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Characterization and Fibrinolytic Activity of *Acetobacter* sp. FP1 Isolated from Fermented Pine Needle Extract

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The strain KCTC 11629BP, isolated from spontaneously fermented pine needle extract (FPE), showed fibrinolysis activity. The isolated strain was analyzed in physiological and biochemical experiments. Based on 16S rDNA sequencing and phylogenic tree analysis, the strain was identified to be a part of the genus *Acetobacter*, with *Acetobacter senegalensis* and *Acetobacter tropicalis* as the closest phylogenetic neighbors. Based on genotypic and phenotypic results, it was proposed that bacterial strain KCTC 11629BP represents a species of the genus *Acetobacter*. The strain was thusly named *Acetobacter* sp. FP1. In conclusion, *Acetobacter* sp. FP1 isolated from FPE possesses fibrinolytic activity.

Keywords: *Acetobacter*, fermented pine needle extract, 16S rDNA, fibrinolytic activity

Acetic acid bacteria (AAB) are present in fruits, flowers, palm sap, garden soil, and honeybees. The optimum growth pH range of AAB is from 5.5 to 6.3 [2], but they can grow at the low pH of 3.0 to 4.0 [24]. Most AAB convert ethanol into acetic acid, and AAB are often detected in fermented foods such as vinegar, beer, chocolate, and cocoa bean [3, 18]. The vinegar, also called acetic acid, is specially produced by AAB. *Gluconacetobacter europaeus*, *Acetobacter pasteruianus*, and *Acetobacter aceti* species are mainly present in vinegars [11]. The AAB recovered from vinegar fermentation are mainly distributed in the genera *Acetobacter* and *Gluconacetobacter*, and the most frequently isolated species are *Acetobacter pasteurianus*, *Acetobacter polyoxogenes*, *Gluconacetobacter xylinus*, *Gluconacetobacter hansenii*, *Gluconacetobacter europaeus*, *Gluconacetobacter oboediens*, *Gluconacetobacter intermedius*, and *Gluconacetobacter entanii* [28].

Pine needle extracts (PEs) have been found to have effects in several cancer cell lines [6] as well as protective effects against LDL oxidation [29] and antibacterial activity [14]. Previously, we reported that spontaneous fermentation increases the physiological effects of PE, including reduction of cholesterol, gastrointestinal motility, and fibrinolytic activity [19, 20]. Variable effects *via* fermentation were also identified, such as fibrinolytic activity [5, 17], antithrombotic activity [27], and antimicrobial activity [13]. Based on recent papers, we isolated AAB having excellent fibrinolytic activity from fermented pine needle extract (FPE) and also identified the culture media, optimal growth conditions, phylogeny, and biochemical characteristics of the isolated microorganism. We confirmed that the isolated microorganism had high similarity to *Acetobacter tropicalis* NRIC 0312 (AB03254). Based on the genotypic and phenotypic data, the strain KCTC 11629BP represents a novel species of the genus *Acetobacter*, for which the name *Acetobacter* sp. FP1 is proposed.

Material and Methods

Plant Material and Preparation of Fermented Pine Needle Extract

Fresh pine needles were selected and harvested from pine trees (*Pinus densiflora* Sieb. *et* Zucc.) in Gok-seong, Jeollanam-Do, Korea. Harvested pine needles were cleaned with tap water, dipped with charcoal in water, dried, and ground for 1 min. The preparation was allowed to settle at 4°C for 3 h, after which the supernatant was recovered. Pine needle extract (PE) was stored at room temperature for years to carry out fermentation.

Culture Conditions and Isolation of Strain

To screen for bacterial strains having high fibrinolytic activity, 100 μl of PE and FPEs were dissolved in 10 ml of distilled water. After serial dilution, samples were spread onto Nutrient Agar media [(g/l): 5.0 peptone, 3.0 beef extract, and 20 agar] and GPAY agar [(g/l): 40.0 glucose, 5.0 peptone, and 5.0 yeast extract, pH 5.0–5.2]. The plate was incubated at 30°C for 2 days. Single colonies on the plates were purified by transferring them onto fresh plates, followed by re-incubation. Strain *Acetobacter* sp. FP1 was one of the isolates that appeared on the nutrient agar plates. The isolated strain was

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cultured in acetic acid bacteria (AAB) medium [(g/l): 5.0 bactopeptone, 5.0 yeast extract, and 1 $MgSO_4 \cdot 7H_2O$, pH 6.6–7.0]. The liquid culture was incubated at 30°C for 2 days, and maintained in glycerol [100% (v/v)] at −70°C.

Fibrinolysis Assays Using Fibrin Plate and UV Spectrophotometer

Fibrinolytic activity of the bacteria was measured on a fibrin plate, and the turbidity changes (A_{350}) were measured using a UV spectrophotometer. The fibrin plate was composed of 5 ml of 1% (w/v) fibrinogen (Sigma, F8630) solution in distilled water mixed with 10 ml of 1.2% agarose solution and 20 µl of thrombin (Sigma, T7009) solution (0.1 U/µl). The solution was then poured into a Petri dish and allowed to stand for 1 h at room temperature to form a fibrin clot. The plate was incubated at room temperature for 1 h, after which holes were made on the fibrin plate using a capillary glass tube (5 mm in diameter). Then, 20 µl of bacterial liquid was dropped onto the plate, which was incubated at 37°C for 1 h. The diameter of the clear region was measured [1].

Fibrinolytic activity was also measured on a UV spectrophotometer [4, 22]. To measure fibrinolytic activity with a UV spectrophotometer, turbidity changes (A_{350}) in 96-well plates were monitored using a microplate reader (Molecular Devices). The fibrinogen solutions were buffered with 50 mM Tris, pH 7.4, containing 1 mM calcium chloride, 50 mM sodium chloride, and 0.01 U/ml of thrombin. After 100 µl of fibrinogen solution (1 mg/ml) was injected onto the plate, turbidity changes (A_{350}) were measured for 30 min.

To identify protease inhibitors, PMAF (phenylmethysulfonyl fluoride) and EDTA (ethylenediaminetetraacetic acid) were examined [12]. The supernatant sample was incubated in triple distilled water (DW) containing 5 mM PMSF (a known inhibitor of serine protease) and EDTA (a known inhibitor of metalloprotease) for 10 min at 37°C. Then, turbidity changes (A_{350}) in 96-well plates were monitored using a microplate reader (Molecular Devices). The fibrinogen solutions were buffered with 50 mM Tris, pH 7.4, containing 1 mM calcium chloride, 50 mM sodium chloride, and 0.01 U/ml of thrombin. After 100 µl of fibrinogen solution (1 mg/ml) was injected onto the plate, turbidity changes (A_{350}) were measured for 30 min.

Morphological and Biochemical Characteristics

Gram staining was performed using a Gram staining kit (Fluka, 77730). Oxidase activity was tested by determining the oxidation of Oxidase Reagent acc (Fluka, 18502). Catalase activity was examined by measuring the production of oxygen bubbles in aqueous hydrogen peroxide solution. To identify the carbon sources used by the bacteria, the bacteria were grown on basal mineral salt medium with glycerol, maltose, methanol, D-mannose, D-glucose, D-galactose, D-fructose, D-arabinose, and D-xylose as carbon sources. Carbon sources were added to the basal mineral salt agar to a final concentration of 1% [21].

PCR Amplification of 16S rDNA and Sequencing

The 16S rDNA was PCR amplified using the 27F primer 5'-AGA GTTTGATCMTGG-CTCAG-3' and the 1492R primer 5'-TACGGY TACCTTGTTACGACTT-3' [26]. 16S rDNA PCR amplification was performed using an MJ Research PTC 225 (Ramsey) in a final volume of 50 µl, containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 0.2 uM of each primer, 1.25 U of *Taq* DNA polymerase (Roche iagnostics), and 3 µl of extracted DNA [8]. The PCR product was purified using a QIA quick PCR purification kit (Qiagen), according to the manufacturer's instructions. 16S rDNA sequence analysis was performed using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and ABI 310 DNA sequencer (Applied Biosystems) following the protocols of the manufacturer.

Phylogenetic Analysis

The 16S rRNA sequence of *Acetobacter* sp. FP1 was aligned with the 16S rRNA sequences of *Gluconacetobacter diazotrophicus* LMG7603T and the reference *Acetobacter* species using the CLUSTAL W2 software [15].

Results and Discussion

Isolation of *Acetobacter* sp. FP1 and Phylogenetic Tree for Strain AAB Based on 16S rRNA Sequence

The 16S rRNA was 1,362 bases in length, and BLAST searches indicated that the 16S rRNA gene sequence was most similar to sequences of other organisms in the family Acetobacteraceae. BLAST search results showed that the bacterium was identified as a similar strain of *Acetobacter tropicalis*, and nineteen 16S rRNA sequences of representative type strains were collected from NCBI.

The sequences were aligned using the CLUSTAL_W program, version 2.0 [15, 23] and phylogenic analysis, including neighbor-joining, maximum-parsimony, and maximum-likelihood trees of DNA.

The phylogenic tree showed that the bacteria belonged to the genus *Acetobacter*, based on the 16S rRNA sequence. The 16S rRNA showed high sequence similarity with *Acetobacter tropicalis* AB032354 (99.5%), whereas lower similarity was observed with *Acetobacter pasteurianu* X71863 (96.2%). The phylogenic tree also showed sequence similarities with *Acetobacter senegalensis* AY883036 (97.8%), *Acetobacter cerevisiae* AJ419843 (98.9%), *Acetobacter malorum* AJ419844 (98.8%), *Acetobacter indonesiensis* AB032356 (98.8%), *Acetobacter orientalis* AB052706 (98.7%), *Acetobacter orleanensis* AJ419845 (98.7%), *Acetobacter cibinongenis* AB052706 (98.3%), *Acetobacter oeni* AY829472 (97.4%), *Acetobacter aceti* X74066 (97.9%), *Acetobacter estunensis* AJ419838 (97.7%), *Acetobacter ghanensis* EF030713 (97.7%), *Acetobacter syzygii* AB052712 (97,6%), *Acetobacter lovaniensis* AJ419838 (97.5%), *Acetobacter fabarum* AM905849 (96.5%), *Acetobacter nitrogenifigens* AY669513 (96.5%), *Acetobacter peroxydans* AB032352 (96.7%), *Acetobacter pomorum* AJ001632 (96.7%), and *Acetobacter pasteurianu* X71863 (96.2%) (Fig. 4). The phylogenic tree further showed that the bacteria belong to the *Acetobacter* family, and 16S RNA sequence similarity was 96–99% between representative type strains. Specifically, similarity with *Acetobacter tropicalis* AB032354 representative type strain was 99.5%.

Table 1. Differential characteristics of *Acetobacter* sp. FP1 and the genus *Acetobacter.*

Characterization	1	2	3
Growth on carbon sources			
Glycerol	+	+	+
Maltose	-	+	-
Methanol	-	-	-
D-Mannose	-	+	ND
D-Glucose	+	+	ND
D-Galactose	-	+	ND
D-Fructose	+	+	ND
D-Arabinose	-	-	ND
D-Xylose	+	+	ND
Growth on YE−30% D-Glucose	-	-	+
Catalase	+	+	+
Oxidase	-	-	-
Gram staining	-	-	-

Strain: 1, *A*. sp. FP1; 2, *A. tropicalis* (AB 032354.1 and AB 032355.1); 3, *A. senegalensis* (AY 883036). +, Positive; -, negative; ND, not determined. Data for strain 2 were taken from Lisdiyanti *et al*. [16], and data for strain 3 were taken from Ndoye *et al*. [18].

Physiological and Biochemical Characteristics of *Acetobacter* sp. FP1

The biochemical characteristics of *Acetobacter* sp. FP1, including its optimum carbon sources, optimum growth temperature, Gram staining, catalase test, and oxidase test, were determined. *Acetobacter* sp. FP1 was also measured for its ability to grow on glycerol (positive), maltose (negative), and methanol (negative), as described previously [7]. However, the strain was unable to grow on yeast extract with 30% glucose. *Acetobacter* sp. FP1 was determined to be Gram-negative, negative in the oxidase test, and positive in the catalase test. *Acetobacter* sp. FP1 was grown at different temperatures on GY agar. Growth at 28°C to 37°C was increased, but growth at 42°C was decreased. In addition, the carbon sources of *Acetobacter* sp. FP1 analyzed were glucose (OD_{600}: 0.759), fructose (OD_{600}: 0.613), and xylose (OD_{600}: 0.625). In addition, the growth rate of *Acetobacter* sp. FP1 was compared between GY and acetic acid medium (ABM). The OD_{600} value in GY liquid was 1.1326667 after 18 h of culture, whereas that in ABM was 1.215667 after 38 h (Table 1).

The predominant fatty acid found in the bacteria was straight-chain unsaturated $C_{18:1\omega7c}$ (43.74%) fatty acid. Other fatty acids were $C_{14:0}$ (1.21%), $C_{16:0}$ (9.39%), $C_{18:0}$ (0.33%), $C_{14:0}$ 2-OH (11.14%), $C_{16:0}$ 2-OH (9.39%), $C_{16:0}$ 3-OH (5.49%), $C_{18:0}$ 3-OH (4.39%), and $C_{19:1\ cyclo\omega8c}$ (2.82%). The major cellular fatty acid was $C_{18:1\omega7c}$, but its content was lower compared with other *Acetobacter* strains. In a comparison with other *Acetobacter* strains, the major hydroxyl acids of *Acetobacter* sp. FP1 were 3-OH $C_{16:0}$ and 2-OH $C_{14:0}$ (Table 2) [8−10].

Table 2. Cellular fatty acid composition (%) of isolates *Acetobacter* sp. FP1.

Fatty acid	*Acetobacter* sp. FP1	*Gluconacetobacter xylinus* LMG 1515^T
	Fatty acid composition (%)	
$C_{14:0}$	1.21	5.94
$C_{16:0}$	9.39	17.16
$C_{18:0}$	0.33	2.13
$C_{14:0}$ 2OH	11.14	3.15
$C_{16:0}$ 2OH	13.97	4.93
$C_{16:0}$ 3OH	5.49	2.16
$C_{18:0}$ 3OH	4.39	-
$C_{18:1}$ ω7*c*	43.74	-
CYCLO-C 19:1ω8*c*	2.82	-

For fatty acid methyl ester (FAME) analysis, *Acetobacter* sp. FP1 was grown for 24 h on MYP agar, at 28°C under aerobic conditions. The predominant fatty acid was the straight-chain, unsaturated $C_{18:1}\omega7c$ (43.74%) fatty acid.

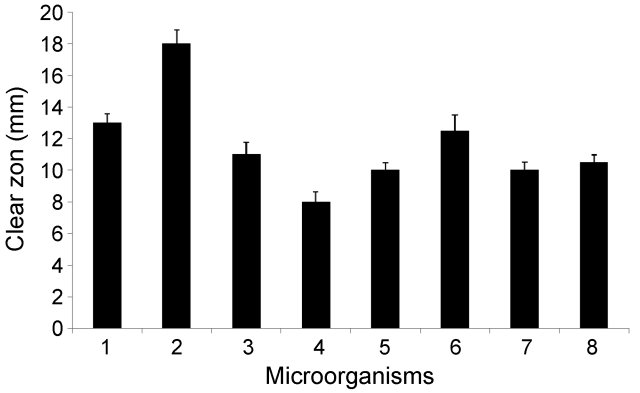

Fig. 1. The fibrinolytic activity of microorganism isolated from PE and FPEs.
Fibrinolysis activity was tested on fibrin plate. Microorganism 2, *Acetobacter* sp. FP1, was identified as having the strongest fibrinolytic activity.

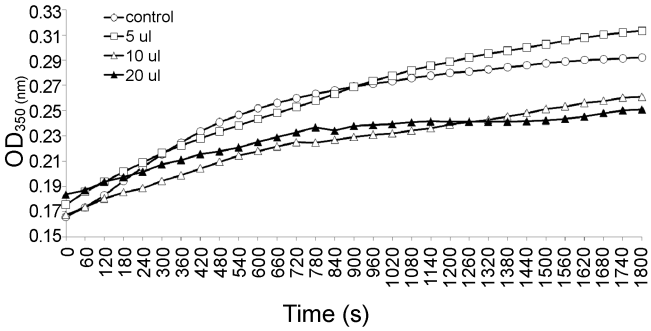

Fig. 2. Fibrinolytic activity of *Acetobacter* sp. FP1 according to concentration.
Fibrinolytic activity of *Acetobacter* sp. FP1 was measured with a UV spectrophotometer (350 nm). The 5 µl sample of bacteria supernatant had an increased $OD_{350\ nm}$ after 900 s. The 10 µl and 20 µl samples did not have increased $OD_{350\ nm}$ after 780 s.

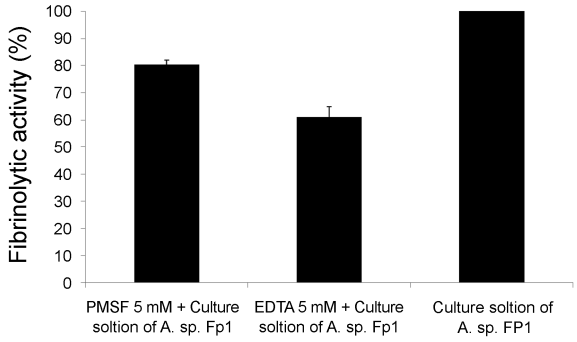

Fig. 3. Fibrinolytic activity of *Acetobacte*r sp. FP1 with effect of PMSF and EDTA treatment.
The culture solution of *Acetobacter* sp. FP1 was treated with PMSF and EDTA. The fibrinolytic activity was 80% with 5 mM PMSF and 60% with 5 mM EDTA. Control is culture solution of *Acetobacter* sp. FP1.

Identification of Fibrinolytic Activity in *Acetobacter* sp. FP1

We isolated eight microorganisms from PE and FPEs using Nutrient and GPAY media. Five microorganisms were selected in PE, three microorganisms in FPE 1, two microorganisms in FPE 2, two microorganisms in FPE 3, three microorganisms in FPE 4, and three microorganisms in FPE 6. The fibrinolytic activity of the microorganisms was identified by the presence of a clear zone on the fibrin plate (Fig. 1), and the turbidity ($A_{350\ nm}$) was measured using a UV spectrophotometer (Fig. 2). We confirmed that isolated microorganism 2 from FPE 2 and FPE 4 having the strongest fibrinolytic activity among the selected microorganisms was *Acetobacter* sp. FP1. The strain was identified as having high similarity with *Acetobacter tropicalis* through phylogenic analysis, but there was no evidence concerning fibrinolytic activity. Identification of protease inhibitors effect on the fibrinolytic activity was carried out using PMSF and EDTA. The fibrinolytic activity of the bacteria was not inhibited by the typical serine protease inhibitor PMSF. Additionally, the metalloproteinase inhibitor EDTA had a weak inhibitory effect (Fig. 3). We also quantified the fibrinolytic activity of culture solution *Acetobacter* sp. FP1 using a UV spectrophotometer. UV spectrophotometry results showed that the culture solution of *Acetobacter* sp. FP1 had fibrinolytic activity and but no protease function. Although we did not find the active components for the fibrinolytic activity of this bacterium, we know that the active components are substances other than protease and the active components are confirmed to be secreted into the culture medium.

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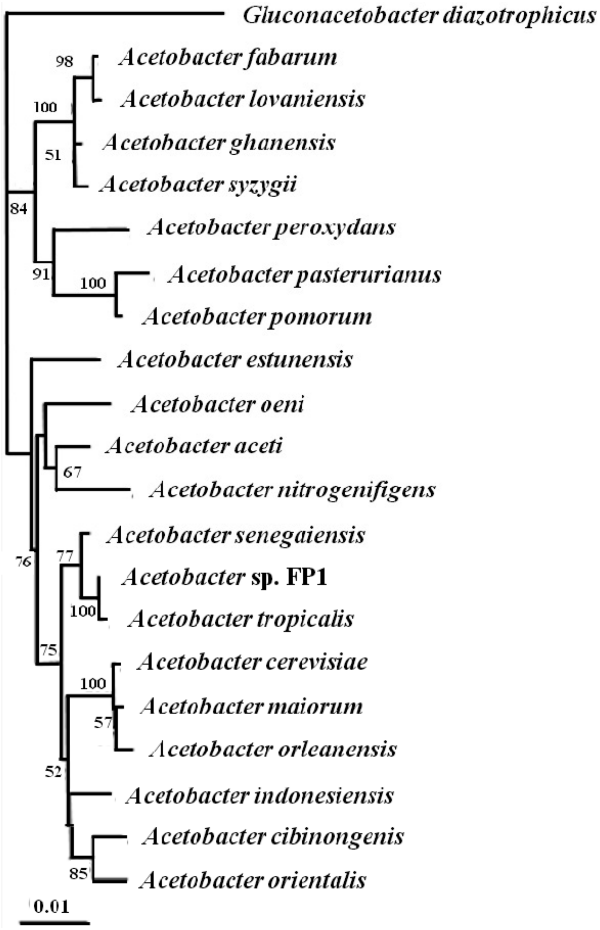

Fig. 4. Phylogenic tree of strain *Acetobacter* sp. FP1 based on its 16S rDNA sequence.
The numbers at the branch points are bootstrap values (based on 1,000 samplings), and only values greater than 50% are shown. The GenBank accession numbers are given. *Gluconacetobacter diazotrophicus* LMG7603^T (X75618) was used as an out-group.

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