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Isolation and identification of a variant strain of *Pseudomonas aeruginosa* PR3 with enhanced production of 7,10-dihydroxy-8(*E*)-octadecenoic acid

Joel B. Ellamar[a], In Hwan Kim[b], Ching T. Hou[c], Hee-Su Park[a], Hak-Ryul Kim[a,d,*]

[a] School of Food Science and Biotechnology, Kyungpook National University, Daegu, Republic of Korea
[b] Department of Public Health Sciences, Graduate School, Korea University, Seoul, Republic of Korea
[c] Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, IL, USA
[d] Institute of Agricultural Science & Technology, Kyungpook National University, Daegu, Republic of Korea

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ABSTRACT

Oxylipins are known to have several biological functions. The oxylipin 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), produced from oleic acid by *Pseudomonas aeruginosa*, was reported to have strong antibacterial activities against broad range of food-borne and plant pathogenic bacteria. Among DOD producers, *P. aeruginosa* PR3 shows efficient production of DOD from oleic acid, triolein, and olive oil. In this study, we report the serendipitous isolation and characterization of a variant strain of PR3, which upon examination showed higher efficiency for DOD production from olive oil than the original PR3 strain. Compared with the PR3 strain, the variant strain displayed enhanced DOD production by 47.6% and different carbon requirements for maximum DOD production. Considering the advantageous differences seen in this study, we propose the further characterization of this new strain and optimization of culture conditions for efficient DOD production.

1. Introduction

Modification of lipids by enzymatic or non-enzymatic processes can create novel functionalities. Among those modifications, oxygenation of lipid is well known to produce oxylipins that show various important biological functions (Howe and Schilmiller, 2002). Hydroxy fatty acid (HFA) is an example of functional oxylipins known to have special properties such as higher viscosity and reactivity than those of normal fatty acids, owing to the hydroxyl groups on the fatty acid backbone. These properties facilitate their use in a broad range of industrial applications (Hou and Forman, 2000; Kato et al., 1984).

The dihydroxy fatty acid, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), was first reported to be produced from oleic acid by the bacterial strain *Pseudomonas aeruginosa* PR3 (Hou et al., 1991). The production of DOD was performed through a two-step hydroxylation process. One hydroxyl group was introduced at carbon 10 with rearrangement of the 9-*cis* double bond of oleic acid molecule into the 8-*trans* configuration, and then the second hydroxyl group was introduced at carbon 7 resulting in the formation of 7,10-dihydroxy-8(*E*)-octadecenoic acid (Kim et al., 2000b). However, triolein, olive oil, and high oleic safflower oil were also found to be good substrates for efficient DOD production by strain PR3 (Bae et al., 2010; Chang et al., 2007, 2008; Suh et al., 2011). Production of DOD from olive oil was optimized to present a maximum of 65% yield (Bae et al., 2010). Recently, DOD was revealed to have strong antimicrobial activities against broad range of food-borne and plant pathogenic microorganisms (Sohn et al., 2013a, 2013b). Therefore, more efficient and higher yields of DOD production is desirable. In this study, we report the chance isolation and characterization of a new variant strain of *P. aeruginosa* PR3, which showed high efficiency for DOD production.

2. Materials and methods

2.1. Chemicals

All purified fatty acids (> 95% by GC) including standard elaidic acid (C17:0) were purchased from Nu-Chek Prep Inc. (Elysian, MN, USA). Virgin olive oil was purchased from a local market in Daegu, Korea. Mixture of trimethylsilylimidazole (TMSI) and pyridine was purchased from Supelco Inc. (Bellefonte, PA, USA). All other chemicals were reagent grade and were used without further purification. Thin layer pre-coated Kieselgel $60F_{254}$ plates were purchased from EM Science (Cherry Hill, NJ, USA). Other chemicals were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless mentioned otherwise.

* Corresponding author. School of Food Science and Biotechnology, Kyungpook National University, Daegu, 41566, Republic of Korea.
E-mail address: hakrkim@knu.ac.kr (H.-R. Kim).

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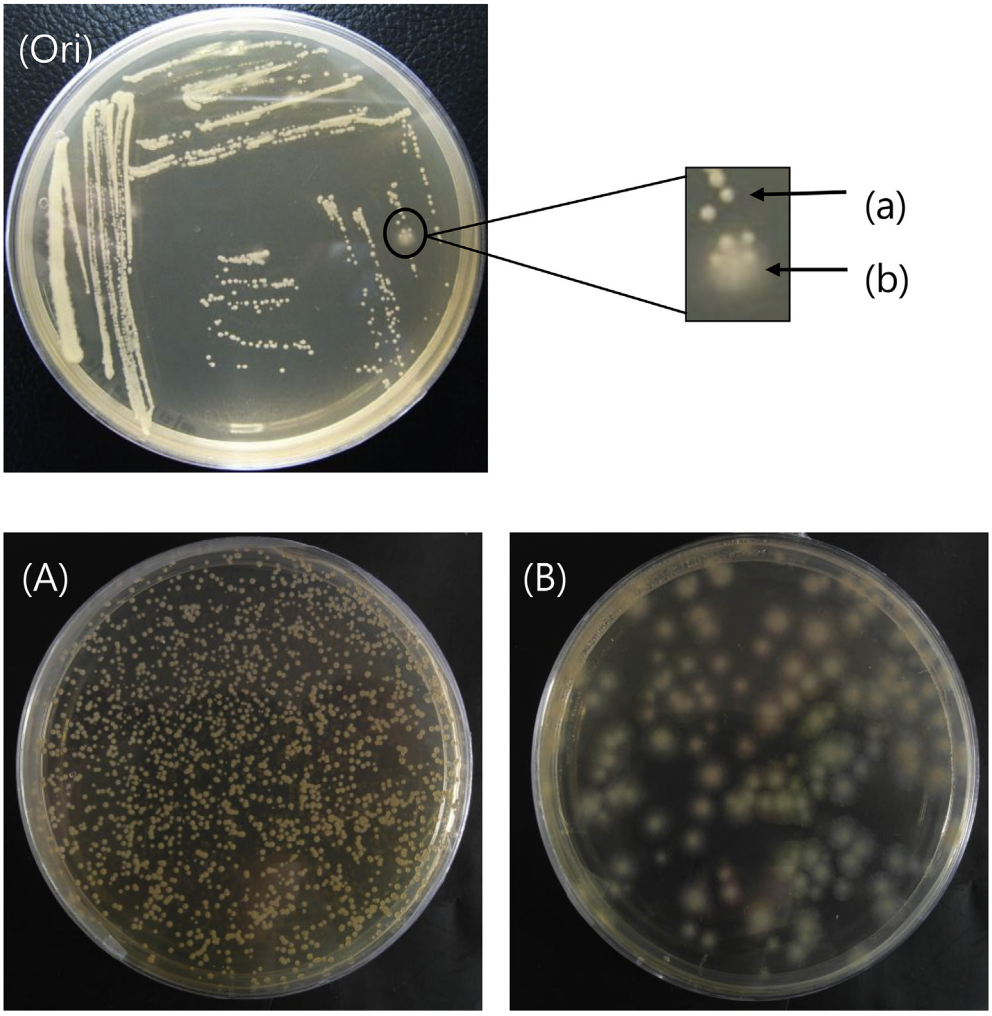

Fig. 1. Isolation of a new strain from *P. aeruginosa* PR3. Plate (Ori) shows the colonies formed from the culture of the original PR3 strain. Two different colonies were picked and shown as 'a' and 'b' in the separate magnified picture. Colony 'a' and 'b' were cultured and re-colonized in plate (A) and (B), respectively.

2.2. Microorganism and bioconversion

Pseudomonas aeruginosa NRRL strain B-18602 (PR3) was obtained from NCAUR Culture Collection (USDA/ARS/NCAUR, Peoria, IL, USA) and was grown aerobically in a 125-mL Erlenmeyer flask containing 50 mL of standard medium at 28 °C, 200 rpm in a shaking incubator. The standard medium used hereafter contained (per liter) 4 g dextrose, 4 g K_2HPO_4, 1 g $(NH_4)_2HPO_4$, 1 g yeast extract, 0.056 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MgSO_4{\cdot}7H_2O$, and 0.001 g $MnSO_4{\cdot}H_2O$. The pH of the medium was adjusted to 8.0 with diluted phosphoric acid.

For isolation of the variant strain, a loop of PR3 strain culture was streaked over the agar plate prepared with the standard medium containing 1.2% of potato agar. After 48 h of incubation at 28 °C for colony formation, select colonies were picked from the plate and transferred into new broth medium. After overnight incubation at 28 °C, 200 rpm in a shaking incubator, the cultures were diluted accordingly and poured over separate agar plates to confirm the colonies.

For production of the hydroxy fatty acids, oil substrate (1%, v/v) was added to 24 h-old culture followed by additional incubation for 72 h under standard conditions. At the end of incubation, the culture was acidified to pH 2.0 with 6 N HCl followed by immediate extraction (twice) with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extract with a rotary evaporator. All the experiments were duplicated with the error range being within 10%, unless specified otherwise.

2.3. Analysis of products

Reaction products were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC). TLC was developed with a solvent system consisting of toluene:dioxane:acetic acid (79:14:7 v/v/v). Spots were visualized by spraying the plate with 40% sulfuric acid followed by heating in a 100 °C oven for 10 min. For GC analysis, proper amount of samples were first methylated with diazomethane for 5 min at room temperature followed by derivatization with a mixture of TMSI and pyridine (1:4 v/v) for at least 30 min at room temperature. The TMSI-derivatized sample was analyzed by GC system (ACME 6100 series, Younlin Co., Korea) equipped with a flame ionization detector (FID) and a capillary column (SPB-1, 15 m 0.32 mm i.d., 0.25-μm thickness, Supelco Inc., Bellefonte, PA). The GC run was performed with temperature gradients of 20 °C/min from 100 °C to 150 °C, 5 °C/min from 150 °C to 200 °C, and then 0.5 °C/min from 200 °C to 210 °C followed by a 10 min hold at 300 °C (nitrogen gas flow rate: 0.67 mL/min). Injector and detector temperatures were held at 270 °C and 280 °C, respectively. Elaidic acid (C17:0) was added to the sample before derivatization as an internal standard for quantification. Structure confirmation of the product was performed by comparison of GC/mass spectrum of the sample with that of standard DOD. For GC/mass spectrometry (GC/MS), electron-impact (EI) mass spectra were obtained with a Hewlett Packard (Avondale, PA, USA) 5890 GC coupled to a Hewlett Packard 5972 Series Mass Selective Detector. The column outlet was connected directly to the ion source. Separation was carried out in a

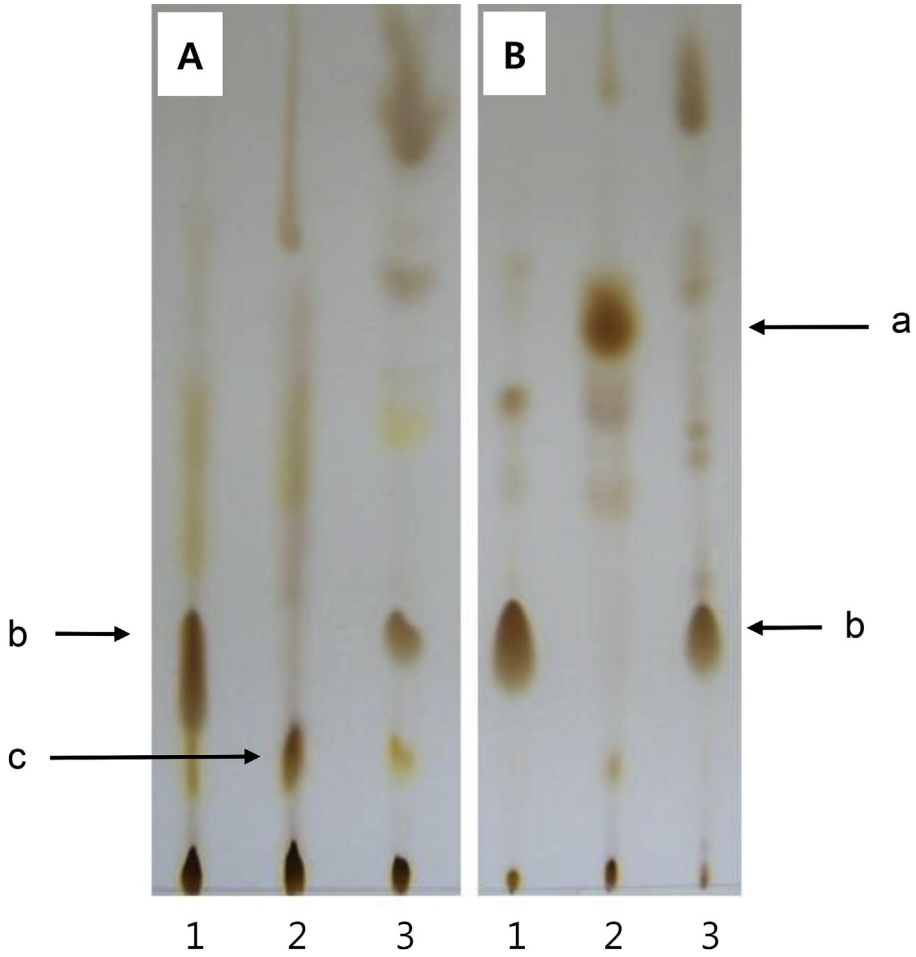

Fig. 2. TLC analysis of the crude extracts from bioconversion of the oil substrates. Bioconversion products by (A) *P. aeruginosa* PR3 and (B) the newly isolated strain. Conversion was performed for 72 h under standard conditions. Lane 1, crude extract from oleic acid; lane 2, crude extract from linoleic acid; lane 3, crude extract from olive oil. Spots a, b, and c represent linoleic acid, DOD, and THOD, respectively. Analytical conditions are given in materials and methods section.

methylsilicone column (30 m × 0.25 mm i.d., 0.25-μm film thickness) with a temperature gradient of 20 °C/min from 70 °C to 170 °C, holding 1 min at 170 °C and 5 °C/min up to 250 °C followed by holding for 15 min (Helium flow rate = 0.67 mL/min). Trimethylsilyl (TMS) derivatives of the product pre-methylated with diazomethane were prepared for GC/MS as mentioned above.

2.4. Analysis of phylogenetic tree of 16S rRNA sequences

Analysis of the phylogenetic tree of the isolated bacteria was conducted by comparison of the 16S rRNA sequences. Briefly, 16S rRNA sequence analysis was conducted using the iQ5 real-time PCR detection system (Bio-Rad Laboratories, California, USA). The phylogenetic tree was generated through a molecular phylogenic analysis based on the 16S rRNA gene base sequence of the isolated strain. The primers used to amplify the 16S rRNA gene were 27F (5′-AGAGTTTGTCMTGGCT CAG-3′) primer and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) primer synthesized based on the conserved sequence of the *E. coli* 16S rRNA gene. The RT-PCR was conducted by performing 33 cycles of denaturation (94 °C, 30 s), annealing (60 °C, 30 s), and elongation (72 °C, 45 s), followed by incubation for 15 min at 72 °C (Baek et al., 2010). After determining the 16S rRNA gene nucleotide sequences, each base sequence was compared with the base sequences of similar strains in the GenBank database to determine the phylogenetic locations of the strains. Phylogenetic analysis was performed using the Neighboring-Joint (NJ) method with the MEGA 5.1 software (Tamura et al., 2007).

3. Results and discussions

3.1. Isolation and characterization of a new strain

An abnormal colony was found during the regular maintenance of *Pseudomonas aeruginosa* NRRL strain B-18602 (PR3) for HFA production. Upon streaking on agar plate, the colonies formed of the normal PR3 strain were relatively small and showed a clear edge (Fig. 1, plate Ori and colony ‘a’ in the magnified picture). In contrast, a few colonies differed in size and shape from the normal PR3 colonies on the same plate. The size of the abnormal colony was relatively big and the colony showed dispersed and smeared edge (Fig. 1, plate Ori and colony ‘b’ in the magnified picture). Each such abnormal colony was picked and transferred into new broth medium followed by overnight incubation under standard conditions. The original PR3 strain and the newly isolated strain were rod-shaped (data not shown). After proper dilution of the individual cultures, they were streaked on separate agar plates. The shapes of colonies of the normal PR3 strain (Fig. 1A) and the newly isolated strain (Fig. 1B) were confirmed to be same as those in the original plate.

For further characterization of the new strain, we evaluated the production of hydroxy fatty acids from lipid substrates. Three different substrates, namely oleic acid, linoleic acid, and olive oil were tested and their products were compared with those produced by the original PR3 strain. PR3 strain showed normal DOD production from oleic acid and olive oil and THOD from linoleic acid, which corresponded to spot b and c, respectively, in Fig. 2A (Hou et al., 1991; Kim et al., 2000a; Suh

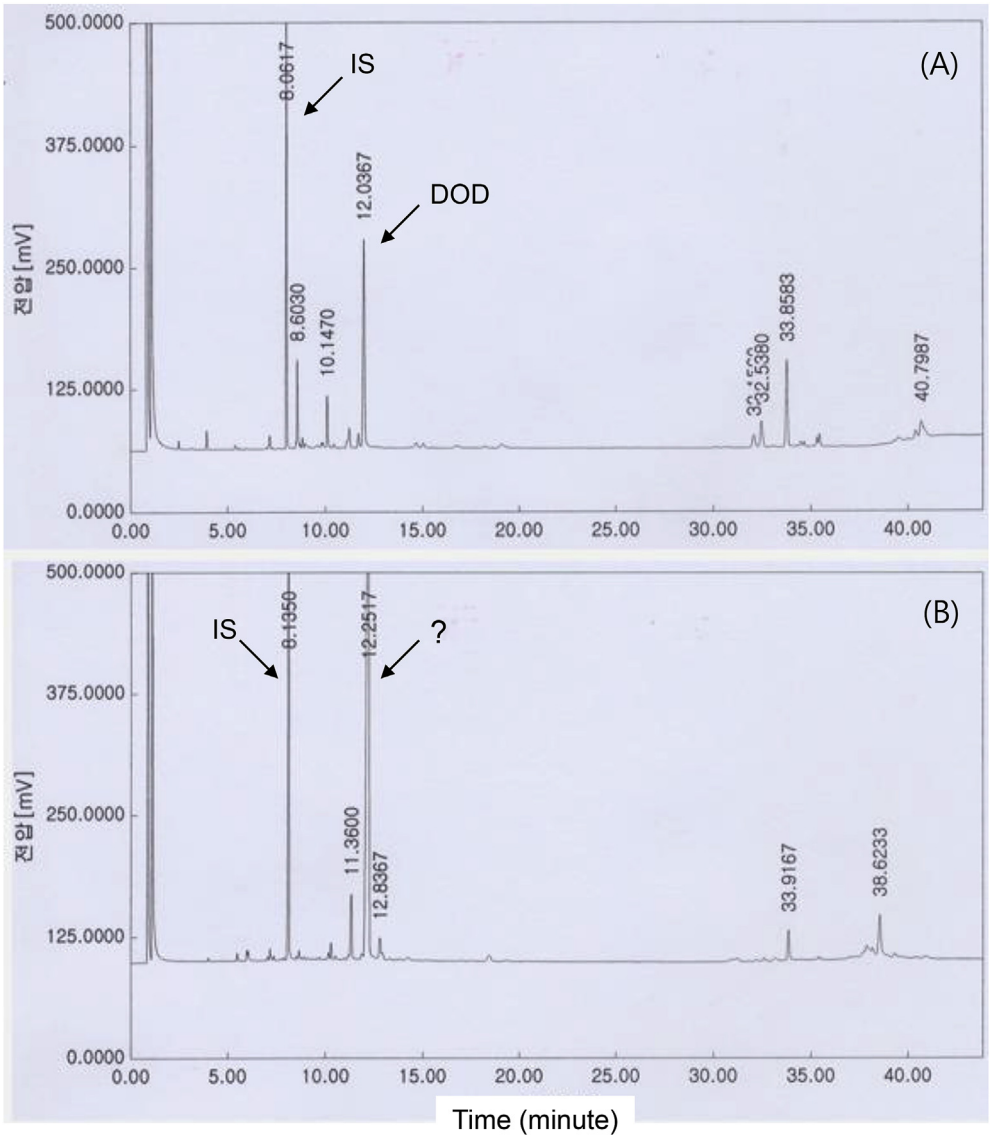

Fig. 3. GC analysis of the crude extracts from olive oil. Products by (A) PR3 and (B) KNU-2B. IS represents the internal standard and other analytical conditions are given in materials and methods section.

et al., 2011). However, production of HFAs from same substrates by the new strain showed quite different patterns in quantity and efficiency. Spots of products from oleic acid and olive oil (spot b' in Fig. 2B) migrated to the same positions on TLC plate as those by the PR3 strain, suggesting that the new strain could possibly produce DOD from corresponding substrates. However, the size of spots by the new strain was bigger than those produced by PR3, suggesting that production of DOD-like compound by the new strain was more efficient than that by PR3. Unexpectedly HFA production from linoleic acid by the new strain was very negligible while PR3 strain showed eminent production of THOD from the same substrate (spot c in Fig. 2A). Quantitative GC analysis of the crude extracts upon use of olive oil as substrate by both strains was performed and compared with each other to confirm DOD production by the new strain (Fig. 3). As expected, the new strain showed a distinct peak at the same retention time (question mark in Fig. 3B) as the DOD peak by PR3 strain (Fig. 3A), and the size of the peak was much bigger than that obtained with PR3. These results were in good agreement with those from TLC analysis.

GC/MS analysis of the major peak of Fig. 3B was performed to confirm the structure of the product. The electron impact (EI) GC/MS data of TMS derivative of the methylated product corresponded to the major peak in Fig. 3B as shown in Fig. 4. This was consistent with the TMS derivative of a methylated C18 dihydroxy monoenoic fatty acid with a molecular mass of 472. The locations of hydroxyl group were apparent from the fragments observed in the EI spectrum of the TMS derivative of the methylated fatty acid. The intense fragment arising from alpha cleavage of the derivatized hydroxyl group toward methyl end gave fragments containing TMS at m/z 215 and two TMS and a double bond at 343 m/z. Other two intense fragments arising from alpha cleavage of the derivatized hydroxyl group toward the methylated carboxyl end were observed at 231 m/z containing TMS and at 359 m/z containing two TMS and a double bond. These fragments allocated the hydroxyl groups at C7 and C10 and a double bond at C8-9. This spectrum profile was exactly matched to that of DOD (Bae et al., 2010; Chang et al., 2007). The data obtained from TLC, GC, and GC/MS analysis confirmed that the new strain produced DOD from oleic acid and olive oil and the productivity was more efficient than that of PR3 strain.

3.2. Phylogenetic tree analysis of the 16S rRNA sequence of the new strain

To identify the newly isolated strain, the base sequence of the 16S

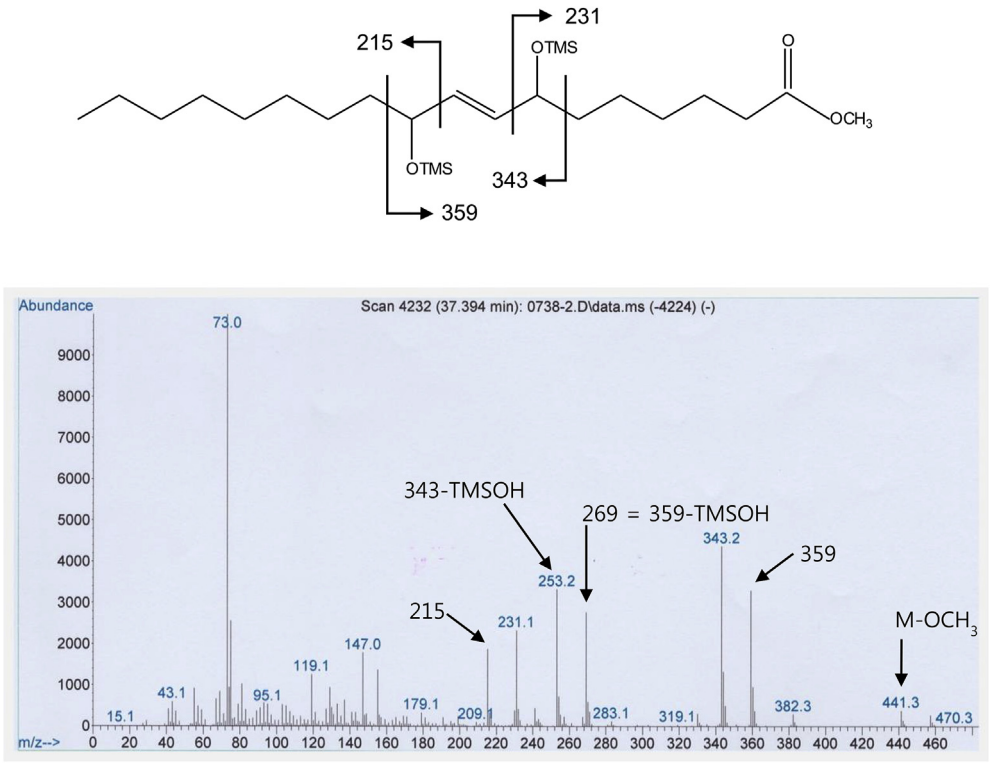

Fig. 4. Electron-impact mass spectrum of TMS derivatives of the methylated product shown as question mark in Fig. 3B. Sample preparation and running conditions are given in materials and methods section.

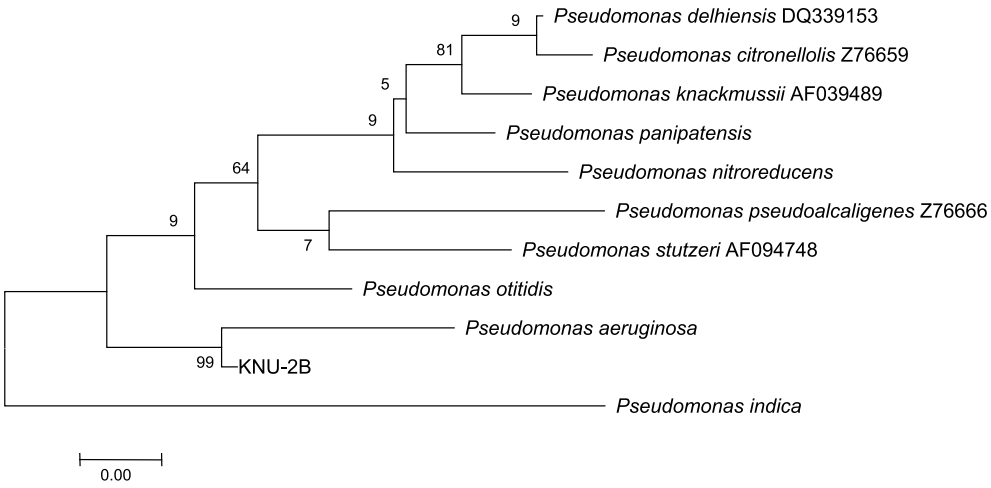

Fig. 5. Phylogenetic tree of the strain KNU-2B based on 16S rDNA gene sequences. The phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA 5.1 software. The homology values are shown at branch points.

Table 1
Effect of carbon sources on DOD production from olive oil by *P. aeruginosa* PR3 and *P. aeruginosa* KNU-2B.

Carbon source (0.4%)	DOD production by KNU-2B (mg/50 mL culture)	DOD production by PR3 (mg/50 mL culture)[a]
Glucose	203 ± 12	180
Galactose	55 ± 04	210
Fructose	310 ± 19	135
Lactose	110 ± 06	117

[a] Values were cited from the reference (Suh et al., 2011).

rRNA gene was analyzed and compared with those of similar strains registered in the GenBank database. Based on the results, the isolated new strain had 99% homology with *P. aeruginosa* X06684. The phylogenetic tree was prepared with the existing *Pseudomonas* group based on the structure of the 16S rRNA gene and the results are shown in Fig. 5. Based on the results from 16S rRNA analysis, molecular phylogenic analysis, and different shape of colony, we concluded that the newly isolated strain was a variant strain of *P. aeruginosa* PR3. Therefore, the new strain was named as *P. aeruginosa* KNU-2B and registered in the Korea Culture Center of Microorganisms (KCCM, Seoul, Korea) as [KFCC11544P].

3.3. Effect of carbon sources on DOD production from olive oil

Effect of the different carbon sources on DOD production from olive oil by KNU-2B strain was determined and compared with that by PR3 strain under the same culture conditions (Table 1). Among four different carbon sources that resulted in effective DOD production from olive oil by PR3 (Suh et al., 2011), fructose was the most effective for DOD production by KNU-2B, while galactose was most effective in case of PR3. The maximum DOD productivity (310 mg/50 mL culture) with fructose by KNU-2B was 47.6% higher than that with galactose by PR3 strain (210 mg/50 mL culture). However, DOD production with galactose by KNU-2B was very low (55 mg/50 mL culture). Glucose and lactose resulted in similar DOD production by both strains. These results suggested that the maximum DOD production from olive oil by KNU-2B was much higher than that by PR3 and the requirement of carbon source for DOD production by KNU-2B was quite different from that by the PR3 strain. Since KNU-2B showed different requirement of carbon source for DOD production, further study should be focused on determination of the optimal culture conditions including various environmental and nutritional factors.

4. Conclusion

In this study, we report the isolation and characterization of a new variant strain of *P. aeruginosa* strain PR3. The new strain showed different outer shape and size of colony compared with PR3. However, the base sequence of the 16S rRNA gene showed 99% homology with *P. aeruginosa* X06684. Therefore, we concluded that the new strain is a variant strain of *P. aeruginosa* PR3 and named the new strain as *P. aeruginosa* KNU-2B. DOD production by KNU-2B was more efficient than that by strain PR3, but THOD production was negligible. Carbon source requirement for DOD production was quite different from that of PR3.

Conflicts of interest

We have no conflict of interest to declare.

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