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Environmental optimization for bioconversion of triolein into 7,10-dihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas aeruginosa* PR3

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Abstract Hydroxy fatty acids (HFAs), originally found in small amount mainly from plant systems, are well known to have special properties such as higher viscosity and reactivity compared with other normal fatty acids. Recently, various microbial strains were tested to produce HFAs from different unsaturated fatty acids. Among those microbial strains tested, *Pseudomonas aeruginosa* PR3 are well known to utilize various unsaturated fatty acids to produce mono-, di-, and tri-HFAs. Previously, we reported that strain PR3 could utilize triolein as a substrate for the production of 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) via the induction of lipase activity (Chang et al., *Appl Microbiol Biotechnol*, 74:301–306, 2007). In this study, we focused on the development of the optimal environmental conditions for DOD production from triolein by PR3. Optimal initial medium pH and incubation temperature were pH 8.0 and 25°C, respectively. Magnesium ion was essentially required for DOD production. Optimal inoculum size, time for substrate addition, and substrate concentration were 1%, 12 to 24 h, and 300 mg, respectively.

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Introduction

Lipids are ubiquitously present multifunctional compounds in nature. They exert biologically critical roles among living things and are used in broad range of industrial applications as well. Occasionally, structural modifications of lipids via chemical method or microbial bioconversion can change their properties or even create novel functionalities. Hydroxy fatty acids (HFAs), originally found in small amount mainly from plant systems (Zimmerman 1966), are well known to have special properties such as higher viscosity and reactivity compared with other normal fatty acids (Bagby and Calson 1989). Based on these structural peculiarities, they contain high industrial potentials in a wide range of applications including resins, waxes, nylons, plastics, lubricants, cosmetics, and additives in coatings and paintings. In addition, HFAs are reported to contain antimicrobial activities against plant pathogenic fungi and some of food-borne bacteria (Bajpai et al. 2004; Hou and Forman 2000; Kato et al. 1984; Shin et al. 2004).

Recently, various microbial strains were tested to produce HFAs from different unsaturated fatty acids. Among those microbial strains tested, *Pseudomonas aeruginosa* PR3 are well known to utilize various unsaturated fatty acids to produce mono-, di-, and tri-HFAs. Conversion of oleic acid to 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) with 60% yield by strain PR3 was reported (Hou et al. 1991), and its production was improved to more than 80% yield through modifying culture conditions (Kuo et al. 1998). 10-hydroxy-8(*E*)-octadecenoic acid was identified as an intermediate in this bioconversion (Hou and Bagby 1992; Kim et al. 2000b).

Strain PR3 also converted linoleic acid to equimolar mixture of 9,10,13-trihydroxy-11(*E*)-octadecenoic acid (9,10,13-THOD), and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (9,12,13-THOD; Kim et al. 2000a). Ricinoleic acid [12-hydroxy-9(*Z*)-octadecenoic acid] and palmitoleic acid were used by PR3 to produce 7,10,12-trihydroxy-8(*E*)-octadecenoic acid (TOD) and 7,10-dihydroxy-8(*E*)-hexadecenoic acid, respectively (Kuo et al. 1998; Bae et al. 2007). Recently, we firstly reported that triolein, the triacylglyceride form of oleic acid, could be efficiently utilized by PR3 to produce DOD through the triolein-induced lipase activity under the optimized carbon and nitrogen sources (Chang et al. 2007). According to the report, triolein was first hydrolyzed into oleic acid by the triolein-induced lipase activity, and then the released oleic acid was converted to DOD by PR3. In this study, we focused on the development of the optimal environmental conditions for the production of DOD from triolein by PR3 for further application of large-scale production in the scaled-up fermenter.

Materials and methods

Chemicals

Triolein with greater than 99% purity and elaidic acid (C17:0) were purchased from Nu-Chek Prep (Elysian, MN). The mixture of trimethylsilylimidazole (TMSI) and pyridine was purchased from Supelco (Bellefonte, PA). All other chemicals were reagent grade and were used without further purification. Thin-layer precoated Kieselgel 60F_{254} plates were obtained from EM Science (Cherry Hill, NJ). Other chemicals were purchased from Sigma Chemical (St Louis, MO), unless mentioned otherwise.

Microorganism and bioconversion

P. aeruginosa NRRL strain B-18602 (PR3) was kindly provided by National Center for Agricultural Utilization Research (NCAUR) Culture Collection (USDA/ARS/NCAUR, Peoria, IL). The strain was grown at 28°C aerobically in a 125-ml Erlenmeyer flask containing 50 ml of standard medium with shaking at 200 rpm. 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 $FeSO_4$ $7H_2O$, 0.01 g $MgSO_4$ $7H_2O$, and 0.001 g $MnSO_4$. H_2O. The medium pH was adjusted to 7.0 with diluted phosphoric acid. Other environmental conditions were adjusted as needed on the basis of the standard condition, unless mentioned elsewhere. For the production of HFAs, triolein (0.3 g) was added as a substrate to 24-h-old culture followed by additional incubation for 72 h. At the end of cultivation, 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 extracts with a rotary evaporator. All the experiments were duplicated with the error range being within 10%.

Analysis of products

The extracted reaction products were analyzed by thin-layer chromatography (TLC) and gas chromatography (GC). The TLC was developed with a solvent system consisting of toluene/dioxane/acetic acid (79:14:7 v/v/v). Spots were visualized first by iodine vapor and then by spraying the plate with 40% sulfuric acid followed by heating in a 100°C oven for 10 min. For GC analysis, the 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 20 min at room temperature. The TMS-derivatized sample was analyzed with Shimadzu GC-17A (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (SPB-1™, 15 m×0.32 mm inner diameter, 0.25 μm thickness [Supelco]). GC was run with a temperature gradient of 20°C/min from 70 to 200°C, holding 1 min at 200°C, and then 0.7°C/min to 240°C, followed by holding for 15 min at 240°C (nitrogen flow rate=0.67 ml/min). Injector and detector temperatures were held at 250 and 270°C, respectively. Elaidic acid (C17:0) was added to the sample before derivatization as an internal standard for quantification.

Results

Effect of initial medium pH on DOD production

pH is one of the most important environmental factors affecting microbial growth and enzyme reactions. The effect of initial medium pH was studied in the range of from 3.0 to 10.0 on the production of DOD from triolein by PR3. Substrate was added to the 24-h-old culture of which pH was initially adjusted to the desired value, and then incubation continued for an additional 72 h. As shown in the Fig. 1, cell growth before substrate addition was efficient with minor variation in the range of pH 5.0 to 10.0. However, DOD production was very sensitive to pH values in the same range. DOD production was maximized at pH 8.0 and decreased thereafter up to 50% of the maximum value until pH 10.0. Below pH 6.0, there was no detected DOD production. Therefore, all the further experiments were carried out at pH 8.0.

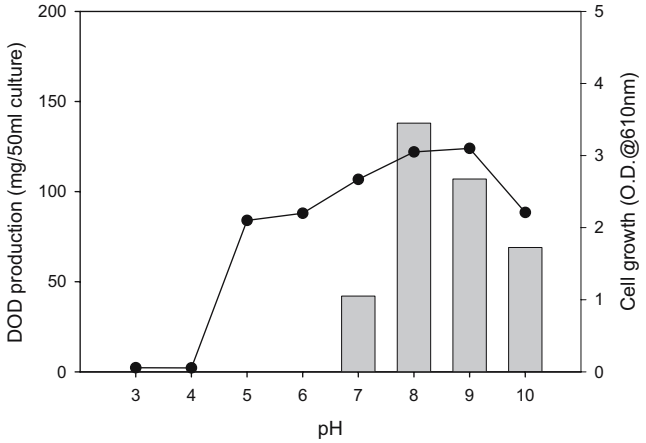

Fig. 1 Effect of initial medium pH on DOD production from triolein by PR3. Initial pH was adjusted using 1 N HCl and 1 N KOH. Triolein (300 mg) was added as a substrate to the 24-h-old culture followed by additional incubation for 72 h. Cell density was obtained from multiplication of the original optical density value by the dilution factor. *Bar* and *line graphs* represented DOD production and cell growth before substrate addition, respectively. All reaction conditions were the same as the standard condition explained in "Materials and methods"

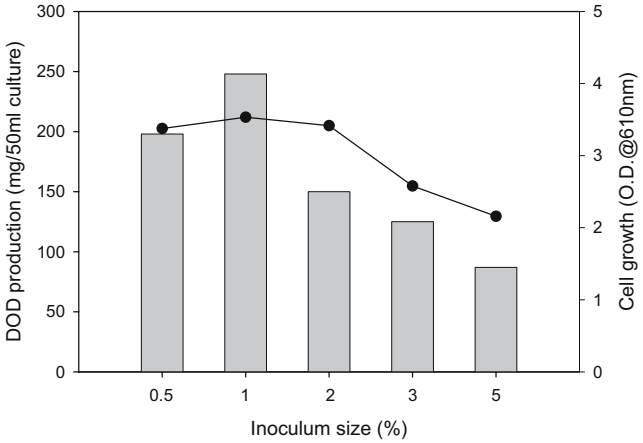

Fig. 3 Effect of inoculum size of the culture on DOD production from triolein by PR3. *Bar* and *line graphs* represented DOD production and cell growth before substrate addition, respectively. All reaction conditions were the same as the standard condition explained in "Materials and methods"

Effect of incubation temperature on DOD production

The effect of incubation temperature on the production of DOD was studied in the range of from 15 to 40°C (Fig. 2). All the incubation conditions except temperature were maintained as same as the standard condition. The DOD production increased significantly as the temperature increased from 20 to 25°C, at which DOD production reached a maximum and gradually decreased thereafter up to 60% of maximum value at 30°C. At 35°C, DOD production dropped to 2.6% of the maximum value. However, cell growth before substrate addition was sufficient at between 20 and 30°C indicating that temperature influenced more importantly DOD production rather than cell growth.

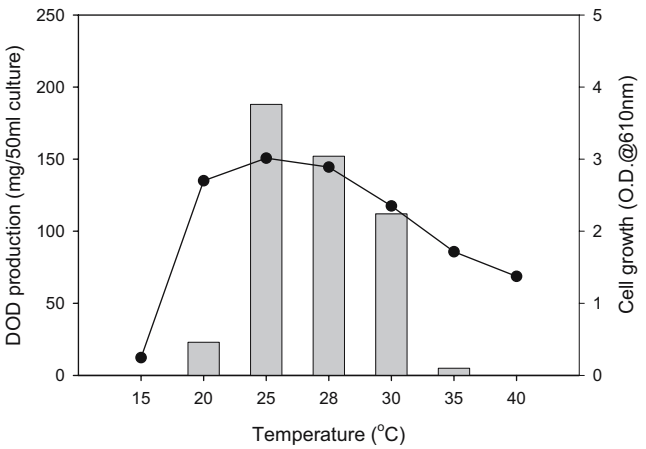

Fig. 2 Effect of incubation temperature on DOD production from triolein by PR3. *Bar* and *line graphs* represented DOD production and cell growth before substrate addition, respectively. All reaction conditions were the same as the standard condition explained in "Materials and methods"

Effect of inoculum size on DOD production

At a given time period, microbial inoculum size of a culture becomes an important factor for microbial growth in terms of cell mass production and status of the growth curve. Upon this fact, inoculum size might give effects on DOD production by PR3 because the substrate was added to the culture after cell growth continued previously for 24 h. When the inoculum size increased from 0.5 to 5% of the culture volume, DOD production increased and peaked at 1.0% and then decreased up to 35% of maximum value at 5.0% (Fig. 3). Cell growth showed similar pattern with DOD production. Hence, 1.0% of inoculum size was determined as the optimal condition for DOD production by PR3.

Effect of time of substrate addition on DOD production

As the optimum inoculum size of the culture for DOD production by PR3 was selected to be 1%, we determined the effect of time of substrate addition on DOD production because total cell mass and cell viability changing time-dependently according to the inoculum size could influence the condition for DOD production. As shown in Fig. 4, cell growth reached stationary phase after 24 h incubation. However, DOD production was maximal when the substrate was added at between 12 and 24 h after microbial cultivation started. This time period corresponded to the late exponential phase of microbial growth. When the substrate was added beyond the 36 h cultivation, DOD

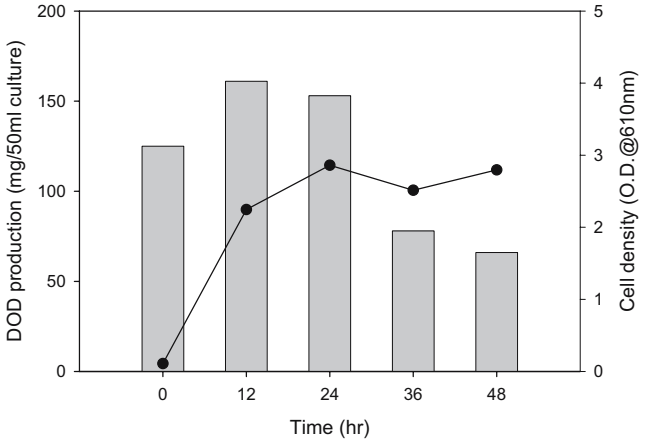

Fig. 4 Effect of time of substrate addition to the culture on DOD production from triolein by PR3. Substrate was added to the culture after the culture was incubated for different time. *Bar* and *line graphs* represented DOD production and cell growth before substrate addition, respectively. All reaction conditions were the same as the standard condition explained in Materials and methods

production dropped significantly below 50% of the maximum value.

Effect of substrate concentration on DOD production

Time-coursed DOD production from oleic acid and triolein by PR3 represented a similar peaked profile indicating that the produced DOD was further consumed by bacterial cells (Hou et al. 1991; Chang et al. 2007). This prompted us to address the effect of substrate concentration on DOD production from triolein by PR3. As the substrate concentration changed in the range of between 100 and 1,000 mg, the amount of total lipid extracted from the culture

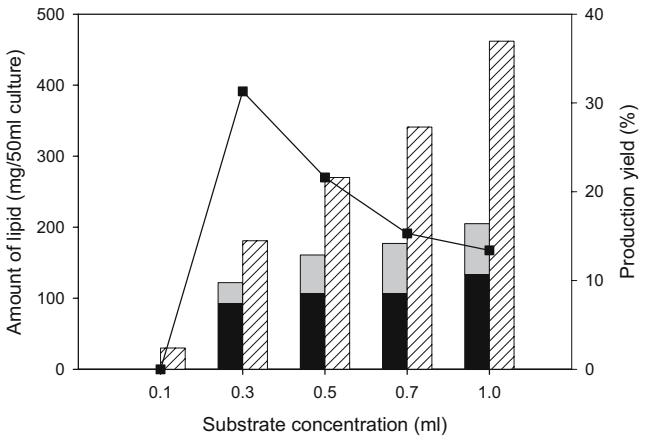

Fig. 5 Effect of substrate concentration on DOD production from triolein by PR3. Different amounts of substrate were added to the 24-h-old culture. *Black*, *gray*, and *striped bars* represented DOD production, residual oleic acid, and the amount of total lipid, respectively. *Line graph* represented the calculated production yield of DOD over triolein. All reaction conditions were the same as the standard condition explained in "Materials and methods"

Table 1 Effect of metal ions on DOD production from triolein by PR3

Metal ions	Cell growth (optical density at 610 nm)[b]	Total DOD production (mg/50 ml culture)
$FeSO_4$	0.65	4.0
$ZnSO_4$	0.74	18.0
$CuSO_4$	0.39	n.d.[c]
$MgSO_4$	2.35	65.0
$MnSO_4$	0.18	n.d.[c]
$CaCl_2$	0.10	n.d.[c]
NaCl	0.81	n.d.[c]
Control[a]	3.29	94.0

[a] Control represented the standard medium.
[b] Final optical density values were obtained by calculation from the original values of the diluted samples.
[c] Not detected

increased proportionally to the concentration (Fig. 5). However, DOD production increased up to 300 mg of substrate and then saturated gradually thereafter. DOD production with at 300 mg was about 76% of the maximum value at 1,000 mg. The amount of residual oleic acid increased according to the substrate concentration up to 700 mg and then saturated thereafter. As a consequence, the calculated production yield of DOD over triolein was peaked at 300 mg (31.3%) and decreased significantly thereafter indicating that there was a certain limit of substrate concentration for efficient DOD production from triolein by PR3.

Effect of metal ions on DOD production

In general, metal ions play important role as cofactors in enzyme reaction and cell growth as well. The effects of several metal ions were studied on the production of DOD

Table 2 Effect of combined metal ions on DOD production from triolein by PR3

Metal ions	Cell growth (optical density at 610 nm)[b]	Total DOD production (mg/50 ml culture)
Fe+Mn+Mg[a]	3.42	126.0
Fe	1.02	n.d.[c]
Mn	1.11	n.d.[c]
Mg	3.15	89.0
Fe+Mg	3.23	122.0
Fe+Mn	1.23	n.d.[c]
Mn+Mg	3.29	124.0

[a] This combination represented the control medium. Concentrations of all the individual metal ions were the same as those in the standard control medium.
[b] Final optical density values were obtained by calculation from the original values of the diluted samples.
[c] Not detected

by PR3. As shown in Table 1, DOD production and cell growth were significantly influenced by the kind of metal ions tested. All the metal ions were used as the sole metal ion source in the same amount used in the original control medium. Among eight metal ions tested, magnesium ion was most effective for DOD production and cell growth followed by zinc and iron ions. Cell growth with sodium ion was similar to those with iron and zinc ions, but DOD production was not detected. However, all the metal ions tested produced less amount of DOD than the control medium. DOD production with magnesium was about 70% of the control medium. When each metal ion was combined individually, all the cases with magnesium showed a similar higher level of DOD production and cell growth than the single usage of magnesium ion representing about 40% higher DOD productivity (Table 2). Without magnesium, cell growth decreased significantly, and DOD production was not detected. These results indicated that magnesium was essential for cell growth and DOD production from triolein by PR3.

Discussion

Optimization of environmental conditions for the microbial bioconversion processes in laboratory scale is important to achieve necessary scientific information for further scaled-up production of the target product in the large-scale fermenter. In this study, we obtained the optimized environmental conditions for DOD production from triolein by *P. aeruginosa* PR3.

Optimal medium pH value of 8.0 for DOD production from triolein by PR3 was quite different from those for the productions of other HFAs from different fatty acid substrates using the same strain. Optimal pH value for DOD production from oleic acid and TOD production from ricinoleic acid by PR3 were 7.0 when the medium pHs were adjusted before substrate addition to the cultures (Hou et al. 1991; Kuo et al. 2001). In the case of DOD production from triolein, pH was adjusted initially at the time of microbial inoculation. However, it was assumed that the optimal pH values for the conversion reaction of DOD production for all the cases mentioned above could be similar because pH of the culture of the latter case became acidic after 24 h incubation by 0.6 pH units, resulting in that the actual pH value of the culture at the time of substrate addition dropped to 7.4. In the industrial microbial bioprocesses, simplification of steps is important because it can protect the reaction culture from any possible microbial contamination during the steps and also can lower production cost. In this point of view, results from this study suggested that additional pH adjustment of the culture before substrate addition was not necessary. Instead, one-time adjustment of the initial medium pH with alkaline condition was sufficient in this process.

In general, high substrate concentration accelerates reaction rate in the simple enzyme reaction process. However, the concentration of substrate for microbial bioconversion can influence the overall process differently because, in a certain case, especially when fatty acid was used as the substrate, the high concentration of substrate can carry toxic effects on the microorganism. DOD production from triolein by PR3 was saturated above 300 mg of triolein. This is in good agreement with DOD production from oleic acid by the same strain (Hou et al. 1991). This result could be explained by the fact that DOD production from triolein was initiated from the release of oleic acid from triolein via the triolein-induced lipase activity (Chang et al. 2007). However, we can assume that the released oleic acid was used as an actual substrate for DOD formation, and at the same time, it could possibly act as toxic factor against microbial growth resulting in delayed or saturated DOD production.

Requirement of metal ions for DOD production from triolein by PR3 was different from that for THOD production from linoleic acid by the same strain. Iron or copper ion was essential for THOD production from linoleic acid, while magnesium was essentially required for DOD production from triolein. It has been reported that the catalytic amount of iron ion was required for the induction of lipid peroxidation catalyzed by lipoxygenase (Gardner et al. 1976). Lipid peroxidation of linoleic acid is essential intermediate step for THOD production from linoleic acid. These results suggested that different enzyme system, other than lipoxygenase, could possibly be involved in the formation of DOD from triolein.

In conclusion, the overall environmental conditions for DOD production from triolein by PR3 were optimized based on the results from this study and from our previous report. Results from our studies demonstrated that DOD could be efficiently produced by PR3 from natural vegetable oils without being intentionally hydrolyzed. Further work should be done to develop the large-scaled system for DOD production from natural vegetable oils by PR3.

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