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Characterization of *Acetobacter pomorum* KJY8 Isolated from Korean Traditional Vinegar

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Acetobacter sp. strains were isolated from traditional vinegar collected in Daegu city and Gyeongbuk province. The strain KJY8 showing a high acetic acid productivity was isolated and characterized by phenotypic, chemotaxonomic, and phylogenetic inference based on 16S rRNA sequence analysis. The chemotaxonomic and phylogenetic analyses revealed the isolate to be a strain of *Acetobacter pomorum*. The isolate showed a G+C content of 60.8 mol%. It contained LL-diaminopimelic acid (LL-A_2pm) as the cell wall amino acid and ubiquinone Q_9 (H6) as the major quinone. The predominant cellular fatty acids were $C_{18:1}$w9c, w12t, and w7c. Strain KJY8 grew rapidly on glucose-yeast extract (GYC) agar and formed pale white colonies with smooth to rough surfaces. The optimum cultivation conditions for acetic acid production by the KJY8 strain were 20°C and pH 3.0, with an initial ethanol concentration of 9% (w/v) to produce an acetic acid concentration of 8% (w/v).

Keywords: Vinegar, acetic acid bacteria, *Acetobacter pomorum*

Introduction

Acetic acid bacteria are involved in food spoilage and in indigenous fermentations, where they produce vinegar from alcohol-containing liquids such as wine and beer [2]. In general, they are characterized by their ability to oxidize ethanol to acetic acid and by their resistance to acetic acid and ethanol. Modern acetic acid fermentations are performed in submerged processes, which can achieve final acetic acid concentrations of up to 20% [13]. However, the microbiology of vinegar fermentation is not adequately understood, in particular the taxonomic position of the organisms involved, their responses to ecological factors, and their physiology and genetics [12].

Acetic acid bacteria are Gram-negative, strict aerobic bacteria consisting of four genera: *Acetobacter*, *Acidomonas*, *Gluconobacter*, and *Gluconacetobacter* [5]. The classification of acetic acid bacteria is still a subject of controversy: Sokollek *et al.* [13] described *Acetobacter oboediens* LTH 2460^T and *Acetobacter pomorum* LTH 2458^T; Boesch *et al.* [3] described *Acetobacter intermedius* TF2^T.

In addition, the type strains of 19 other species are also located in the cluster of the genus *Acetobacter*, including *A. aceti*, *A. hansenii*, *A. pasteurianus* [21], *A. xylinus* [18], *A. oboediens*, and *A. pomorum* [13]. Previously, a method for starter preparation was described [12] and the applicability of this approach to cultivate and preserve various isolates from industrial vinegar fermentations was reported [13].

In this study, we isolated an *Acetobacter*, from vinegar samples collected in Daegu city and Gyeongbuk province, capable of producing acetic acid with high productivity. The chemotaxonomic characteristics of the isolate KJY8, identified as *A. pomorum*, were investigated, and a phylogenetic analysis was performed based on the nucleotide sequences of its 16S rRNA genes. In addition, the optimal cultivation conditions for acetic acid production were studied.

Materials and Methods

Bacterial Strains and Culture Conditions

Farm-made vinegar samples were collected in Daegu city and Gyeongbuk province and were plated on GYC agar (5% glucose, 1% yeast extract, 0.5% $CaCO_3$, and 2% agar) according to De Ley *et al*. [5, 24]. Bacterial strains that formed a clear halo around the colonies were isolated at 30°C. For liquid culture, the isolated strains were cultivated at 30°C on a horizontal shaker at 150 rpm. The initial medium acidity (pH) was adjusted to a desired level using 2N HCl.

Determination of the G+C Content of Chromosomal DNA

The chromosomal DNA was prepared according to the method of Stackebrandt and Liesack [14], and the G+C content was determined using the method of Tamaoka and Komagata [17].

Phylogenetic Analysis

Two primers, 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1542R (5′-AGAAAGGAGGTGATCCAGCC-3′), were used for amplification of the 16S rRNA gene [22], and the amplified product was purified, sequenced, and aligned with 16S rRNA sequences of *Acetobacter* species and other related strains using Clustal W software [19]. A phylogenetic tree was constructed using the neighbor-joining method [9]. The GenBank accession number for the 16S rRNA nucleotide sequence of strain KJY8 is AB569643.

Morphological and Physiological Characteristics

The morphology of the cells was examined by scanning electron microscopy (SEM). The *A. pomorum* KJY8 grown in GYC medium was incubated in 4% glutaraldehyde at room temperature for 2 h, dehydrated in a graded series of ethanol, and immersed in isoamyl acetate for 20 min [6]. SEM was accomplished using an Ultra High Resolution Scanning Electron Microscope (Hitachi, Japan) at an accelerating voltage of 5.0 kV.

The morphological properties of strain KJY8 were also determined according to *Bergey's Manual of Systematic Bacteriology* [5]. The gram staining was performed using a Gram-color kit (Merck Chemicals, Germany). To observe the motility, the hanging-drop technique was used [20]. The ketogenesis-forming glycerol, growth on glutamate agar, and assimilation of ammoniacal nitrogen were investigated based on the methods described by Asai *et al*. [1]. The utilization of various substrates as carbon source was tested as described by Shirling and Gottlieb [11]. The acid production from D-glucose, D-sorbitol, and D-mannose was determined as described by Takeuchi and Hatano [15]. An API 20E kit (bioMérieux, France) was used to examine the catalase, oxidase, citrate, lactose, arginine, and nitrate liquefaction.

Analyses of Cellular Fatty Acids and Quinines

The whole-cell fatty acids were extracted and analyzed according to the instructions suggested by Microbial Identification System (MIDI Inc., USA).

After extraction, the ubiquinones were isolated according to the method described by Yamada and Kondo [23], and identified using the methods described by Tamaoka *et al.* [16].

Effects of Cultivation Conditions on Acetic Acid Production

Effects of temperature and pH on the growth and acetic acid production by strain KJY8 were examined in a GYC broth for 7 days at 30°C. The ethanol content, temperature, and pH were varied during the cultivation of strain KJY8 in liquid GYC medium. The concentration of acetic acid was determined by titratable acidity [4].

Results and Discussion

Screening of Acetic-Acid-Producing Bacteria from Vinegar

Acetic-acid-producing bacteria were isolated from traditional vinegar manufactured in Daegu city and Gyeongbuk province. Several hundred colonies were grown on a selective plate. Ten strains that generated a large, clear halo were selected as acetic-acid-producing bacteria. A prominent strain (KJY8) was selected based on the size of the clear halo formed on the GYC plate (Table 1). Compared with the control strain *A. pomorum* KCTC 22319, the KJY8 strain was superior in the production of acetic acid as determined by the size of the clear halo produced around the colonies.

Strain Identification

The complete sequence of the 16S rRNA gene was determined for the isolated KJY8 strain. Fig. 1 shows the phylogenetic tree derived from the 16S rRNA sequences of 19 type strains of *Acetobacter* species and other members in the family *Acetobacteraceae*. The phylogenetic analysis revealed that strain KJY8 is a member of the genus *Acetobacter*, and most closely related to *A. pomorum* LHT 2458^T, which exhibited the highest similarity in 16S rRNA sequence (99.7%) to KJY8. However, the phylogenetic analysis also gave us the idea that *A. pomorum* LHT 2458^T

Table 1. Isolation of acetic acid-producing *Acetobacter* strains from Korean traditional vinegar.

Sample	Control[a]	KJY1	KJY2	KJY3	KJY4	KJY5	KJY6	KJY7	KJY8	KJY9	KJY10
Decomposition of calcium carbonate	+	++	+	++	+	+	+	++	+++	++	+

[a]*A. pomorum* (KCTC 22319).

Symbols (size of halo): +++ (6.6–11.0), strong; ++ (3.1–6.5), good; + (1.0–3.0), normal.

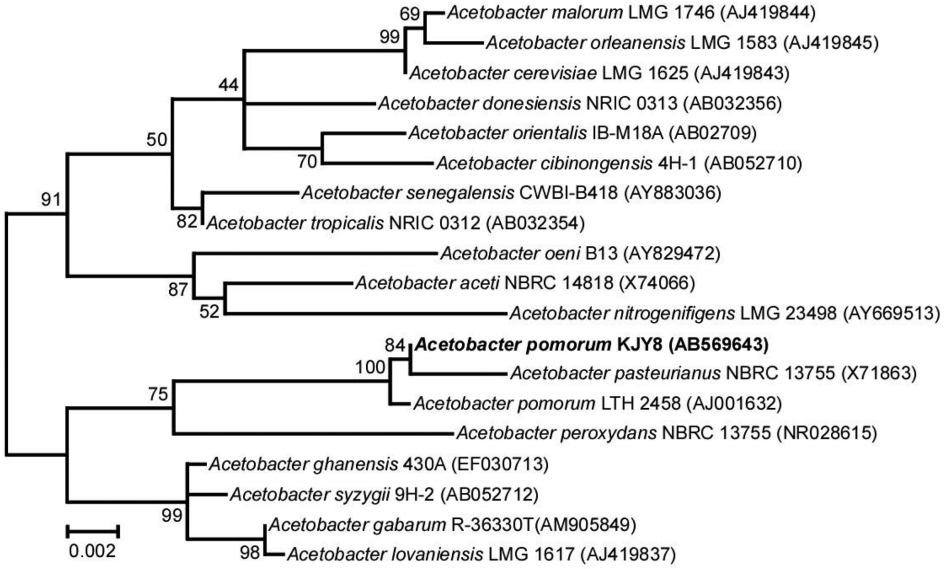

Fig. 1. Phylogenetic tree showing relationships between strain KJY8 and related species.
The tree was constructed from an alignment of the full-length sequence of 16S rRNA from various species using the neighbor-joining method. The number on the nodes corresponds to the bootstrap percentages based on 1,000 pseudoreplicates. The bar denotes the relative branch length. The 16S rRNA sequences are identified by their GenBank accession number in parentheses.

might be transferred to *A. pasteurianus* LMG 1262^T, as it oxidizes acetate and lactate, has Q_9 as its major ubiquinone, and exhibits a high level of 16S rRNA sequence similarity with the type strain of *A. pasteurianus* LMG 1262^T [13]. Based on our results, strain KJY8 is distinct from *G. oxydans* DSM 3503^T, *A. syzygii* 9H-2^T, *A. aceti* NCIMB 8621^T, and *A. indonesiensis* NRIC 0313^T.

Morphological and Physiological Characteristics of KJY8 Strain

Strain KJY8 was Gram-negative, non-spore-forming, and non-motile. The cells were comprised of rods (Table 2, Fig. 2) in GYC medium at 30°C and occurred singly, in pairs, or occasionally in short chains. After incubation on GYC medium for 7 days, the colonies were pale white, smooth to rough, and convex to flat (Table 2).

Among the various compounds usable as carbon and energy sources, strain KJY8 utilized ethanol, D-glucose, D-mannitol, and D-fructose. Acetic acid was produced from all substrates tested (D-glucose, D-mannose, and D-sorbitol). A negative reaction was recorded for glycerol, sorbitol, methanol, *n*-propanol, *meso*-xylitol, L-arginine, L-asparagine, L-glutamic acid, and L-lysine. In GYC broth, the strain grew at temperatures ranging from 20°C to 35°C, at an optimum pH of 3.0. The KJY8 strain oxidized acetate and lactate into CO_2 and H_2O. However, the oxidase test was positive and nitrate was not reduced to nitrite. Strain KJY8 had a 60.8 mol% G+C content, as determined by HPLC. The genomic DNA G+C content of other strains from the genus *Acetobacter* is 53–63 mol%. All these characteristics suggest that strain KJY8 is very similar to *A. pomorum* LHT 2458^T [13] (Table 3).

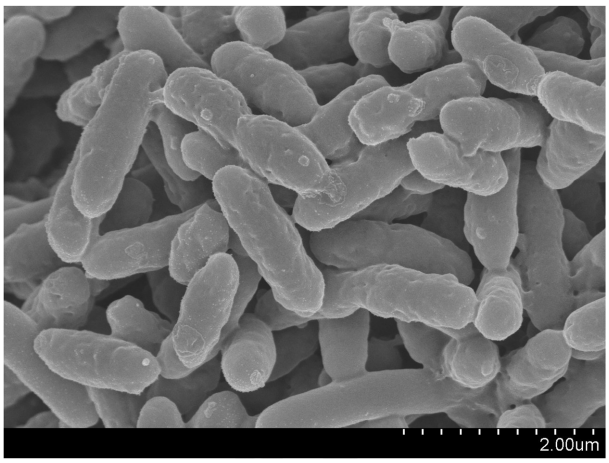

Fig. 2. Scanning electron micrograph of *A. pomorum* strain KJY8 grown on GYC agar for 7 days at 30°C.

Table 2. Morphological characteristics of strain KJY8 and type strains of *Acetobacter* species.

Characterization	*A. hansenii*[a]	*A. pasteurianus*[a]	KJY8
Gram staining	-	-	-
Cell shape	Long rod	Rod	Rod
Cell size (μm)	0.6~0.7 × 1.4~1.8	0.3~0.4 × 0.7~0.8	0.2~0.3 × 0.6~0.8
Motility	-	-	-
Colony characteristics			
Shape	Entire, circular convex to flat	Entire, circular convex to flat	Entire, circular convex to flat
Color[b]	Pale white	Pale white	Pale white
Surface	Smooth to rough	Smooth to rough	Smooth to rough
Transparency	Opaque	Opaque	Opaque

[a]Data from De Ley *et al*. [5].

[b]The color codes correspond to those in the National Bureau of Standards [7].

Table 3. Comparison of physiological characteristics of strain KJY8 and type strains of *Acetobacter* species.

Characterization	*A. hansenii* [a]	*A. pasteurianus*[b]	Strain KJY8
Biochemical reaction			
Catalase	+	+	+
Nitrate reaction	-	-	-
Ketogenesis from			
Glycerol	+	-	-
D-Mannitol	+	+	+
Sorbitol	+	-	-
Oxidation of Ca-DL-lactate	+	+	+
Growth on carbon sources			
Methanol	-	-	-
Ethanol	-	+	+
n-Propanol	-	+	-
meso-xylitol	-	-	-
D-Fructose	+	d	+
D-Glucose	+	d	+
Na-Acetate	-	+	+
Na-L-malate	-	+	+
L-Alanine	+	d	+
L-Arginine	+	-	-
L-Asparagine	+	-	-
L-Glutamic acid	+	-	-
L-Lysine	+	-	-
Ubiquinone	-	Q_9	Q_9
G+C content	58.1~62.6	52.8~62.5	60.8

[a]Data from Lisdiyanti *et al*. [8].

[b]Data from Sokollek *et al*. [13].

Symbols: +, positive; -, negative; d, weak positive.

Chemotaxonomic Characteristics of KJY8 Strain

Strain KJY8 contained a dehydrogenated ubiquinone, with Q_9 as the major quinone. The ubiquinone profile of KJY8 was similar to that of *A. pasteurianus* LMG 1262^T and *A. pomorum* LHT 2458^T [13] as regards the isoprenoid composition.

Strain KJY8 contained summed feature 7 (66.49%, $C_{18:1}$ w9c, w12t, w7c), $C_{16:0}$, $C_{16:0}$2OH, and $C_{14:0}$2OH as the major cellular fatty acids, with small amounts of $C_{16:0}$3OH, $C_{14:0}$, $C_{18:0}$, $C_{20:3}$ w6, 9, 12c, $C_{19:0}$ cyclo w8c, and summed feature 3 (*iso*-$C_{16:1}$ and/or $C_{14:0}$3OH).

Effects of Temperature, pH, and Ethanol Concentration on Acetic Acid Production by KJY8 Strain

The effects of temperature, pH, and ethanol concentration on the production of acetic acid by *A. pomorum* KJY8 were examined in shake flask cultivations (Fig. 3). As shown in Figs. 3A and 3B, the growth temperature and pH of the medium did not profoundly influence the acetic acid production with a given initial ethanol concentration of 8%. With the exception of medium acidity of pH 2.0, the maximum acetic acid concentration of approximately 6% was obtained after 6 days of cultivation, regardless of the temperature or pH at constant initial ethanol concentration. The production of acetic acid was enhanced by increasing the initial ethanol concentration (Fig. 3C). The maximum acetic acid concentration of 8% was achieved with an initial ethanol concentration of 9%, corresponding to a productivity of 0.13 ± 0.01 (g acetic acid produced/l·day). Yield of acetic acid achieved by strain KJY8 might be comparable with the previous report [10] in which an approximate acetic acid yield of 62% was obtained from optimized rice vinegar fermentation.

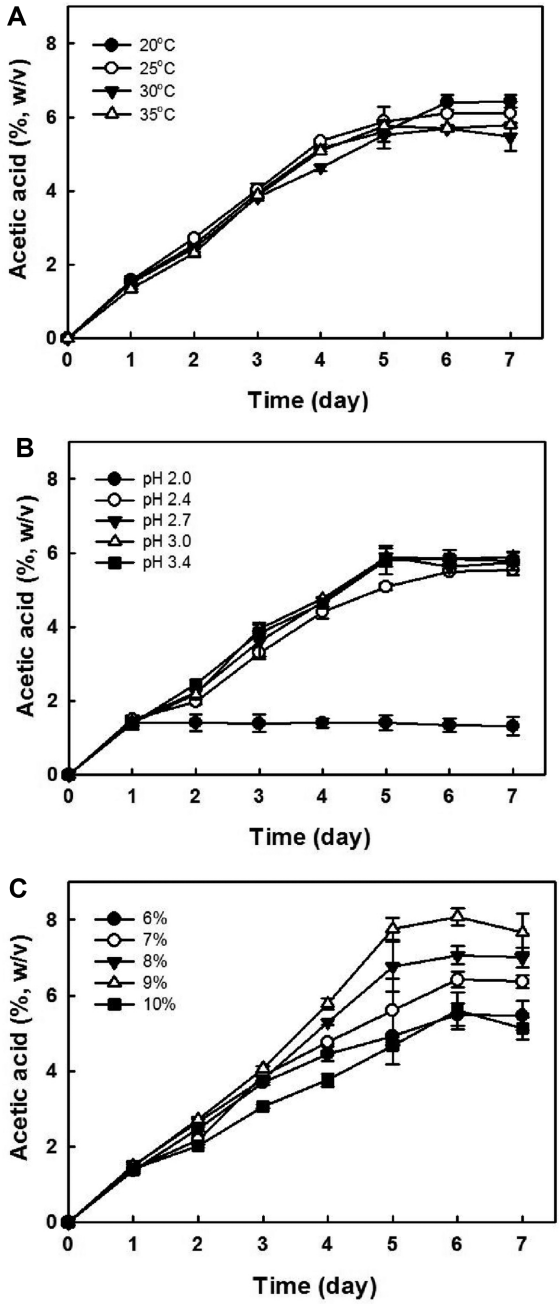

Fig. 3. Effects of temperature (**A**), medium pH (**B**), and ethanol concentration (**C**) on the production of acetic acid by *A. pomorum* KJY8.
Cells were grown at 30°C for 7 days in GYC medium supplemented with 8% ethanol (**A** and **B**), or with the indicated concentration of ethanol (**C**).

In conclusion, this is the first study characterizing a new *A. pomorum* strain isolated from Korean traditional vinegar. Further research into the medium and operation optimization for scale-up cultivation is now under way in order to utilize this prominent acetobacter.

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References

1. Asai T, Iizuka K, Komagata K. 1964. The flagellation and taxonomy of the genera *Gluconobacter* and *Acetobacter* with reference to the existence of intermediate strains. *J. Gen. Appl. Microbiol.* **10:** 95-126.
2. Bartowsky EJ, Xia D, Gibson RL, Fleet GH, Henschke PA. 2003. Spoilage of bottled red wine by acetic acid bacteria. *Lett. Appl. Microbiol.* **36:** 307-314.
3. Boesch C, Trcek J, Sievers M, Teuber M. 1998. *Acetobacter intermedius* sp. nov. *Syst. Appl. Microbiol.* **21:** 220-229.
4. Cairns AM, Watson M, Creanor SL, Foye RH. 2002. The pH and titratable acidity of a range of diluting drinks and their potential effect on dental erosion. *J. Dent.* **30:** 313-317.
5. De Ley J, Gillis M, Swings J. 1984. *In: Bergey´s Manual of Systematic Bacteriology*. Williams & Wilkins, Baltimore.
6. Escalante A, Giles-Gomez M, Hernandez G, Cordova-Aguilar MS, Lopez-Munguia A, Gosset G, Bolivar F. 2008. Analysis of bacterial community during the fermentation of pulque, a traditional Mexican alcoholic beverage, using a polyphasic approach. *Int. J. Food Microbiol.* **124:** 126-134.
7. Kelly KL, Judd DB. 1976. *Color: Universal Language and Dictionary of Names. Special Publication 440*. National Bureau of Standards, Washington, DC.
8. Lisdiyanti P, Navarro RR, Uchimura T, Komagata K. 2006. Reclassification of *Gluconacetobacter hansenii* strains and proposals of *Gluconacetobacter saccharivorans* sp. nov. and *Gluconacetobacter nataicola* sp. nov. *Int. J. Syst. Evol. Microbiol.* **56:** 2101-2111.
9. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4:** 406-425.
10. Shin JS, Jeong YJ. 2003. Changes in the components of acetic acid fermentation of brown rice using raw starch digesting enzyme. *J. Korean Soc. Food Sci. Nutr.* **32:** 381-387.
11. Shirling EB, Gottlieb D. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16:** 313-340.
12. Sokollek SJ, Hammes WP. 1997. Description of a starter culture preparation for vinegar fermentation. *Syst. Appl. Microbiol.* **20:** 481-491.
13. Sokollek SJ, Hertel C, Hammes WP. 1998. Description of

Acetobacter oboediens sp. nov. and *Acetobacter pomorum* sp. nov., two new species isolated from industrial vinegar fermentations. *Int. J. Syst. Bacteriol.* **48:** 935-940.
14. Stackebrandt E, Liesack W. 1993. *Nucleic Acids and Classification: Modern Approaches in Bacterial Systematics*. Academic Press, London.
15. Takeuchi M, Hatano K. 1998. *Gordonia rhizosphera* sp. nov. isolated from the mangrove rhizosphere. *Int. J. Syst. Bacteriol.* **48:** 907-912.
16. Tamaoka J, Katayama-Fujimura Y, Kuraishi H. 1983. Analysis of bacterial menaquinone mixtures by high performance liquid chromatography. *J. Appl. Microbiol.* **54:** 31-36.
17. Tamaoka J, Komagata K. 1984. Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol. Lett.* **25:** 125-128.
18. Tanaka M, Murakami S, Shinke R, Aoki K. 2000. Genetic characteristics of cellulose-forming acetic acid bacteria identified phenotypically as *Gluconacetobacter xylinus*. *Biosci. Biotechnol. Biochem.* **64:** 757-760.
19. Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22:** 4673-4680.
20. Tittsler RP, Sandholzer LA. 1936. The use of semi-solid agar for the detection of bacterial motility. *J. Bacteriol.* **31:** 575-580.
21. Wu J, Gullo M, Chen F, Giudici P. 2010. Diversity of *Acetobacter pasteurianus* strains isolated from solid-state fermentation of cereal vinegars. *Curr. Microbiol.* **60:** 280-286.
22. Yamada Y, Hoshino K, Ishikawa T. 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. *Biosci. Biotechnol. Biochem.* **61:** 1244-1251.
23. Yamada Y, Kondo K. 1973. Coenzyme Q system in the classification of the yeast genera *Rhodotorula* and *Cryptococcus*, and the yeast-like genera *Sporobolomyces* and *Rhodosporidium*. *J. Gen. Appl. Microbiol.* **19:** 59-77.
24. Yeo SH, Lee OS, Lee IS, Kim HS, Yu TS, Jeong YJ. 2004. *Gluconacetobacter persimmonis* sp. nov., isolated from Korean traditional persimmon vinegar. *J. Microbiol. Biotechnol.* **14:** 276-283.