

## Surface Film Formation in Static-Fermented Rice Vinegar: A Case Study

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### ABSTRACT

In the present study, we aimed to determine the cause of surface film formation in three rice vinegars fermented using the traditional static fermentation method. The pH and total acidity of vinegar were 3.0–3.3 and 3.0–8.7%, respectively, and acetic acid was the predominant organic acid present. Colonies showing a clear halo on GYC medium were isolated from the surface film of all vinegars. Via 16S rDNA sequencing, all of the isolates were identified as *Acetobacter pasteurianus*. Furthermore, field-emission scanning electron microscopy analysis showed that the bacterial cells had a rough surface, were rod-shaped, and were  $\sim 1 \times 2 \mu\text{m}$  in size. Interestingly, cells of the isolate from one of the vinegars were surrounded with an extremely fine threadlike structure. Thus, our results suggest that formation of the surface film in rice vinegar was attributable not to external contamination, to the production of bacterial cellulose by *A. pasteurianus* to withstand the high concentrations of acetic acid generated during fermentation. However, because of the formation of a surface film in vinegar is undesirable from an industrial perspective, further studies should focus on devising a modified fermentation process to prevent surface film formation and consequent quality degradation.

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### 1. Introduction

Vinegar is a liquid condiment that contains bioactive compounds such as organic acids, mainly acetic acid, and phenolic compounds. Owing to the characteristic sourness of acetic acid and the health benefits, vinegar has been used not only as an essential condiment, but also as a pharmaceutical agent since ancient times [1]. Vinegar is produced via a two-stage fermentation process. The first stage is anaerobic alcohol fermentation by yeast, in which fermentable sugars are converted to ethanol [2]. In this stage, if the raw material for vinegar is cereals such as rice, malt, or sorghum, saccharification of starch into fermentable sugars using mold is necessary as the preceding step to alcohol fermentation [3]. The second stage is aerobic acetification by acetic acid bacteria (AABs), which involves oxidation of ethanol to acetic acid via acetaldehyde intermediates [2].

Based on the processing technology employed, vinegar production can be broadly divided into two categories, surface fermentation and submerged fermentation processes. Although the surface fermentation method is preferred to the submerged fermentation method because it is economical and it preserves some volatile aromatic compounds, it

sometimes leads to the formation of a pellicle-like film on the surface. Formation of such a film causes overoxidation and turbidity in vinegar. Surface fermentation is also called the static method, because the AABs are fixed on the surface of the material to allow contact with atmospheric oxygen. Therefore, it requires a long time to yield the final product, vinegar. In contrast, the submerged fermentation process involves direct injection of oxygen into the substrate liquid using a generator or an acetator to increase the surface area, and therefore, the fermentation is faster. The surface fermentation method is employed to produce traditional and selected vinegar, despite disadvantages such as a long fermentation period, a risk of contamination, and difficulty of quality control. This is not only because volatile aromatic compounds are retained, but also because the initial investment is more economical than that for the submerged fermentation process [2,4].

However, in static-fermented vinegar, surface film formation is often found, for example, Shanxi aged [3], brown rice [5], wine [6], and strawberry vinegar [7]. The surface film of vinegar was first reported in 1886 to be made of cellulose produced by *Gluconacetobacter xylinus* (formerly *Bacterium xylinum* and *Acetobacter xylinum*) [8]. In addition,

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other AABs such as *A. tropicalis* SKU1100 and *A. lovaniensis* (formerly *A. pasteurianus* IFO3284) have also been reported to produce cellulose [9,10]. It is known that the bacterial cellulose produced by *G. xylinus* plays multiple roles such as protecting the cell from desiccation, UV light, and coexisting competitors, and aiding in adherence to surfaces to allow maximal use of atmospheric oxygen [11]. However, in the vinegar industry, AABs and cellulose are targeted for removal because they cause overoxidation and turbidity in vinegar [12].

Owing to increased awareness of the functional and health properties of vinegar, recently, there has been an increasing demand for high-quality vinegar naturally fermented using conventional starter culture and fermentation methods. Nonetheless, only a few studies have examined the contamination of, and cellulose film formation in, vinegar in Korea [12]. Therefore, the present study aimed to determine the cause of the occurrence of surface in static-fermented rice vinegar by analyzing the characteristics of the vinegar and the responsible strains.

## 2. Materials and methods

### 2.1. Vinegar collection and culture media

Three rice vinegars with surface film were obtained from a vinegar farmhouse. The vinegar was produced in the same brewery but not at the same time and processed by surface fermentation in a pot inoculated with *A. pasteurianus*. The selected strain was isolated from farmhouse vinegar in Chungbuk province, Korea, because it has high acid-producing ability. The collected vinegars were categorized as vinegar 1 (V1), vinegar 2 (V2), and vinegar 3 (V3) on the basis of the morphological characteristics of their surface films. Glucose, yeast extract, and calcium carbonate (GYC) culture medium was used to culture causative species of surface film formation isolated from the vinegar samples. The GYC medium was prepared using 10.0% glucose, 1.0% yeast extract, 2.0% CaCO<sub>2</sub>, and 1.5% agar with a pH of 6.8.

### 2.2. pH and total acidity

The pH of the vinegar was measured using a pH meter (D-71G, Horiba, Kyoto, Japan). Total acidity was calculated using the acetic acid coefficient (0.006) and 20 mL of 20-fold diluted vinegar solution titrated with a 0.1 N NaOH standard solution ( $f = 1.000$ ) to pH 8.3.

### 2.3. Organic acid analysis

Organic acids were analyzed using HPLC Agilent 1200 series (Agilent, Santa Clara, CA) system. For separation, a Zorbax SB-AG column (250 × 4.6 mm, Agilent) was used at a setting of 35 °C oven temperature. The mobile phase was 20 mM phosphate buffer adjusted to pH 2.0 using phosphoric acid, with a flow rate of 1.0 mL/min for all the chromatographic separations. For chromatography, 10 µL of a 50-fold diluted sample was injected and analyzed for 7 min, after which the diode array detector was used for detection at 210 nm. Organic acid concentrations in the sample were determined using the calibration curve calculated from six standard substances: acetic acid, citric acid, succinic acid, lactic acid, malic acid, and oxalic acid.

### 2.4. Bacterial strain isolation and identification

A proportion of the floating mass and the surface film from the vinegar was spread on the culture medium plates for primary cell isolation. Single-colony isolation was carried out by consecutive pure isolation using a single isolated strain. Culture conditions were maintained at 27 °C for 5 days. The PCR primers used for identification were 27 F 5'-(AGA GTT TGA TCM TGG CTC AG)-3' and 1492 R 5'-(TAC GGY TAC CTT GTT ACG ACT T)-3'. PCR was carried out using EF-Taq (SolGent, Daejeon, Korea) with 20 ng of genomic DNA as the template in a 30 µL reaction mixture. First, Taq polymerase was activated at 95 °C for 2 min; followed by 35 cycles for 1 min at 95 °C, 55 °C, and 72 °C; and extension for 10 min at 72 °C. The amplified products were purified using a multiscreen filter plate (Millipore Corp., Billerica, MA). Sequencing was carried out using the PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. A DNA sample containing the extension products was mixed with Hi-Di formamide (Applied Biosystems, Foster City, CA), and the mixture was cultured at 95 °C for 5 min before it was incubated on ice for 5 min. An ABI Prism 3730XLDNA analyzer (Applied Biosystems) was used for analysis. The phylogenetic analysis was conducted in MEGA7 using Maximum Likelihood method.

### 2.5. Field-emission scanning electron microscopes

To observe the morphology of AABs, isolated from surface film of vinegar, via scanning electron microscopy (SEM), bacterial samples were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate solution (pH 7.2) for 90 min at 4 °C. The immobilized cells

were washed three times in 0.05 M sodium cacodylate buffer (pH 7.2), and fixed with 1% osmium tetroxide solution in 0.05 M sodium cacodylate buffer (pH 7.2) for 90 min at 4 °C. Subsequently, the cells were washed once with 0.05 M sodium cacodylate buffer and dehydrated using a graded series (50%, 70%, 80%, 90%, 100%, 100%, and 100% (v/v)) of ethanol. Dehydrated cells were suspended in 100% hexamethyldisilazane (HMDS) solution, and 10 µL of partial working sample was re-suspended on a micro slide and dried. To increase the electrical conductivity of the sample, platinum (Pt) coating was applied using a vacuum coater (EM ACE200, Leica, Vienna, Austria) for 100 s at 20 mA. SEM observations were performed at 10,000× magnification at 5 kV using SUPRA 40VP (Oberkochen, Carl Zeiss, Germany).

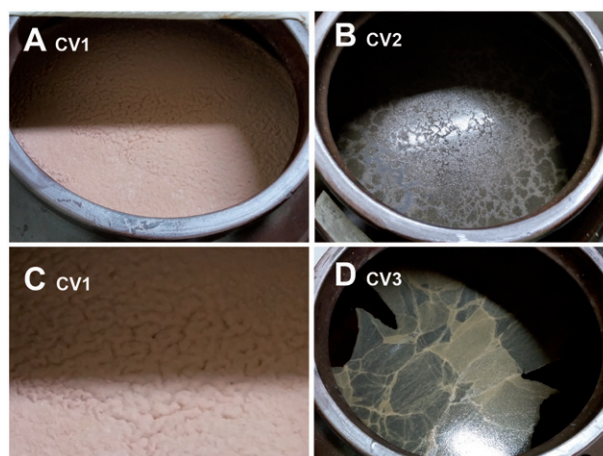
### 3. Results

#### 3.1. Morphological characteristics of the surface film

The surface films formed in the three static-fermented rice vinegars are shown in Figure 1. The morphological characteristics of the surface film of V1 were different from those of the films of V2 and V3. Macroscopic observation indicated that a creased layer of thickly stacked pink suspension was formed on the surface of V1, the shape of which was easy to disintegrate and was easily dispersed by a physical force. In contrast, a transparent pellicle was formed on the surface of V2 and V3, which could maintain its shape even after application of physical force.

#### 3.2. Physicochemical properties of the vinegar

The pH, total acidity, and organic acid concentrations of the three static fermented rice vinegars are presented in Table 1. Consistent with the findings for morphological characteristics, the physicochemical properties of V1 were different from those of V2 and V3. pH analysis indicated that V1 had a pH of 3.3, higher than that of V2 (pH 3.1) and V3 (pH 3.0). This was consistent with the findings for total acidity: V1 showed an acidity of 3.0%, lower than that of both V2 and V3 (~8.0%). Total organic acid content also showed complementary results, as V1 showed a total value of 3766.3 ± 72.1 mg/100 mL, which was approximately three-fold lower than that of either V2 (10025.2 ± 90.4 mg/100 mL) or V3 (11291.0 ± 279.9 mg/100 mL). V2 and V3 showed very similar organic acid compositions. Acetic acid – the main organic acid in all three vinegars – was three-fold higher in V2 and V3 than in V1. Malic



**Figure 1.** Appearance of pellicle forms on the top of rice vinegar produced by static fermentation method. (A, C) Vinegar covered with a pink and thick layer, V1; (B) Vinegar covered with clear amber and thin layer, V2; (D) Vinegar covered with clear amber and thin layer, V3.

**Table 1.** pH, total acidity and organic acid of rice vinegar produced by static fermentation method.

	V1	V2	V3
pH	3.3 ± 0.0	3.1 ± 0.0	3.0 ± 0.0
Total acidity (%)	3.0 ± 0.0	8.1 ± 0.1	8.7 ± 0.0
Organic acids (mg/100mL)			
Acetic acid	2845.1 ± 70.7	8266.2 ± 80.9	8710.7 ± 121.4
Succinic acid	568.6 ± 11.6	1058.0 ± 7.1	1742.8 ± 417.9
Lactic acid	346.2 ± 8.9	669.6 ± 3.3	641.6 ± 2.9
Malic acid	ND	17.4 ± 3.0	181.4 ± 16.8
Oxalic acid	6.3 ± 0.7	14.0 ± 0.7	13.0 ± 1.1
Citric acid	ND	ND	1.5 ± 0.2
Total	3766.3 ± 72.1	10025.2 ± 90.4	11291.0 ± 279.9

Values are mean ± SD (n = 3).

ND: not detected.

acid was detected in V2 (17.4 ± 3.0 mg/100 mL) and V3 (181.4 ± 16.8 mg/100 mL), whereas citric acid was detected only in V3 (1.5 ± 0.2 mg/100 mL).

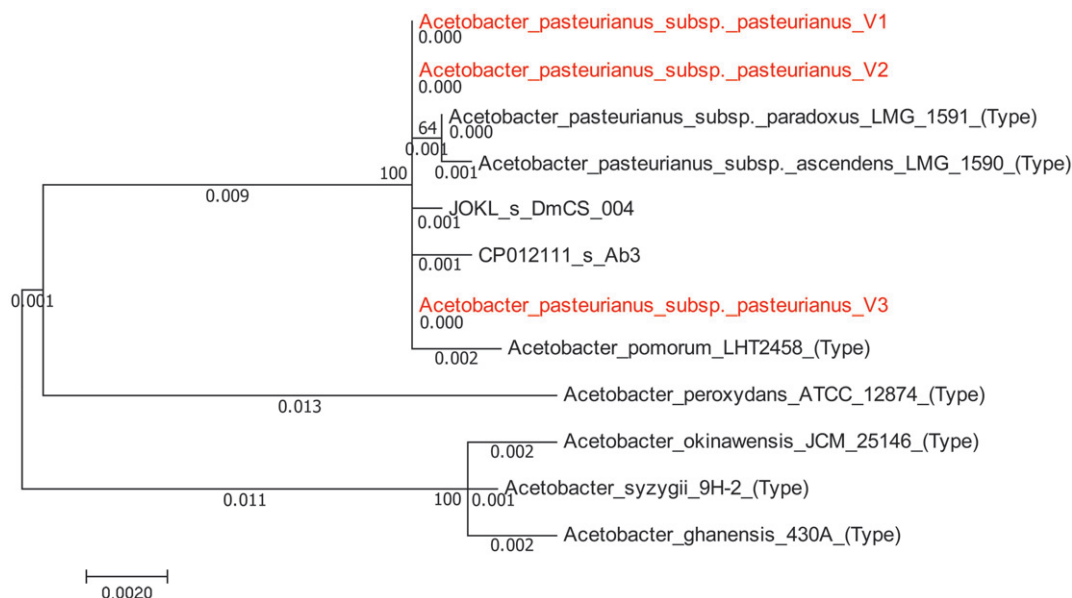
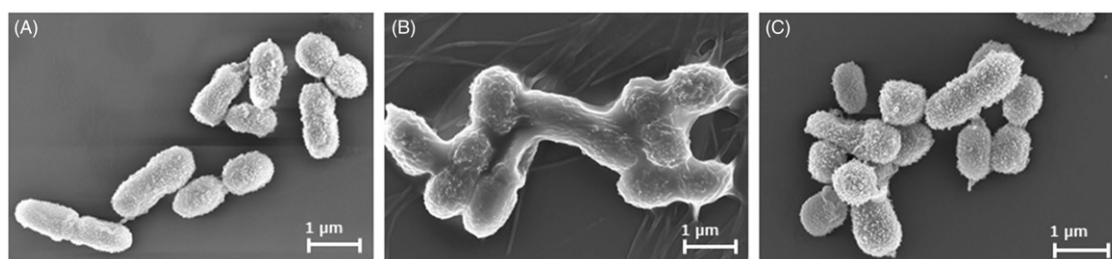
#### 3.3. Identification and FE-SEM analysis of the isolates from the surface film

Only three pure cultures were isolated from the surface film in the rice vinegar samples. They were found to form a clear halo on GYC medium. The 16S rDNA sequencing results for the isolates are shown in Table 2, and the genetic relatedness was displayed through the phylogenetic tree in Figure 2. All isolates were identified as *A. pasteurianus* subsp. *pasteurianus* with >100% sequence similarity based on EZcloud database. The tree indicated that the three *A. pasteurianus* is the same species. All isolates were identified as *A. pasteurianus* with >99.9% sequence similarity. As shown in Figure 3, cells of the three *A. pasteurianus* isolates observed through FE-SEM were rod-shaped with a rough surface, and smaller than 1 × 2 µm in size. Interestingly, a cell

**Table 2.** 16S rDNA gene analysis of the microorganism isolated from three rice vinegar produced by static fermentation method.

Strain no.	Sample	Media <sup>a</sup>	Taxon name	Accession no.	Similarity (%)
1	V1	GYC	<i>Acetobacter pasteurianus</i>	NR_104959.1	99.9
2	V2	GYC	<i>Acetobacter pasteurianus</i>	NR_104959.1	99.9
3	V3	GYC	<i>Acetobacter pasteurianus</i>	NR_104959.1	99.9

<sup>a</sup>The cultivation media used for the isolation are represented by the abbreviation indicated in the text.

**Figure 2.** Molecular Phylogenetic analysis by Maximum Likelihood method.**Figure 3.** Field emission scanning electron micrographs of *Acetobacter pasteurianus* collected from pellicle forms on the top of rice vinegar 1 (A); 2 (B); and 3 (C).

of strains isolated from V2 was surrounded with an extremely fine threadlike structure.

#### 4. Discussion

The final total acidity of V1 is too low to be called vinegar. In addition appearance of pellicle forms of V1 is different from those of V2 and V3. Total acidity of vinegar could be decreased by the over-oxidation which converts acetic acid to carbon dioxide and water. It is considered that the V1 vinegar was contaminated with undesirable microbial growth as the resulting of total acidity decreasing caused by over-oxidation. Therefore it is regarded that the shape of the surface film of V1 is quite different from other two vinegars, because of suspension due to microbial contamination.

AABs are gram-negative or gram-variable, non-spore forming, ellipsoidal to rod-shaped, and obligate aerobic bacteria belonging to the family *Acetobacteraceae* of class *Alphaproteobacteria* [13]. To date, more than 100 species belonging to 14 genera of AAB have been identified: *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia*, *Ameyamaea*, *Neokomagataea*, and *Komagataeibacter* [14]. Of these, the genera *Acetobacter*, *Gluconacetobacter*, and *Komagataeibacter* are employed in industrial vinegar production because of their extremely high tolerance to ethanol and acetic acid as well as their acetic acid-producing ability. Acid tolerance is a very important criterion for vinegar bacteria, because acetic acid can be toxic to cells, even at concentrations as low as 0.5% (v/v) [7,15].

In the present study, a creased surface film was found in all vinegar samples (Figure 1). *A. pasteurianus* was the sole isolate from the layers, with 100% similarity based on 16S rDNA sequencing (Table 2) and they are same species (Figure 2). This finding is consistent with that of Nanda et al. [4] who isolated *A. pasteurianus* with 99% similarity from a crepe pellicle of moromi, an alcoholic liquid containing acetic acid in Japanese rice and brown rice vinegar produced by traditional static fermentation. *A. pasteurianus* is a strain found in cereal, cider, and wine vinegar produced using both static and submerged fermentation processes [16]. It is reported that *A. pasteurianus* is predominant in vinegar with an acetic acid content of <6% (v/v); however, a certain strain of *A. pasteurianus*, AB3, has been isolated from rice vinegar with an acetic acid content of >9% (v/v) [15]. Based on these findings, we proposed that the vinegar sample contained the inoculated pure strain, *A. pasteurianus*, that the surface film formation in vinegar was not attributable to contamination by external factors.

According to Figure 3, FE-SEM images showed that the cells of *A. pasteurianus* isolated from the surface film in all vinegar samples were rod-shaped, with a rough surface, and  $\sim 1 \times 2 \mu\text{m}$  in size. Of these, cells of the V2 isolate were surrounded by an extremely fine threadlike structure. Kanchanarach et al. [17] corroborated that an amorphous layer surrounding *A. pasteurianus* cells during acetic acid fermentation was a type of pellicle polysaccharide, and proposed that it prevents the permeation of acetic acid into the cytoplasm by acting like a bio-film-barrier. Polysaccharides are classified as homopolysaccharide and heteropolysaccharide according to their constituents. *Gluconacetobacter xylinus* produces a homopolysaccharide composed of glucose units linked by  $\beta$ -1,4 glycosidic bonds, whereas *A. tropicalis* and *A. lovaniensis* produce heteropolysaccharides composed of glucose, galactose, and rhamnose [8,18]. Büyük and Çoban [19] reported that cellulose produced by *A. pasteurianus* isolated from wine is net-shaped and that it has an unbranched polymer linked via  $\beta$ -1,4-glycosidic bonds.

As an additional perspective, Andrés-Barrao et al. [20] reported that the cell response and morphology of *A. pasteurianus* vary at different stages of acetic acid fermentation. The cell surface of *A. pasteurianus* is smooth in a medium with no ethanol and acetic acid in the early stages of fermentation. In contrast, the cell surface becomes rough in a medium with 4% ethanol and it becomes smooth in the medium with 4% acetic acid and 0% ethanol at the end of fermentation. In addition, the results of their study suggested that formation of a rough cell surface in *A. pasteurianus* was associated with a

decrease in the content of capsular polysaccharides (CPSs) which are a type of polysaccharide observed on the surface of gram-negative bacteria along with exopolysaccharides, and are strongly resistant to acetic acid. It has been found that dTDP-rhamnose, a constituent of CPSs, is not synthesized when dTDP-4-dehydrorhamnose reductase is down-regulated. Therefore, ethanol and acetic acid can be through the outer membrane when the content of CPS decreases.

In conclusion, the findings of the present study indicate that *A. pasteurianus* cells in rice vinegar produced biofilm to withstand the high acetic acid concentration (above 8%), and that the surface film in the vinegar samples was formed by the aggregation of the bacteria and polysaccharides. However, it is speculated that some other factors might have been responsible for the formation of the pink floating matter found in V1, because of its unique morphological characteristics and the physicochemical properties of V1. Formation of the pellicle might be a survival strategy employed by the bacteria. However, its presence is undesirable if high-quality vinegar needs to be produced, because it causes turbidity and overoxidation. Thus, further studies should focus on devising a modified fermentation process to prevent surface film formation and consequent quality degradation, and determine the cause of pellicle formation through further analyses.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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