucose, with a productivity of 5.2 g $L^{-1}$ $h^{-1}$.

**Table 3.** Data reported on batch fermentations for lactic acid production from glucose

| Microorganisms used | Lactic acid (g/L) | Yield (g/g) | Productivity (g $L^{-1}$ $h^{-1}$) | Ref. |
|---|---|---|---|---|
| *Lactobacillus casei* NRRL-B441 | 82 | 0.91 | 5.6 | [18] |
| *Lactobacillus rhamnosus* ATCC 10863 | 68 | 0.76 | 3.5 | [18] |
| *Lactobacillus amylophilus* ATCC 49845 | 21 | 0.95 | 1.6 | [19] |
| *Lactobacillus delbrueckii* IFO 3534 | 81 | 0.78 | 4.5 | [20] |
| *Lactobacillus delbrueckii* NRRL-B445 | 47 | 0.98 | 1.8 | [21] |
| *Lactococcus lactis* ATCC 19435 | 70 | 0.89 | 4.0 | [22] |
| *Lactobacillus* sp. RKY2 | 95 | 0.95 | 5.2 | this work |

Therefore, *Lactobacillus* sp. RKY2 seems to be an efficient lactic acid bacterium for the production of lactic acid, because it could produce lactic acid, with high concentration and productivity.

## CONCLUSION

A new lactic acid bacterium was isolated from Korean traditional soybean paste. This bacterium exhibited efficient production of lactic acid, and was a gram-positive rod-cell-type bacterium. Based on the results of the partial 16S rDNA sequence analysis, and similarity index comparisons with several reference strains, the newly isolated strain was determined to belong to the genus *Lactobacillus*, and was dubbed *Lactobacillus* sp. RKY2, which has been deposited in the Korean Collection for Type Cultures as KCTC 10353BP. Morphologically, the strain RKY2 was rod-shaped, and occurring singly, in pairs, or short chains. The bacterium was 1.2 μm in length, and 0.5 μm in width. *Lactobacillus* sp. RKY2 could efficiently produce lactic acid, with a productivity of 5.2 g $L^{-1}$ $h^{-1}$ and cell growth of 16.5 optical density units, on MRS medium supplemented with 100 g/L of glucose. *Lactobacillus* sp. RKY2 appears to be beneficial to industrial lactic acid production, in that it is able to convert glucose into lactic acid through the homofermentative pathway, resulting in a high productivity and yield.

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