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Isolation and Characterization of *Acetobacter* Species from a Traditionally Prepared Vinegar

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Acetic acid bacteria (AAB) were isolated from vinegar fermented through traditional methods in Namhae county, Gyeongnam, the Republic of Korea. The isolated strains were Gram negative, non-motile, and short-rods. Three selected strains were identified as either *Acetobacter pasteurianus* or *Acetobacter aceti* by 16S rRNA gene sequencing. *A. pasteurianus* NH2 and *A. pasteurianus* NH6 utilized ethanol, glycerol, D-fructose, D-glucose, D-mannitol, D-sorbitol, L-glutamic acid and Na-acetate. *A. aceti* NH12 utilized ethanol, *n*-propanol, glycerol, D-mannitol and Na-acetate. These strains grew best at 30°C and an initial pH of 3.4. They were tolerant against acetic acid at up to 3% of initial concentration (v/v). The optimum conditions for acetic acid production were 30°C and pH 3.4, with an initial ethanol concentration of 5%, resulting in an acetic acid concentration of 7.3–7.7%.

Keywords: Vinegar, acetic acid bacteria, *Acetobacter pasteurianus, Acetobacter aceti*

Introduction

Acetic acid bacteria (AAB) are Gram-negative, non-spore forming, catalase-positive, and strictly aerobic bacteria [1]. AAB oxidize ethanol to acetic acid and thus they are the key microorganisms for vinegar production [10, 14]. Vinegars are popular fermented foods produced by AAB and traditionally, vinegar fermentations have been carried out by AAB present in natural environments [8]. Natural fermentation results in vinegars with diverse metabolites, especially different amount of acetic acid depending upon the major AAB involved for the fermentation [20]. Unique flavor and taste of many traditional vinegars are the combined results from natural fermentations. In recent years, vinegars are consumed not just as a seasoning but also a health drink [5]. Due to the increased interests in health-promoting effects of vinegars, diverse vinegar products have been produced from many different raw materials (fruits, vegetables, and cereals) and different types of microorganisms have been involved for fermentations [5, 21]. Naturally fermented vinegars often have good flavor and tastes [21]. Controlled fermentation is preferred when vinegars with uniform qualities are required or vinegars with higher concentration of acetic acid are desired in a short period of time. Vinegars have been produced at Namhae county, Gyeongnam, Republic of Korea, by traditional ways. But no report has been made on the quality of Namhae vinegar nor the microorganisms involved in the fermentation known. In this study, we isolated 3 *Acetobacter* strains from a Namhae vinegar, and examined the optimal cultivation conditions for the isolates to produce acetic acid. This report is the first step to understand the AAB present in Namhae vinegar and to improve the quality of Namhae vinegar in the future.

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Materials and Methods

Bacterial strains and culture conditions

A vinegar prepared by traditional ways at Namhae

county, Gyeongnam, Republic of Korea, in the fall of 2014 was used as the source for AAB. Makgeolli (traditional Korean rice wine, 6% alcohol) was stored at room temperature for 4–6 days and then inoculated with mother vinegar (10%, v/v). Acetic acid fermentation (AAF) was carried out for 30 days at room temperature statically. Vinegar was serially diluted with 0.1% peptone water and diluted samples were spreaded on GYC agar plates (3% glucose, 0.5% yeast extract, 1.0% $CaCO_3$, 3% (v/v) ethanol, and 1.5% agar) and YCM agar plates (0.5% yeast extract, 2.5% mannitol, 1.0% $CaCO_3$, 3% (v/v) ethanol, and 1.5% agar) [21]. Plates were incubated at 30°C. The AAB cultures were cultivated in GYC broth (3% glucose, 0.5% yeast extract, 3% (v/v) ethanol) and YCM broth (0.5% yeast extract, 2.5% mannitol, 3% (v/v) ethanol) at 30°C with vigorous shaking (220 rpm).

Identification of isolated strains

Isolates were identified by 16S rRNA gene sequencing. The universal primers were used to amplify 16S rRNA gene fragments from 3 isolates. The primer sequences are as follows: 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 337 F (5'-GACTCCTACGGGAGGCWGCAG-3'), 1100R (5'-GGGTTGCGCTCGTTG-3') and 1492R (5'-GGTTAC CTTGTTACGACTT-3'). PCR reactions were done using a MJ Mini personal thermal cycler (BioRad, Hercules, CA, USA) and the PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. Amplified fragments were purified from an agarose gel using a PCR purification kit (FavorPrep PCR purification mini kit, Favorgen, Ping-Tung, Taiwan). DNA sequences were determined at Cosmogenetech (Seoul, Korea) and analyzed by BLAST provided by National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) and EZ_TAXON database (http://www.ezbiocloud.net/eztaxon) for similarity searches. The morphologies of the isolates were examined by a microscope, and Gram staining was performed using a kit (BD Diagnostic, Franklin Lakes, NJ, USA). To observe the motility, the hanging-drop method was used [18].

Culture-independent method for detection of AAB from a Namhae vinegar

Total DNA was prepared from a Namhae vinegar sample using a EZ-10 spin column soil DNA miniprep kit (Bio Basic Inc., Markham, ON, Canada). The primer set, 337F and 800R (5'-TACCAGGGTATCTAATCC-3'), was used to amplify 16S rRNA genes. PCR products were purified by using a gel extraction kit (Expin^{TM} Gel SV kit, GeneAll Biotechnology, Seoul, Korea), ligated with pGEM T-easy vector (Promega, Madison, WI, USA), and then introduced into *E. coli* DH5α competent cells by electroporation. Five white colonies were selected randomly from LB plates with ampicillin (50 μg/ml) and X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside, 40 μg/ml). Nucleotide sequences of the inserts were determined at Cosmogenetech (Seoul, Korea), and analyzed by BLAST program.

Growth of *Acetobacter* strains

A. pasteurianus NH2 and NH6 were cultivated in GYC broth and *A. aceti* NH12 was cultivated in YCM broth at 30°C with vigorous shaking. Utilization of various carbon sources was tested as described previously [13]. Each *Acetobacter* strain was first propagated in 10 ml GYC or YCM broth for 3 days at 30°C and then used to inoculate fresh culture broth (0.5% yeast extract and 1% acetic acid) with a carbon source (methanol, ethanol, *n*-propanol, glycerol, D-fructose, D-glucose, D-mannitol, D-sorbitol, L-arginine, L-glutamic acid, L-lysine and Na-acetate, 2%, w/v or v/v). Methanol, ethanol, and *n*-propanol were filter sterilized and added to the sterile broth to a final concentration of 2% (v/v). Inoculated cultures were incubated at 30°C for 7 days.

A. pasteurianus NH2 and NH6 were cultivated in GYC broth and *A. aceti* NH12 was cultivated in YCM broth for 2 days at 30°C with vigorous shaking, and then 0.5 ml of each culture was used to inoculate 50 ml GYC and YCM broth with different conditions. Growth was examined at different temperature (15, 20, 25, 30, and 37°C), different initial pH (2.0, 2.4, 2.7, 3.0, 3.4, adjusted by 1N HCl), different acetic acid content (1, 3, 5, and 7%, v/v), and different ethanol concentration (0, 3, 5, 7, 9, and 12%, v/v). The culture broth for optimal ethanol concentration was composed of 3% glucose, 0.5% yeast extract and 1% acetic acid (for NH2 and NH6) or 0.5% yeast extract, 2.5% mannitol and 1% acetic acid (for NH12). Inoculated cultures were incubated for 6 days and the OD_{600} values were measured. The amount of acetic acid was determined by measuring the titratable acidity (TA) of culture.

Results and Discussion

Isolation of *Acetobacter* species from a Namhae vinegar

AAB were isolated from a vinegar fermented by traditional methods at Namhae county, Gyeongnam, Republic of Korea, during 2014. Vinegar samples were spreaded on GYC and YCM agar plates containing $CaCO_3$. After incubation, colonies surrounded by clear halo zones were obtained. The isolates were tested for their abilities of ethanol tolerance. Finally, three isolates (NH2, NH6, and NH12) were selected for further studies. In general, isolation and cultivation of AAB, especially from fermented beverages such as vinegar, have been described to be problematic, often resulting in underestimation of microbial species richness when culture-dependent methods are applied [6]. Low recovery of species is explained partially due to the presence of organisms which are viable but in non-culturable state [4]. Limitations of culturing were in part overcome by the formulation of appropriate media, which allowed the cultivation of slowly growing organisms. A number of conventional culture media to isolate AAB from different sources are reported in literature, where carbon sources are mainly glucose, mannitol, and ethanol.

Three isolates were identified by 16S rRNA gene sequencing. The universal primer set (9F and 1492R) was used to amplify 16S rRNA genes from NH2 and NH6 and a primer set (337F and 1100R) was used to amplify 16S rRNA gene from NH12. A 16S rRNA gene from NH12 was not amplified when the primer set (9F and 1492R) was used, indicating some mismatches in the primer binding sites. Sequence analyses by NCBI blast confirmed that two strains, NH2 and NH6, were *Acetobacter pasteurianus* and their 16S rRNA gene sequences (1,350 bp) showed 99% homology with the 16S rRNA gene from *Acetobacter pasteurianus* HSZ3-21 (KJ095003.1). The other strain, NH12, was *Acetobacter aceti* and its 16S rRNA gene sequence (421 bp) showed 100% homology with that of *Acetobacter aceti* LMG1496 (JF793948.1). Sequence analyses by EZ_TAXON also confirmed that 2 strains (NH2 and NH6) were *Acetobacter pasteurianus* subsp. *pasteurianus,* and the sequences showed 99.7% similarity with the 16S rRNA gene from *Acetobacter pasteurianus* subsp. *pasteurianus* LMG1262 (BACG01000075). NH12 strain was *Acetobacter aceti* and its sequence showed 99.52% similarity with that of *Acetobacter aceti* NCIMB8621 (X74066). From these results, NH2 and NH6 were identified as *A. pasteurianus,* and NH12 was identified as *A. aceti*. *Acetobacter* species are Gram-negative, non-motile, and short-rod (Fig. 1). Genbank accession numbers for the 16S rRNA genes are KR150440, KR150441, and KR150442 for *A. pasteurianus* NH2, *A. pasteurianus* NH6, and *A. aceti* NH12, respectively.

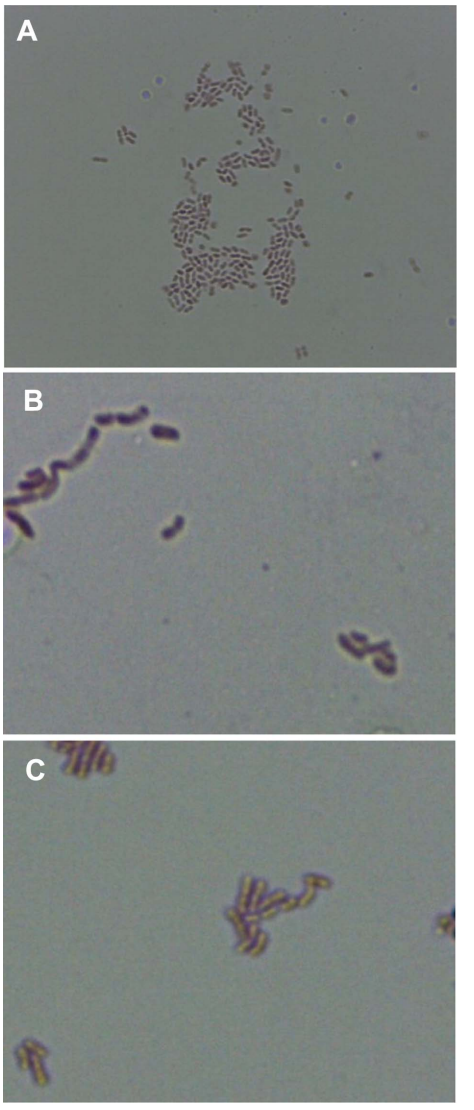

Fig. 1. Microscopic images of isolated 3 AAB. (A) *A. pasteurianus* NH2 (×400), (B) *A. pasteurianus* NH6 (×1000), (C) *A. aceti* NH12 (×1000).

Culture-independent method for detection of AAB

Among the 5 randomly selected clones, 3 were 100% matched with the 16S rRNA gene of *A. pasteurianus*/*A.*

Table 1. Identification of AAB using culture-independent method.

No	Size (bp)	The most matched organism	Identity (%)
1	421	*Acetobacter aceti*/*Acetobacter sicerae*	100
2	415	*Acetobacter pasteurianus*/*Acetobacter pomorum*	100
3	421	*Acetobacter aceti*/*Acetobacter sicerae*	100
4	415	*Acetobacter pasteurianus*/*Acetobacter pomorum*	100
5	416	*Acetobacter pasteurianus*/*Acetobacter pomorum*	100

Table 2. Growth of AAB on GYC or YCM broth containing a different carbon source.

Carbone source	*A. pasteurianus* NH2	*A. pasteurianus* NH6	*A. aceti* NH12
Ethanol	+	+	+
Methanol	–	–	–
Propanol	–	–	+
D-glucose	+	+	–
D-fructose	+	+	–
D-mannitol	+	+	+
Sorbitol	+	+	–
Na-acetate	+	+	+
L-arginine	–	–	–
L-glutamic acid	+	+	–
L-lysine	–	–	–
Glycerol	+	+	+

pomorum and two were 100% matched with that of *A. aceti*/*A. sicerae* (Table 1). The results agreed well with the results from culture-dependent methods described above. Culture-independent methods can complement culture-dependent methods for the more accurate detection of AAB from vinegars [19]. Clearly, *A. pasteurianus* and *A. aceti* are the main AAB for the vinegar sample prepared at Namhae county. Both species are used for vinegar fermentation worldwide and also occur in beer as spoilers [7, 16]. *A. pasteurianus* has been reported as the dominant AAB among rice vinegar (Komesu) and unpolished rice vinegar (Kurosu) in Japan [8].

Growth of *Acetobacter* strains

Utilization of various carbon sources by 3 *Acetobacter* isolates are summarized in Table 2. *A. pasteurianus* NH2 and NH6 utilized ethanol, glycerol, D-fructose, D-glucose, D-mannitol, D-sorbitol, L-glutamic acid, and Na-acetate. *A. aceti* NH12 utilized ethanol, *n*-propanol, glycerol, D-mannitol, and Na-acetate. Most *A. pasteurianus* strains are known to produce acid from ethanol, n-butanol, and n-propanol [14]. But *A. pasteurianus* NH2 and NH6 did not grow on n-propanol. Most *A. pasteurianus* strains require growth factors in the presence of D-mannitol, and a few strains grow on L-alanine, L-glutamine, L-glutamic acid and L-proline as a sole nitrogen source in the presence of D-mannitol [14]. *A. pasteurianus* NH2 and NH6 grew on L-glutamic acid without D-mannitol. *A. pasteurianus* DSM3509 from beer utilized ethanol, glycerol, D-fructose, D-glucose, D-mannitol and D-sorbitol, but the strain was unable to grow on L-glutamic acid [15]. Seven *A. pasteurianus* (LMG 1262, 1555, 1629, 1630, 1658, 1659, 1686) strains were reported not able to grow on methanol, and some of them were not able to grow on glycerol [3]. All *A. aceti* strains are known to acidify ethanol, n-propanol, n-butanol, D-xylose, D-mannose, and D-glucose. *A. aceti* strains also utilize glycerol, D-mannitol, and Na-acetate as carbon sources for growth [14]. *A. aceti* NH12 grew well on ethanol, glycerol, D-mannitol and Na-acetate. Other *A. aceti* (LMG1496, 1504^T, 1531 and 1535) strains were able to grow on glycerol but not on methanol, and the result was similar with *A. aceti* NH12 [3].

Three *Acetobacter* strains were grown for 6 days at different temperatures (Fig. 2). All strains grew at 25 and 30°C, entered into stationary phase (OD_{600} = 0.28–0.35) in 3 days. But they did not grow at 15, 20 and 37°C in 6 days. The effect of initial pH of broth on the growth was examined during 6 days cultivation at 30°C (Fig. 3). All strains grew well at the initial pH of 3.0–3.4, reaching the OD_{600} value of 0.25–0.35 in 3 days. But they did not grow at the initial pH of 2.0, 2.4 and 2.7. Vinegar is obtained by a two-stage fermentation process. In the first step (alcohol fermentation, AF), fermentable sugars are converted into ethanol by yeasts. In the second step (acetic acid fermentation, AAF), AAB oxidize the ethanol to acetic acid. Both stages occur by spontaneous fermentation, and the optimum temperature for AAF is 25–30°C [2, 17]. The vinegar used for this

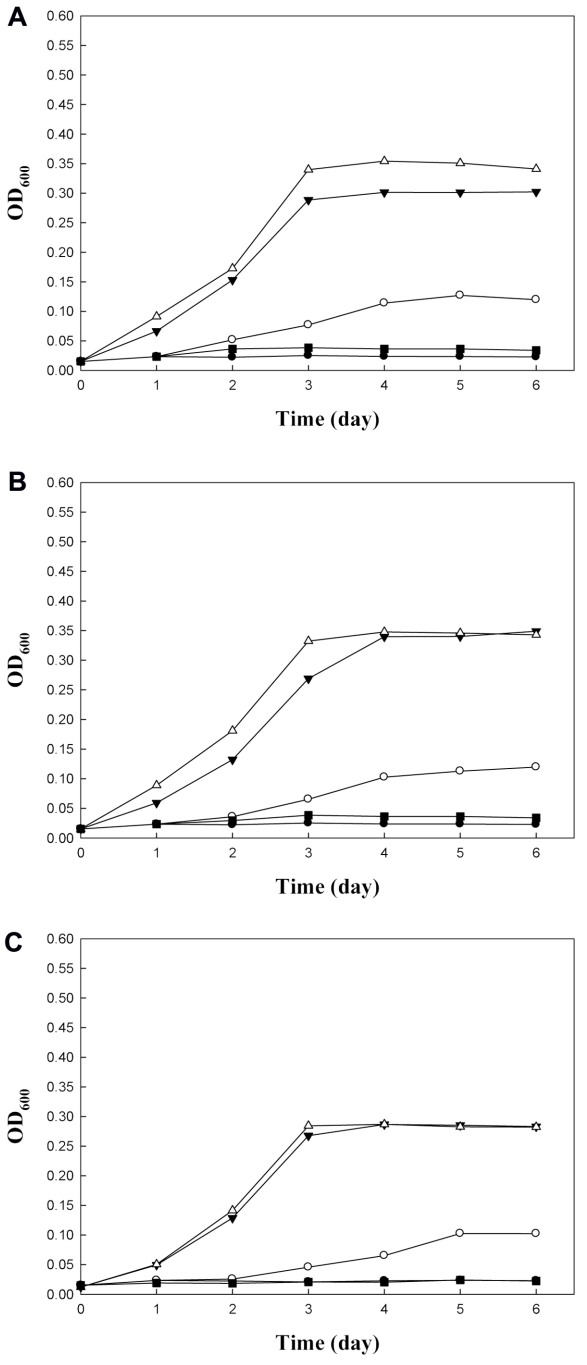

Fig. 2. Growth of AAB at different temperatures. (A) *A. pasteurianus* NH2, (B) *A. pasteurianus* NH6, (C) *A. aceti* NH12. ●, 15°C; ○, 20°C; ▼, 25°C; △, 30°C; ■, 37°C.

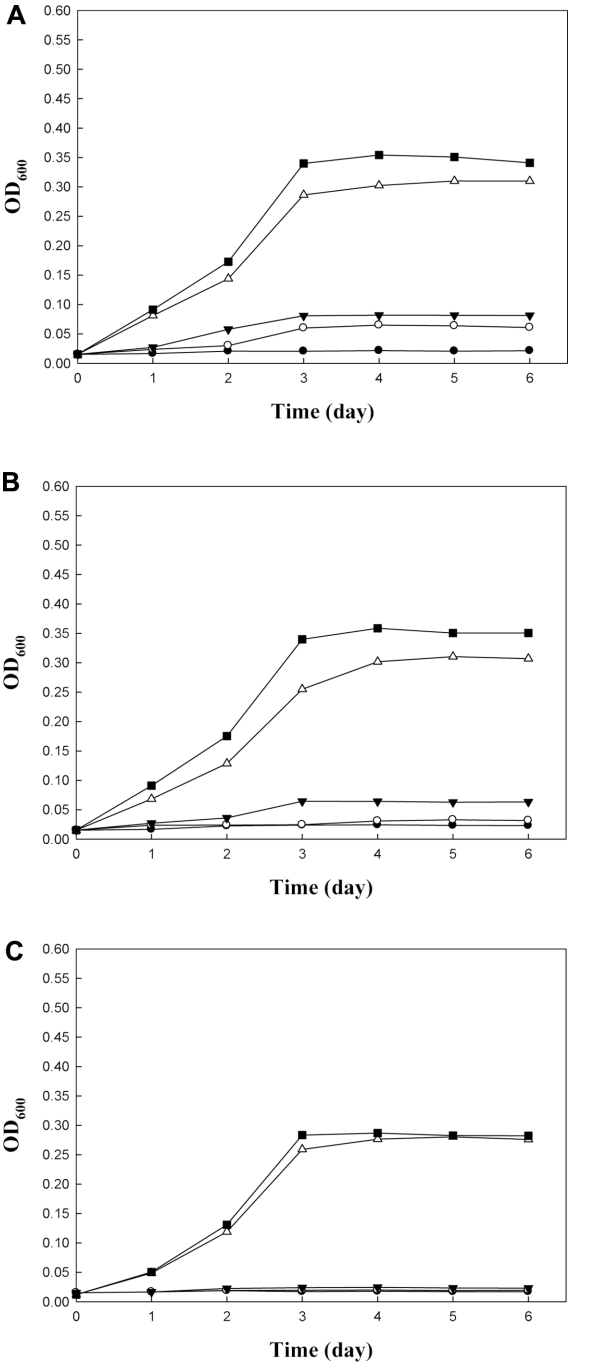

Fig. 3. Growth of AAB at different initial pH. (A) *A. pasteurianus* NH2, (B) *A. pasteurianus* NH6, (C) *A. aceti* NH12. ●, pH 2.0; ○, pH 2.4; ▼, pH 2.7; △, pH 3.0; ■, pH 3.4.

work was prepared by natural fermentation and fermented at room temperature.

Generally, the optimum pH for the growth of AAB is 5.0–6.5, while they can grow at lower pHs (3.0–4.0) [11]. And their optimum temperature is in the range of 28–30°C although some species are recognized as thermotolerant [9, 11].

Three *Acetobacter* strains were tolerant against acetic

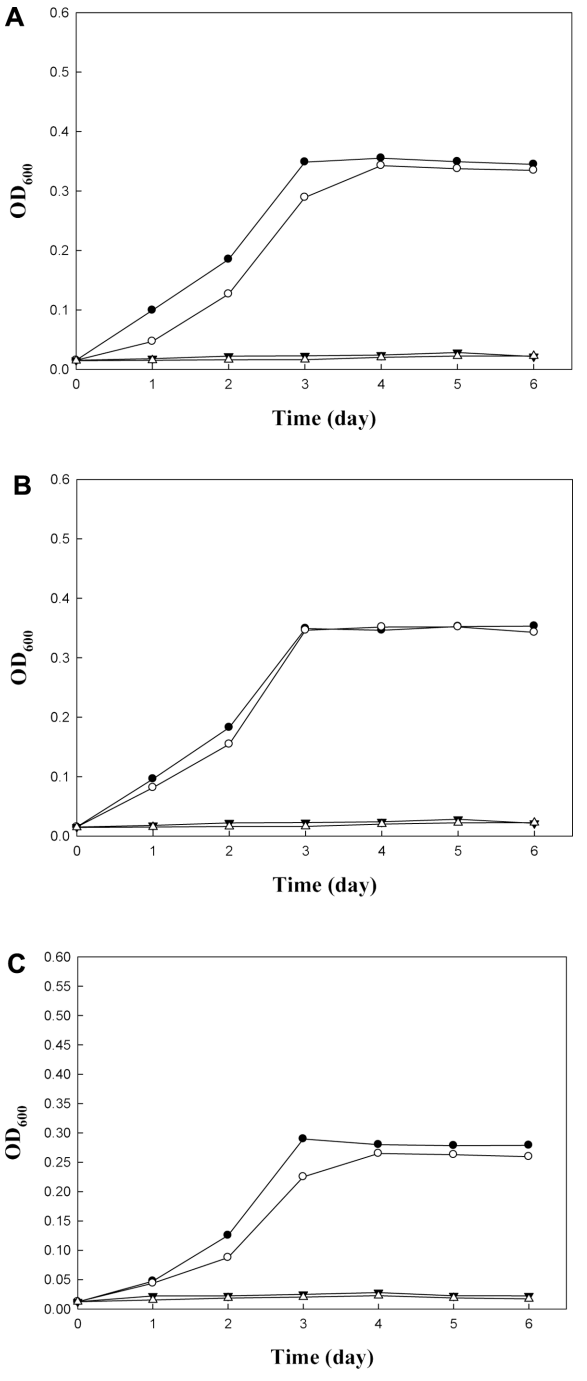

Fig. 4. Growth of AAB at different initial acetic acid concentration. (A) *A. pasteurianus* NH2, (B) *A. pasteurianus* NH6, (C) *A. aceti* NH12. ●, 1%; ○, 3%; ▼, 5%; △, 7%.

acid up to 3% of initial concentration (v/v) (Fig. 4). *A. pasteurianus* NH2 and NH6 were more tolerant and the OD_{600} values reached 0.35 in 3 days at 1% acetic acid. For *A. aceti* NH12, the OD_{600} value reached 0.28 in 3 days at 1% acetic acid. At 3% initial acetic acid concentration, *A. pasteurianus* NH2 and *A. aceti* NH12 grew slowly than in 1% acetic acid concentration. Most strains are unable to grow at 5 and 7% initial acetic acid concentrations. Acetic acid is produced from ethanol by two successive catalytic reactions by membrane-bound alcohol dehydrogenase and aldehyde dehydrogenase [14]. Thus, the ability to oxidize ethanol into acetic acid and the ability to tolerate higher concentration of acetic acid are both required for *Acetobacter* species to produce acetic acid in large quantity.

Effect of ethanol concentration

Effect of initial ethanol concentration on the production of acetic acid was examined (Fig. 5). For *A. pasteurianus* NH2 and NH6, the OD_{600} values reached 0.35 in 3 days at the initial ethanol concentration of 3, 5, 7 and 9%. For *A. aceti* NH12, the OD_{600} value reached 0.28 in 3 days at the initial ethanol concentration of 3, 5 and 7%. *A. pasteurianus* NH2 and NH6 grew rapidly in the absence of ethanol, and the OD_{600} values reached 0.52–0.55 in 3 days. But *A. aceti* NH12 was unable to grow in 6 days.

A. pasteurianus NH2 produced acetic acid in the highest amount (7.72%) with an initial ethanol concentration of 5% in 5 days followed by *A. pasteurianus* NH6 (7.30%) with an initial ethanol concentration of 7% and *A. aceti* NH12 (5.95%) with an initial ethanol concentration of 5%. Generally, *A. pasteurianus* strains oxidize ethanol to acetic acid. Some *A. pasteurianus* strains can produce acetic acid not only from ethanol, but also from glucose [12]. This might be the reason why more than 7% acetic acid was produced from 5% initial ethanol concentration because the media (GYC) contained glucose (3%, w/v).

The optimum culture conditions for acetic acid production by *A. pasteurianus* strains were 30°C and pH 3.4 with an initial ethanol concentration of 5%, resulting in acetic acid concentration of 7.3–7.7%. In other report, a *A. pasteurianus* strain was cultured in RAE (1% yeast extract, 1% peptone, 4% glucose, 0.3% $Na_2HPO_4 \cdot 2H_2O$, 0.2% citric acid, w/v) broth with different ethanol concentration (0–10%, v/v), and the optimal ethanol concentration was 4% (v/v) [1]. Identification of the major AAB involved in Namhae vinegar fermentation is the first step and further studies on their roles for the quality of vinegar are required. Use of isolated strains as starters for vinegar fermentation should be done and the quality of vinegar should be compared with naturally fermented vinegars.

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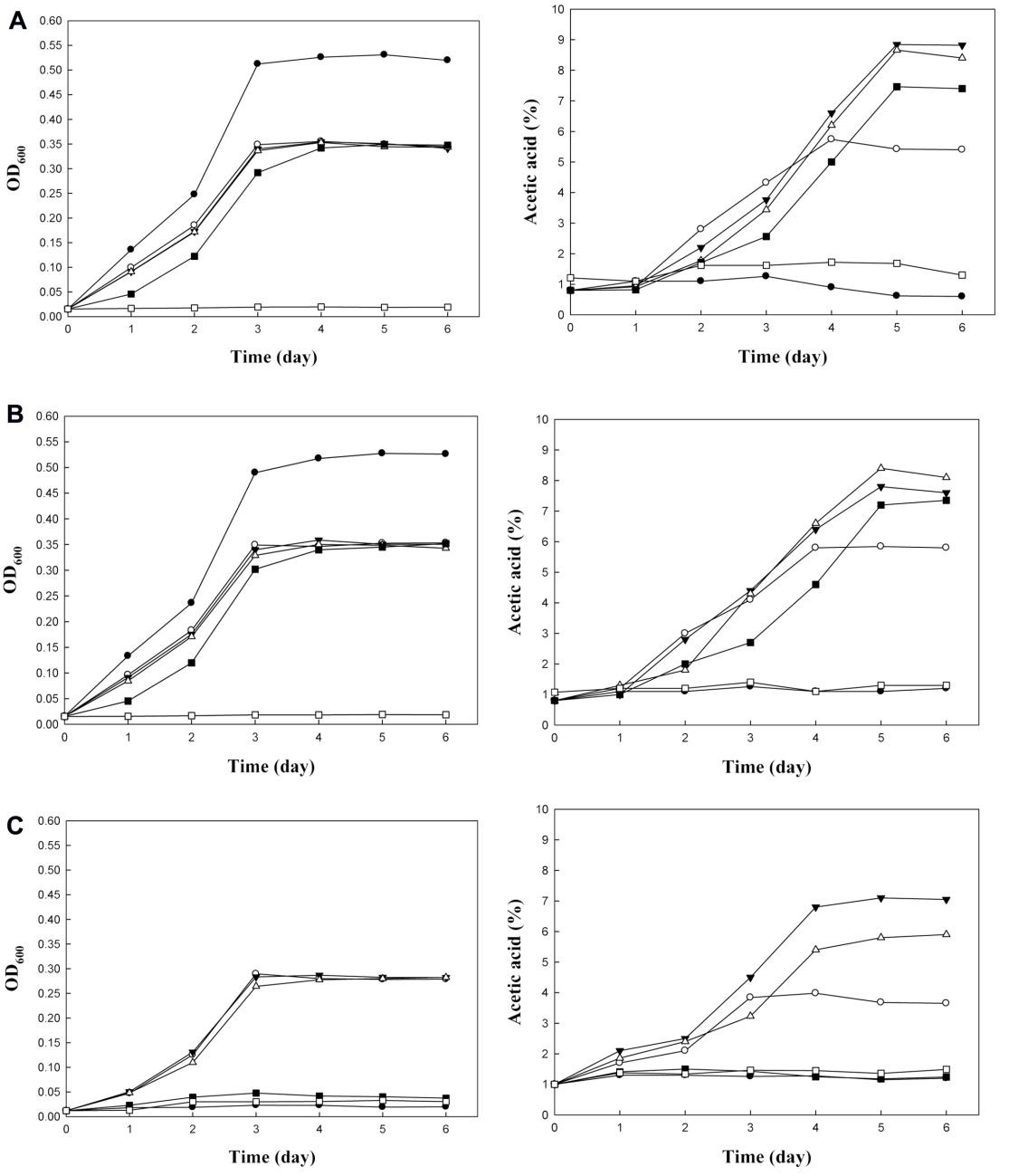

Fig. 5. Growth and TA of AAB at different initial ethanol concentration. (A) *A. pasteurianus* NH2, (B) *A. pasteurianus* NH6, (C) *A. aceti* NH12.

Acknowledgments

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국문초록

전통방식으로 제조한 식초로부터 *Acetobacter* 종들 분리 및 특성 조사

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경남 남해군에서 전통방식으로 제조한 식초에서 초산균들을 분리하였다. 분리균주들은 그램 음성, 비운동성, 단간균으로 이중 선발된 3 균주는 16S rRNA 유전자 염기서열 분석 결과 *Acetobacter pasteurianus* 혹은 *Acetobacter aceti*로 동정되었다. *A. pasteurianus* NH2와 *A. pasteurianus* NH6는 ethanol, glycerol, D-fructose, D-glucose, D-mannitol, D-sorbitol, L-glutamic acid, 그리고 Na-acetate를 이용하였다. *A. aceti* NH12는 ethanol, n-propanol, glycerol, D-mannitol과 Na-acetate를 이용하였다. 이들은 30℃, 초기 pH 3.4에서 가장 잘 자랐고 초기 초산농도 3% (v/v)까지는 생육하였다. 초산 생산을 위한 최적 조건은 30℃, pH 3.4, 초기 ethanol 농도 5%로 이 경우 초산을 7.3–7.7% 생성하였다.

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