Journal of Agricultural Technology 2015 Vol. 11(8): 1857-1873
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

Bioconversion of Philippine Oil to Biologically Active Hydroxy Fatty Acid 7,10-dihydroxy-8(*E*)-octadecnoic acid (DOD) by *Pseudomonas aeruginosa* PR3

Joel B. Ellamar*[1], Hak Ryul Kim[2], Renato G. Reyes[3]

[1]Department of Animal Science, Institute of Agriculture and Forestry, Tarlac College of Agriculture, Philippines
[2]Schools of Applied Biosciences, College of Agriculture, Kyungpook National University, South Korea 702-701
[3]College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines

Ellamar, J.B., Kim, H.K. and Reyes, R.G. (2015). Bioconversion of Philippine Oil to Biologically Active Hydroxy Fatty Acid 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by *Pseudomonas aeruginosa* PR3. Journal of Agricultural Technology 11(8): 1857-1873.

Hydroxy fatty acids (HFAs) are multifunctional compounds with promising agricultural, biomedical and industrial applications. HFAs are produced from free fatty acids via microbial bioconversion and one of which is the 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD). DOD is produced from oleic acid by a bacterial isolate *Pseudomonas aeruginosa* PR3 through hydroxylation and isomerization. DOD possess broad spectrum antimicrobial activity against foodborne and plant pathogenic microorganisms. In recent study, DOD was utilized as substrate for the one-step synthesis of 7, 10 epoxyoctadeca-7, 9 dieonoic acid (EODA), a novel furan fatty acid (FFA). Biological characterization of this FFA revealed that it has antioxidant activity and antimicrobial properties. In continuing screening programs for new industrial chemicals produced from vegetable oil through microbial biotechnology, the future of Philippine oil for the production of value added-hydroxy fatty acids offer promise, hence this research. Results of the present study showed that oil from the Philippines contains significant amounts of free fatty acid. Gas Chromatography and GC-Mass Spectroscopy analyses showed that the major free fatty acid in pili nut oil (PNO) and palm oil (PO) was oleic acid and lauric acid was in virgin coconut oil (VCO) Results of the bioconversion studies revealed that *P. aeruginosa* PR3 could successfully utilized PNO and PO as substrates for DOD production. Time-coursed studies also revealed that PR3 could utilize PNO efficiently and optimum production at 48 hour. Extracellular lipase activity and DOD production using PNO by PR3 revealed that it was time-dependent and varied on the type of medium. Results from this study demonstrated that PNO could also be used as substrate for the production of value-added hydroxy fatty acids by microbial bioconversion.

Keywords: microbial bioconversion, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD), hydroxy fatty acids (HFAs), *Pseudomonas aeruginosa* PR3, Pili Nut Oil

***Corresponding Author:** Joel B. Ellamar **E-mail:** ellamarjoel@gmail.com

Introduction

Hydroxy fatty acids popularly known as HFAs are multifunctional compounds produced by microorganisms. HFAs are used in broad range of industrial applications including novel pesticides, novel medicines, high-functional resins and fibers, biodegradable plastic materials, lubricants, cosmetics and paints (Bajpai *et al*., 2004; Hou and Forman, 2000; Kato *et al*., 1984; Shin *et al*., 2004). There have been many successful studies about HFA production by microorganisms using free fatty acids and triacylglycerols (TGs) as substrates in the fatty acid industry with enhanced functionalities (Kuo *et al*., 2002) however, purified free fatty acids and TGs are too expensive to be applied in industry. Thus, the use of vegetable oil abundantly available in nature containing TGs like oleic acid and linoleic acid would be economical compared to purified fatty acids and TGs. The high viscosity, pour point and freezing point and reactivity to atmospheric oxygen made vegetable oil popular ingredient in industries for the production of cosmetics, lubricants, chemical additives and as fuel. Vegetable oil with enhanced properties by cleaving and adding functional groups by microorganisms are also used in the production of coatings, detergents, polymers, flavors, agricultural chemicals and in pharmaceuticals.

One of the products of microbial bioconversion is 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) from oleic acid (C18:1 9-*cis*) by the bacterial isolate *P. aeruginosa* PR3. DOD was uniquely produced involving both hydroxylation and possibly isomerization involving an addition of two hydroxyl groups at two positions carbon 7 and 10 and a rearrangement of the double bonds of the substrate molecule from carbon 9 to 8. Biological activity test on DOD revealed that it has broad-spectrum antimicrobial activity against foodborne and plant pathogenic microorganisms (Kim *et a*l., 2008). DOD can also reduce surface tension (Knothe *et al*., 1995). In a recent study, DOD was utilized for the one-step synthesis of 7,10 epoxyoctadeca-7,9 dieonoic acid (EODA), a novel furan fatty acid (FFA). Biological characterization of this FFA revealed that it has antioxidant activity (Ellamar *et al.*, 2013) and antimicrobial properties (Ellamar and Kim 2011).

Objective: In continuing screening program for new industrial chemicals produced from vegetable oil through microbial biotechnology, the future of Philippine oil as substrate for the production of value added-hydroxy fatty acids offer promise, thus, utilization of Philippine oil for the production of biologically active hydroxy fatty acid 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by *Pseudomonas aeruginosa* PR3 deemed necessary, hence, this research.

Materials and methods

Microorganism.

Pseudomonas aeruginosa NRRL strain B-18602 (PR3) was kindly provided by Dr. Ching T. Hou of the United States Department of Agriculture/National Center for Agricultural Utilization Research (USDA/NCAUR), Peoria, IL, USA. The strain was aerobically grown at 28ºC in a 125 ml Erlenmeyer flask containing 50 ml of standard medium or SM6 with a shaking speed of 200 revolution per minute (rpm) in a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). The basal semi- synthetic medium was composed of 4 g glucose, 4 g K_2HPO_4, 1 g $(NH_4)_2HPO_4$ and 1 g yeast extract per liter at pH 8.0. Metal ions amounting to 1 mM per ml was incorporated into 50 ml medium as growth cofactors. The metal ion was composed of 0.056 g $FeSO_4.(7H_2O)$, and 0.01 g $MgSO_4.(7H_2O)$ and 0.001 g $MnSO_4(H_2O)$ per liter.

Chemicals.

Olive oil (extra virgin grade) was purchased from CJ Incheon, Korea. Pili nut oil (PNO) from Bicol Region, palm oil (PO) from South Asia Food, Inc. and virgin coconut oil (VCO) were purchased in the Philippines. Heptadecanoic acid was chosen as an internal standard and purchased from Nu-Chek Prep (Elysian MN, USA). A mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 v/v) was purchased from Supelco (Bellefonte, PA. USA). Thin-layer pre-coated Kieselgel $60F_{254}$ plates were obtained from EM Science (Cherry Hill, NJ, USA). Silica gel, Davisil^{TM}, grade 635, 60-100 mesh, and 60A, 99^{+}% and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless mentioned elsewhere. All other chemicals were reagent grade and were used without further purification.

Free Fatty Acid Profile Analysis.

The oil were analysed for free fatty acid content. The fatty acid content was determined by taking 10 mg of the oil sample into a pre-weighed 4 ml clean glass vial using a pipette followed by the addition of 2 ml 5% sulfuric acid in methanol using a glass pipettor. The vial containing the sample was covered tightly to avoid solvent from evaporation and was heated at 90ºC for 1.5 h in a heat block. The sample was cooled down followed by adding 1.5 ml petroleum ether and 1 ml distilled H_2O. The sample was centrifuged at 2,200

revolutions per minute (rpm) for 10 minutes. The supernatant was dispensed into a new 4 ml clean glass vial and the solvent was evaporated using nitrogen gas. After evaporating, 0.2 ml hexane was added to the sample and subsequently transferred to a GC vial with glass insert for analysis.

Screening of Oil for the Production of 7,10-dihydroxy-8(E)-octadecenoic acid by P. aeruginosa PR3.

The bioconversion reaction for the screening of oil for DOD production by *P. aeruginosa* PR3 was carried out in 100 ml baffled flask containing 50 ml SM6 medium. The basal semi-synthetic medium was composed of 4 g glucose, 4 g K_2HPO_4, 1 g $(NH_4)_2HPO_4$, 1 g yeast extract per liter at pH 8.0. After preparing the medium, 1.0 ml of a 24-h-old culture of *P. aeruginosa* PR3 was inoculated into the medium followed by the addition of 1.0 ml pre-prepared metal ion. The metal ion was composed of 0.056 g $FeSO_4.(7H_2O)$, and 0.01 g $MgSO_4.(7H_2O)$ and 0.001 g $MnSO_4(H_2O)$. The cultures were incubated for 24 h and shaken at 150 rpm using a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). Culture broth sample amounting to 0.1 ml were taken out from the sample for cell density analysis prior to the addition of substrate. Each oil samples amounting to 0.5 ml was then added to a 48-h-old culture as substrate followed by continued incubation for an additional 72 h and then continuously shaken at 150 rpm using a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). At the end of the bioconversion, 0.1 ml were taken out from the sample for cell density analysis, and then culture broth was acidified to pH 2 with 5N HCl followed by immediate extraction with an equal volume of ethyl acetate (1:1, v/v). The samples were dispensed into a separatory funnel and shaken using a shaker for three (3) minutes. After shaking, the samples were allowed to stand until two layers appeared. The lower layer was discarded and the supernatant was dispensed into beakers. The solvent was evaporated from the extracted reaction product in fumehood until 2-3 ml of solvents was left. The reaction product was transferred into test tubes and centrifuged (Beckman Coulter Alegra TM 6R Centrifuge) at 2,000 rpm for 10 minutes. After centrifugation, the supernatant from the test tube was transferred into pre-weighed 4 ml glass vial and continuously dried using an air pump (Rocker 3200, Taiwan) equipped with silica as filter. The crude production yield was determined by subtracting the weight of the vial containing the reaction product with the weight of the empty vial. The samples were stored at -20ºC until analysis.

Time-coursed production of 7,10-dihydroxy-8(E)-octadecenoic acid from pili nut, palm oil derived from natural sources by P. aeruginosa PR3.

The time-coursed bioconversion reaction for the screening of oil for DOD production by *P. aeruginosa* PR3 was carried out in 250 ml baffled flask containing 100 ml SM6 medium. The semi-synthetic medium was composed of 4 g glucose, 4 g K_2HPO_4, 1 g $(NH_4)_2HPO_4$, 1 g yeast extract, per liter at pH 8.0. After preparing the medium, 1.0 ml of *P. aeruginosa* PR3 culture grown for 24 h was inoculated into the medium followed by the addition of 1.0 ml pre-prepared metal ion. The metal ion was composed of 0.056 g $FeSO_4.(7H_2O)$, and 0.01 g $MgSO_4.(7H_2O)$ and 0.001 g $MnSO_4(H_2O)$. The cultures were incubated for 24 h shaken at 150 rpm using a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). Culture broth sample amounting to 0.1 ml were taken out from the samples for cell density analysis prior to the addition of substrate. Each oil samples amounting to 0.5 ml was added to a 48-h-old culture as substrate followed by continued incubation for an additional 96 h and then continuously shaken at 150 rpm in a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). Ten (10) ml samples of the culture broth were taken aseptically out from the flask and dispensed into 50 ml Eppendorf tubes every 24 h for analysis and acidified to pH 2 with 5N HCl followed by immediate extraction with an equal volume of ethyl acetate (1:1, v/v). After shaking for 3 min, the samples were centrifuged at 2000 rpm for 10 min. The supernatant were transferred into pre-weighed 4 ml clean glass vials using a micropipette and evaporated the solvents to dryness using an air pump (Rocker 3200, Taiwan) equipped with silica in fume hood. The crude production yield was determined by subtracting the weight of the vial containing the reaction product with the weight of the empty vial. The samples were stored at -20ºC until analysis. The oil having the highest production of 7,10-dihydroxy-8(*E*)-octadecenoic acid was further used for optimization study.

Determination of Lipase Induction Activity.

Lipase induction activity of *P. aeruginosa* PR3 was carried out in 250 ml baffled flask containing 100 ml medium and 150 ml baffled flask containing 50 ml medium. Two different culture media were evaluated for the lipase induction activity. The first medium was the standard medium (SM6) composed of 0.4 g each carbon sources, 0.4g K_2HPO_4, 0.1 g $(NH_4)_2HPO4$, and 0.1 g yeast per 50 ml and the medium with urea as sole nitrogen source (USM6) composed of 0.4 g glucose, 0.4 g K_2HPO_4 and weight percentage of each nitrogen source containing 0.01585 g nitrogen per 50 ml. The medium pH was

adjusted to 8.0. After preparing the medium, 1 ml per 50 ml culture medium and 2.0 ml per 100 ml culture medium of 24-h-old culture of *P. aeruginosa* PR3 was inoculated into the medium followed by the addition of 1 ml per 50 ml culture medium and 2 ml per 100 ml culture medium pre-prepared metal ion. The metal ion was composed of 0.056 g $FeSO_4.(7H_2O)$, and 0.01 g $MgSO_4.(7H_2O)$ and 0.001 g $MnSO_4(H_2O)$.

The cultures were incubated for 24 h, shaken at 150 rpm using a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). Culture broth sample amounting to 1.0 ml were taken out from the sample for cell density analysis prior to the addition of substrate. Pili nut oil amounting to 0.5 ml per 50 ml culture and 1.0 ml per 100 ml culture were added to the 48-h-old culture as substrate followed by continued incubation for an additional 120 h and then continuously shaken at 150 rpm in a shaking incubator (VS-848005R, Vision Scientific Co., LTD, Korea). For the 50 ml culture broth seven (7) ml were taken aseptically out from the flask and dispensed into 50 ml Eppendorf tubes every 24 h for analysis. For the 100 ml culture broth, 10 ml were taken aseptically out from the flask and dispensed into 50 ml Eppendorf tubes every 24 h for analysis. To check the cell density of each treatment, 1.0 ml were taken out from the samples and dispensed into 2 ml Eppendorf tubes.

For the lipase induction activity, 1 ml of broth culture were taken out from the samples and dispensed into 2 ml Eppendorf tubes and put into crushed ice, followed by immediate centrifugation at 4 ºC at 10000 rpm for 10 min. After centrifugation, lipase activity was estimated by spectrophotometric method (Vorderillbecke, *et al.*, 1992) with p-nitrophenylbutyrate (*p*-NPB) as substrate. Enzyme sample of 0.1 ml prepared in 50 nM sodium phosphate buffer at pH 8.0 was mixed with 0.9 ml working substrate solution containing *p*-NPB. The *p*-NPB solution (10mM) was prepared by dissolving *p*-NPB in 100% cold ethanol followed by dilution ten times with the same buffer used for the preparation of enzyme solution. For activity determination, assay mixture was incubated at 37°C, and the increase in the absorbance was measured spectrophotometrically at 410 nm for at least 3 min against an enzyme-free control. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 nmol of *p*-nitrophenol from *p*-NPB per minute. The values in each experiment were the averages of two samples.

The pH concentration of the samples were also determined using a calibrated pH meter. After checking the pH concentration, samples were acidified to pH 2 with 5N HCl followed by immediate extraction with an equal volume of ethyl acetate (1:1, v/v). After shaking for 3 min, the samples were centrifuged at 2,000 rpm for 10 min. The supernatants were transferred into pre-weighed 4 ml clean glass vials using a micropipette and evaporated the

solvents to dryness using an air pump (Rocker 3200, Taiwan) equipped with silica as filter in a fume hood. The crude production yield was determined by subtracting the weight of the vial containing the reaction product with the weight of the empty vial. The samples were stored at -20ºC until analysis.

Analysis of Reaction Product.

All reaction products were analyzed by Thin Layer Chromatography (TLC) and Gas Chromatography (GC) using the method of Bae *et al*., (2010). The TLC plates were developed using a solvent system consisting of toluene: 1, 4 dioxane:acetic acid (79:14:7, v/v/v) and sprayed the plates with 50% sulfuric acid and heating in a 95ºC oven for 10 min. For GC analysis, the 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 35 minutes at room temperature. The TMS-derivatized samples were analyzed with Autosampler ACME 6100, 6000 Series Gas Chromatograph System equipped with a flame-ionization detector and a capillary column (SPB-1^{TM}), 15mx0.32 mm i.d., 0.25 µm thickness (Supelco Inc., Bellefonte, PA, USA). GC was run with a temperature gradient of 20ºC/min from 100 to 150ºC, 5ºC/min from 150 to 200ºC, and then 0.5C/min from 200 to 210ºC, followed by holding for 10 min at 300ºC at a nitrogen flow rate of 0.67ml/min. Injector and detector temperatures were held at 270 and 280ºC, respectively. For quantitative analysis, heptadecanoic acid (17:0) was added as an internal standard prior to methylation. A linear relationship was established between the peak ratios of the products and methyl heptadecanoic acid (Kuo and Nakamura, 2004).

Statistical Analysis.

All experiments were done in duplicates and all data were analyzed using IRRISTAT for all the screening studies and the SAS Program for the time-course studies in completely randomized design (CRD). Analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were used to determine the significant differences among treatment means.

Results

Free fatty acid profile of pili nut oil (PNO), palm oil (PO) and virgin coconut oil (VCO) from Philippines were analyzed by GC and GC-MS. Short and long chain fatty acids were found on virgin coconut oil (VCO) and lauric acid (C12:0) was the highest with 31%. VCO also contains caproic, caprylic,

capric, myristic, palmitic, linoleic, oleic and traces of arachidic acid. PO and PNO contain similar profile of free fatty acids, however, they vary in terms of percentage. PNO contains almost 70% of oleic acid (C18:1Δ^9) while 52% in PO. PNO and PO also contains caproic acid, linoleic acid, stearic acid and traces of arachidonic acid and arachidic acid (Table 1). The fatty acid profile of PO and PNO was similar to olive oil.

Table 1. Free fatty acid profile of three Philippine oil

Systematic Name/ Common Name	**Chemical Formula**	**Percentage[a]**			
		Virgin Coconut Oil	**Palm Oil**	**Pili Nut Oil**	**Olive Oil[b]**
Hexanoic acid/Caproic acid	C6:0	1.13	26.48	19.44	-
Octanoic acid/Caprylic acid	C8:0	10.77	nd	nd	-
Decanoic Acid/Capric acid	C10:0	5.55	nd	nd	-
Dodecanoic acid/Lauric acid	C12:0	30.80	nd	nd	-
Tetradecanoic acid/Myristic acid	C14:0	13.87	nd	nd	0.0-0.50
Hexadecanoic acid/Palmitic acid	C16:0	12.46	nd	nd	7.50-20.00
9,12-Octadecadienoic acid/Linoleic acid	C18:2$\Delta^{9,12}$	2.64	13.62	7.11	3.50-21.00
9-Octadecenoic acid/Oleic acid	C18:1Δ^9	14.47	52.05	67.83	55.00-83.00
Octadecenoic acid/Stearic acid	C18:0	6.90	5.40	4.12	0.50-5.00
11-Eicosenoic acid/Gondoic acid	C20:1Δ^{11}	nd	nd	0.30	-
11-Eicosanoic acid/Arachidonic acid	C20:1	nd	0.33	nd	0.00-0.40
n-Eicosanoic acid/Arachidic acid	C20:0	0.16	0.65	0.22	0.00-0.60
Others		1.08	1.45	0.66	

[a] *10 mg samples were methylated and analyzed in GC and GC-MS*

[b] *Range percentage of virgin olive oil. American Oil Chemist Society. 2009. Codex Standard for Olive Oils and Olive Pomace Oils; nd - Represents not detected; - No data*

The oil were utilized as substrates for the bioconversion into 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) by *P. aeruginosa* PR3. There was no DOD produced in virgin coconut oil, however, several spots which probably

corresponds to new compounds although not yet identified implies that this oil can be used as substrate for the production of other products like biosurfactant and other oxygenated hydroxy fatty acids. Bioconversion of PNO and PO produced DOD. The spots produced after PNO and PO bioconversion were further analysed by GC and then GC-MS for structure determination and confirmed that the spot represented DOD. From these results, it was confirmed that strain PR3 could produce DOD from PNO and PO (Figure 1 and Figure 2).

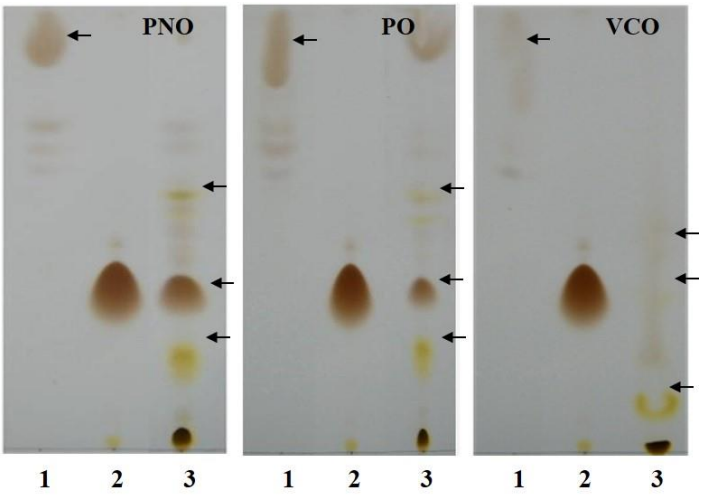

Figure 1. Thin layer chromatography analysis of the crude extract from bioconversion screening of pili nut, palm and virgin coconut oils by *P. aeruginosa* PR3. Lane 1; standard PNO, PO and VCO, lane 2; purified DOD, lane 3; bioconverted PNO, PO and VCO. Lower arrows indicate new products; middle arrows correspond to DOD; and upper arrows correspond to oleic acid

After confirmation, PNO and PO were used for time-coursed production of DOD. DOD production was monitored every 24 h for 4 days after substrates were added to the 48-h-old cultures. DOD production increased with time up to 48 h incubation followed by sharp decrease in the production as shown in Figure 3. Maximum amount of DOD produced at 48 h incubation was 92.25 mg per 50 ml culture, representing 27.13% and 35% production yields from oleic acid contained in olive oil, respectively. Cell density among treatment means was significantly different. Cell density in olive oil and palm oil were maximum at 24 h and declined thereafter. However, cell density in PNO was maximum at 48 h incubation and declined thereafter.

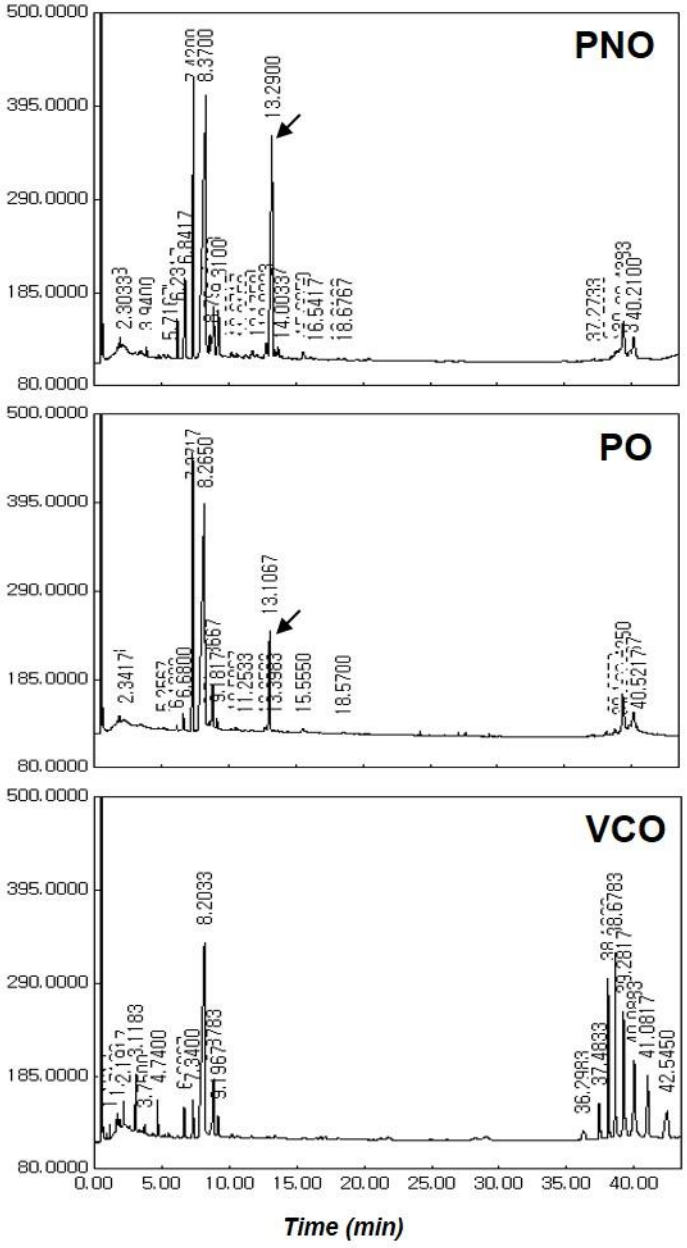

Figure 2. Gas chromatogram of the crude extract produced from the bioconversion of pili nut (PNO) palm (PO) and virgin coconut (VCO) oils by *P. aeruginosa* PR3. Arrow represents DOD peak

In the time-coursed extracellular lipase induction analysis, lipase induction activity and DOD production in 50 and 100 ml SM6 medium

increased significantly after 24 h after substrate addition and peaked at 48 h and 72 h and decreased thereafter while in USM6 medium, lipase induction activity and DOD production significantly increased after 24 h after substrate addition and peaked at 96 h and 120 h. Cell density significantly increased 24 h after the addition of metal ion and highest on the 48 h and slightly declined thereafter both on 50 ml SM6 and 50 ml USM6 media. The final pH concentration and change in pH concentration of the lipase-induced medium were significantly different and became acidic on time specified incubation period by 1 to 2 pH concentration (Figures3, 4, 5, 6, 7, 8 and 9).

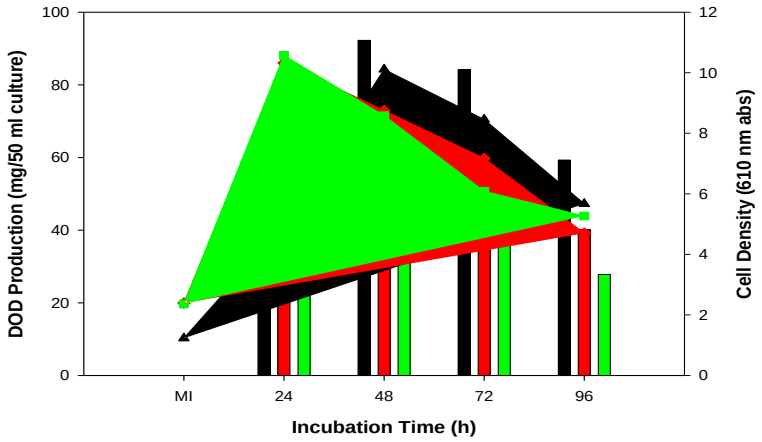

Figure 3. Time-coursed production of DOD from olive oil, pili nut oil and palm oil by *P. aeruginosa* PR3. Bar graph represents DOD production: olive oil (■); PNO (■); PO (■): Line graph represents the cell density: olive oil (▲); PNO (◆); and PO (■). MI means 24 hr after metal ion was added

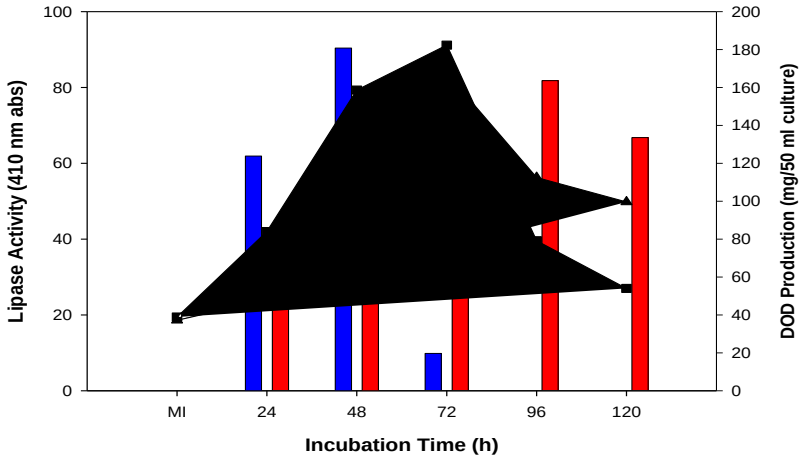

Figure 4. Relationship of time-coursed lipase induction activity and DOD production from pili nut oil by *P. aeruginosa* PR3 in 50 ml culture. Bar graph represents DOD production: SM6 culture medium ( ); USM6 culture medium ( ); Line graph represents the lipase induction activity: SM6 culture medium (■); USM6 culture medium (▲)

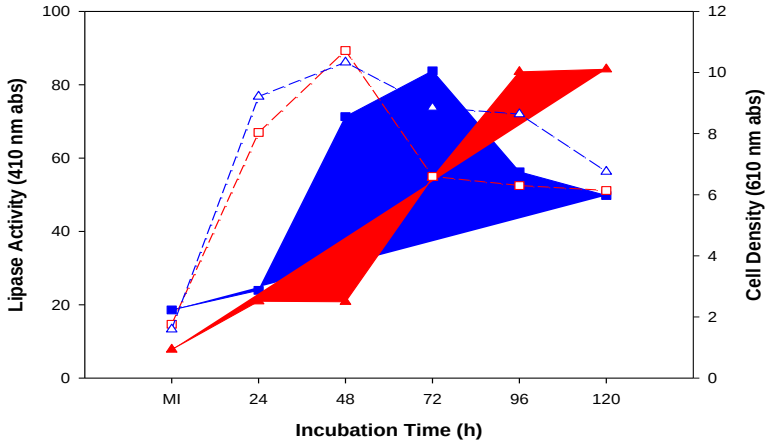

Figure 5. Relationship of time-coursed lipase induction activity and cell density during DOD production from pili nut oil by *P. aeruginosa* PR3 in 50 ml culture. Solid lines with close square and triangle represent lipase induction activity: SM6 ( ); USM6 ( ); Broken lines with open square and triangle represent cell density: SM6 ( ); USM6 ( )

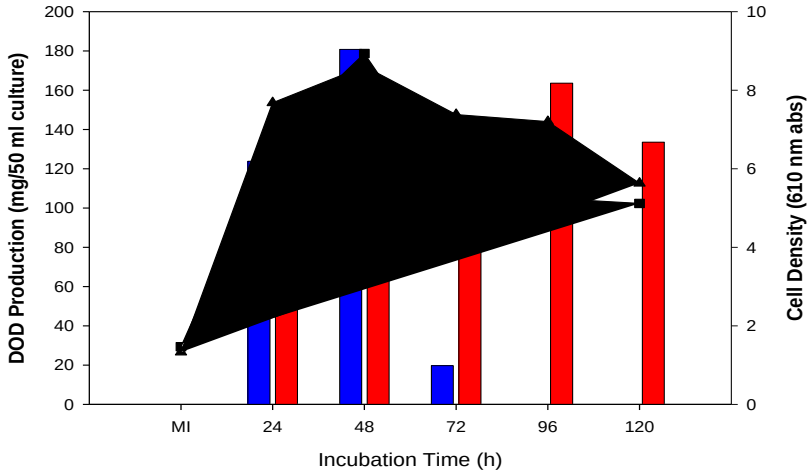

Figure 6. Relationship of DOD production and cell density during lipase induction activity from pili nut oil by *P. aeruginosa* PR3 in 50 ml culture. Bar graph represents DOD production: SM6 culture medium ( ); USM6 culture medium ( ); Line graph represents the lipase induction activity: SM6 culture medium (■); USM6 culture medium (▲)

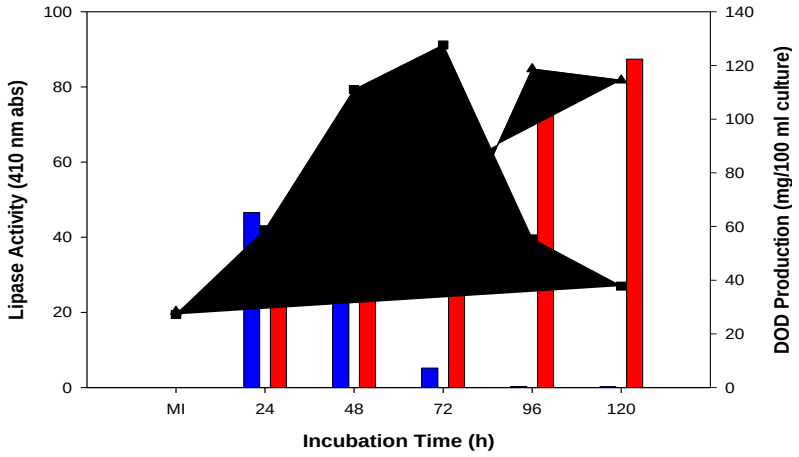

Figure 7. Relationship of time-coursed lipase induction activity and DOD production from pili nut oil by *P. aeruginosa* PR3 in 100 ml culture. Bar graph represents DOD production: SM6 culture medium ( ); USM6 culture medium ( ); Line graph represents the lipase induction activity: SM6 culture medium (■); USM6 culture medium (▲).

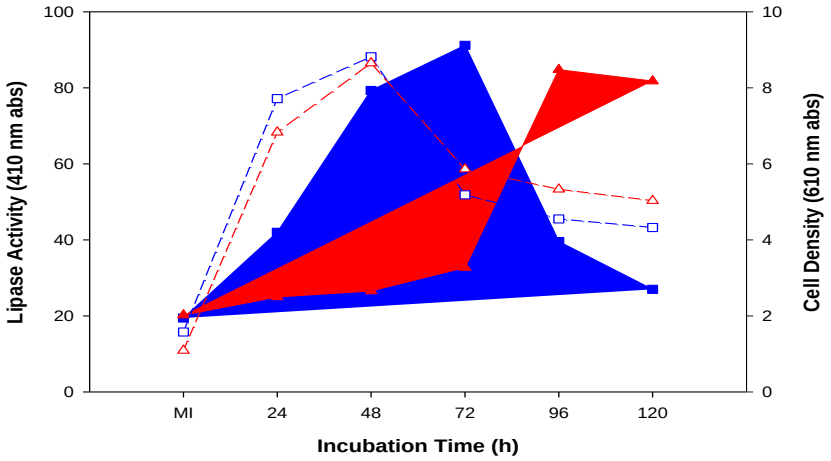

Figure 8. Relationship of time-coursed lipase induction activity and cell density during DOD production from pili nut oil by *P. aeruginosa* PR3 in 100 ml culture. Solid lines with close square and triangle represent lipase induction activity: SM6 (■); USM6 (▲); Broken lines with open square and triangle represent cell density: SM6 (□); USM6 (△).

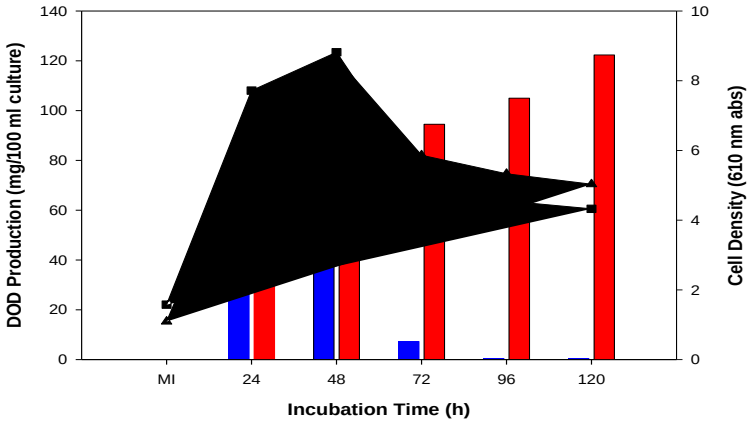

Figure 9. Relationship of DOD production and cell density during lipase induction activity from pili nut oil by *P. aeruginosa* PR3 in 100 ml culture. Bar graph represents DOD production: SM6 culture medium (■); USM6 culture medium (■); Line graph represents the cell density: SM6 culture medium (■); USM6 culture medium (▲)

Discussions

Bioconversion studies for the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid of oil from the Philippines demonstrated positive results. Results confirmed that strain PR3 could produce DOD from PNO and PO in the screening studies. The time-course experiment showed the ability of PR3 to utilize Philippine oil, however, showed decrease in DOD production across incubation period indicating that DOD produced was further metabolized by PR3. The decline in cell density suggests that nutrients in the medium to support bacterial growth were depleted. Optimal incubation time in this study was similar to the case of triolein (Chang *et al*., 2007) at 48 h but different from DOD production using oleic acid as substrate by the same strain PR3 (Hou *et al*., 1991) peaked at 72 h after substrate addition. This discrepancy could be explained by the fact that PR3 may require an earlier time for the induction of lipase activity to release oleic acid from PNO and PO (Chang *et al*., 2007).

Relative to the lipase induction activity of PR3 using PNO as substrates, the results suggest that the lipase induction could be actively sustained in the presence of utilizable substrate in the medium. The decline in DOD production in SM6 medium implied that PR3 utilized the DOD produced as carbon source. During lipase induction, PR3 utilized the nutrients to produced energy in order to convert oleic acid in PNO and as a result, the growth medium became nutrient-limited. DOD production in urea supplemented SM6 medium is different from that of the SM6 medium. It was assumed that lipase activity and DOD production were influenced by urea.

Moreover, the relationship of time-coursed lipase induction activity and cell density during DOD production from pili nut oil using SM6 and urea supplemented media resulted variability compared to earlier reports. Cell density in SM6 and urea supplemented SM6 was optimum at 48 h. The rapid decrease in cell density coincided with the decrease in lipase induction activity in SM6 medium whereas in urea supplemented SM6 medium was different, although there was an observed gradual decline in cell density, the lipase induction activity is still increasing which also corresponds to the increase of DOD production.

On the other hand, lipase induction activity in 100 ml culture upon addition of metal ion ($FeSO_4.(7H_2O)$, $MgSO_4.(7H_2O)$ and $MnSO_4(H_2O)$ to a 24-h-old culture may have also activated lipase induction activity to the 50 ml SM6 medium. Lipase induction activity increased significantly both on different media, however, the peak for the SM6 medium was at 48 h and 72 h and decreased thereafter while in USM6 medium the lipase induction activity peaked at 96 h and 120 h similar to the result in 50 ml USM6 medium.

With regards to the relationship of lipase induction activity and DOD production, the significant increase and decrease of lipase activity corresponded to the increase and decrease of DOD produced. In the case of SM6, production of DOD increased significantly with the lipase induction activity by time dependent manner. For the USM6 medium, production of DOD was different. Optimum productions were attained at 96 h and 120 h similar to the lipase induction activity as shown in Figures 9, 10 and 11.

Furthermore, increase in cell density might have contributed in the lipase induction activity of *P. aeruginosa* PR3 for DOD production. It was observed that DOD production declined in SM6 medium after 48 h which means that PR3 utilized DOD as source of nutrients. It was assumed that the pathway for urea utilization between SM6 and USM6 is different. The decline in DOD production also corresponds to the decline in the number of cells suggesting that as the medium become nutrient-deficient for PR3 its capacity to survive become less, leading to death. Conversely, DOD production in USM6 medium was different from the SM6 medium. It was evident that *P. aeruginosa* PR3 did not consume DOD as energy source. It was assumed that the presence of urea influenced the utilization of nutrients of the bacterium. *P. aeruginosa* PR3 first utilized the available nutrients in the medium to promote cell growth necessary for the bioconversion of oleic acid in pili nut oil instead of the DOD produced during the bioconversion process. This results on the lipase induction activity in SM6 medium was similar to the previous experiments conducted on olive oil and oleic acid as substrates in which lipase activity was optimum at 72 h (Chang *et al*., 2007; Bae *et al*., 2010; Suh *et al*., 2010). However, lipase induction activity in USM6 medium was different. It was assumed that the type of the culture medium influenced lipase induction activity. In previous study on the production of DOD from triolein via lipase induction by *P. aeruginosa* PR3, it was reported that triolein but not oleic acid remarkably induced lipase activity and was observed from 12 h after substrate addition and then peaked at 36 h followed by steep declination (Chang *et al*., 2008). This means that lipase activity plays a significant role in catalyzing the release and conversion of oleic acid from pili nut oil to DOD by *P. aeruginosa* PR3, thus, influencing the production of DOD.

Acknowledgement

To the generosity of Prof. Hak Ryul Kim of the Applied Microbiology Laboratory, Schools of Applied Biosciences, College of Agriculture, Kyungpook National University, Daegu City, South Korea for the conduct of this research under the Basic Science Research Program through the National

Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology.

References

Bae, J.H., Suh, M.J., Lee, N.Y., Hou, C.T. and Kim, H.R.(2010) Production of a value-added hydroxy fatty acid, 7,10-dihydroxy-8(*E*)-octadecenoic acid from high oleic safflower oil by *Pseudomonas aeruginosa* PR3. Applied Microbial Biotechnology.

Bajpai, V., Shin. S.Y., Kim, M.J. and Kim, H.R. (2004) Antifungal activity of bioconverted oil extract of linoleic acid and fractionated dilutions against phytopathogens *Rhizoctonia solani* and *Botrytis cinera*. Agric. Chem. Biotechnol. 47:199-204.

Chang, I.A., Bae, J.H., Suh, M.J., Kim, I.H., Hou, C.T. and Kim, H.R. (2008) Environmental optimization for bioconversion of triolein into 7,10 dihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas aeruginosa* PR3. Appl. Micriobiol. Biotechnol. 78:581-586.

Chang, I.A., Kim, I.H., Kang, S.C., Hou C.T. and Kim, H.R. (2007) Production of 7,10-dihydroxy-8(*E*)-octadecenoic acid from triolein via lipase induction by *Pseudomonas aeruginosa* PR3. Appl. Microbiol. Biotechnol. 74:301-306.

Ellamar, J.B. and Kim, H.R. (2010) Production and biological properties of a novel furan fatty acid from 7,10-dihydroxy-8(E)-octadecenoic acid. Masteral thesis, School of Applied Biosciences. Kyungpook National University, Korea.

Ellamar, J.B., Song, K.S. and Kim, H.R. (2011) One-Step production of a biologically active novel furan fatty acid from 7,10-Dihydroxy-8(E)-octadecenoic acid. J.Agric. Food Chem. 59,8175-8179.

Hou, C.T. and Forman, R.J. (2000) Growth inhibition of plan pathogenic fungi by hydroxy fatty acids. J. Ind. Microbiol. Biotechnol. 24:275-276.

Hou, C.T., Bagby, M.O., Plattner, R.N., Koritala, S. (1991) A novel compound, 7,10-dihydroxy-8(E)-octadecenoic acid from oleic acid by bioconversion. J. Am. Oil Chem. Soc. 68:99-101.

Kato, T., Yamaguchi, Y., Abe, N., Uyehara, T., Nakai, T., Yamanaka, S. and Harada, H. (1984) Unsaturated hydroxy fatty acids, the self-defensive substances in rice plant against rice blast disease. Chem. Lett. 25:409-412.

Kim, H.R., Bae, J.H., Hou, C.T. and Chang, S.C. (2008) Production of biologically active hydroxy fatty acids by *Pseudomonas aeruginosa* PR3. Biocatalysis and Bioenergy. USA. John Wiley& Sons, Inc,.

Knothe, G., Dunn, R.O. and Bagby, M.O. (1995) Surface tension studies on novel allylic mono- and dihydroxy fatty compounds. A method to distinguish erythrolthreo diastereomers. J. Am. Oil Chem. Soc. 72:43-47.

Kuo, T.M., Kaneshiro, T. and Hou, C.T. (2002) Microbiological conversions of fatty acids to value-added products. In: Kuo TM, Gardner HW (eds). Lipid Biotechnology. New York: Marcel Dekker. pp. 606-628.

Shin, S.Y., Kim, H.R., and Kang, S.C. (2004). Antibacterial activity of various hydroxy fatty acids bioconversted by *Pseudomonas aeruginosa* PR3. Agric. Chem. Biotechnol. 47:205-208.

Suh, M.J., Baek, K.Y., Kim, B.S., Hou, C.T. and Kim HR (2010) Production of 7,10-dihydroxy-8(*E*)-octadecenoic acid from olive oil by *Pseudomonas aeruginosa* PR3. Appl. Microbiol. Biotechnol. p 7.