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ORIGINAL PAPER

Microbial Conversion of Vegetable Oil to Rare Unsaturated Fatty Acids and Fatty Alcohols by an *Aeromonas hydrophila* Isolate

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Abstract We isolated two new microorganisms capable of converting vegetable oil to several rare unsaturated fatty acids and rare unsaturated fatty alcohols from a soil sample. The strains were identified as belonging to the same genus and species, *Aeromonas hydrophila*. The rare unsaturated fatty acids and rare unsaturated fatty alcohols were accumulated as a wax ester form by the strains. Compared to other strains, the *A. hydrophila* isolates effectively decreased fatty acid chain lengths and converted rapeseed oil, which is rich in 9-C18:1 fatty acid, into rare fatty acids, such as 7-C16:1 fatty acid and 5-C14:1 fatty acid. Furthermore, the *A. hydrophila* isolates converted the resulting fatty acids to rare unsaturated fatty alcohols, such as 7-C16:1 fatty alcohol and 5-C14:1 fatty alcohol. The isolates also converted safflower oil, which is rich in 9,12-C18:2 fatty acid, to 7,10-C16:2 fatty acid, 5,8-C14:2 fatty acid, 9,12-C18:2 fatty alcohol, 7,10-C16:2 fatty alcohol, and 5,8-C14:2 fatty alcohol. 7,10,13-C16:3 fatty acid, 9,12,15-C18:3 fatty alcohol, and 7,10,13-C16:3 fatty alcohol were also converted from linseed oil, which is rich in 9,12,15-C18:3 fatty acid, by the *A. hydrophila* isolates. These fatty acids and fatty alcohols are rarely found in natural oils. Since decreasing fatty acid carbon chain lengths from the carboxyl end and reducing unsaturated fatty acids to unsaturated fatty alcohols are both difficult reactions to accomplish by chemical means, we suggest that these *A. hydrophila* isolates may facilitate introduction of new bioprocess for producing rare unsaturated fatty acids and rare unsaturated fatty alcohols, especially fatty alcohols harboring more than two double bonds.

Keywords Microbial conversion · Vegetable oil · Wax ester · Unsaturated fatty acid · Unsaturated fatty alcohol · *Aeromonas hydrophila*

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Introduction

Polyunsaturated fatty acids (PUFA) are known to have various important physiological effects. However, natural sources of PUFA are limited. Therefore, considerable resources have been directed toward finding microorganisms capable of producing PUFA, such as arachidonic acid [1], γ-linolenic acid [2], eicosapentaenoic acid [3], dihomo-γ-linolenic acid [4], and docosahexaenoic acid [5–7]. In addition, several studies have discovered microorganisms capable of producing conjugated linoleic acids [8, 9] and unusual fatty acids [10]. These studies indicate that microbial-mediated processes can effectively produce rare and functional fatty acids. However, the types of fatty acids produced by these microorganisms are limited. Therefore, discovery of new microorganisms capable of producing other rare fatty acids is desired.

Many kinds of fatty alcohols are produced industrially by chemical means for use as surfactants. These compounds, however, are limited to several saturated fatty alcohols and oleyl alcohol. This limitation is imposed by the breakdown of the double bonds in the fatty acids under severe conditions necessary for chemical processing to occur (240–330 °C, 20–70 MPa). Therefore, a new method

capable of effectively reducing unsaturated fatty acids to unsaturated fatty alcohols is required.

In this study, we screened microorganisms for their ability to transform vegetable oil to new oil compounds. Among the microorganisms screened, two microorganisms, which belonged to the same genus and species, were found to produce wax esters composed of several rare unsaturated fatty acids and rare unsaturated fatty alcohols. We expect that the isolated microorganisms will be useful for the development of new methods for generating rare fatty acids and rare fatty alcohols, especially unsaturated fatty alcohols harboring more than two double bonds.

Experimental Procedures

Materials

Rapeseed, safflower, and linseed oils were purchased from Nisshin OilliO Group (Tokyo, Japan). The fatty acid composition of these oils is given in Table 1. *n*-Tricaproine was obtained from Tokyo Chemical Industry (Tokyo, Japan). Polypepton and extract bonito were purchased from Wako Pure Chemical Industry (Osaka, Japan), and gall powder was obtained from Nacarai tesque (Kyoto, Japan). All other regents were of analytical grade.

Screening of Microorganisms

The initial screening of microorganisms was conducted using a solid medium consisting of 1% rapeseed oil, 1% gall (bile) powder, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4{\cdot}7H_2O$, and 2% agar (pH 7.0) [11]. The supernatant of a suspension consisting of 5 mL sterilized water and 0.5 g soil was spread on the surface of culture plates. Colonies giving rise to widespread clearing zones following incubation at 28 °C for 3 days were selected for a second round of screening. Each of the isolates was subsequently cultured in 2 mL nutrient broth (NB) liquid medium (1% extract bonito, 2% polypepton, and 0.1% NaCl, pH 7.0) with reciprocal shaking (400 rpm) at 28 °C for 1 day. We added 100 mg rapeseed oil to the culture broth and continued the cultivation for 3 days. Cellular and extracellular oil materials were then extracted using 2 mL chloroform/methanol (2:1, vol/vol), and the organic solvent phase was recovered by centrifugation (8,000×*g*, 10 min). Each organic phase obtained was applied to a silica gel 60 plate (Merck, Darmstadt, Germany), which was then developed using *n*-hexane/ethyl acetate/acetic acid (90:10:1, vol/vol/vol).

Table 1 The fatty acid and fatty alcohol composition of the wax esters produced by strains Nos. 6 and 308

	Rapeseed					Safflower			Linseed		
	Strain no. 308			Strain no. 6		Strain no. 6			Strain no. 6		
	Original[a]	Fatty acid[b]	Fatty alcohol[c]	Fatty acid	Fatty alcohol	Original	Fatty acid	Fatty alcohol	Original	Fatty acid	Fatty alcohol
Composition (wt%)											
C12:1	nd	nd	nd	0.9	nd	nd	0.6	nd	nd	0.5	nd
C14:0	nd	nd	0.8	1.6	0.8	nd	1.9	1.5	nd	1.8	1.2
C14:1	nd	nd	2.3	8.0	4.6	nd	1.3	1.4	nd	1.6	1.8
C14:2	nd	nd	0.6	2.0	0.6	nd	4.8	2.6	nd	0.9	0.5
C14:3	nd	nd	nd	nd	nd	nd	nd	nd	nd	1.0	1.2
C16:0	4.4	4.2	6.4	2.3	3.3	6.7	2.0	3.9	5.7	2.3	4.5
C16:1	0.2	11.1	15.5	28.1	32.4	nd	5.1	6.9	nd	7.5	11.3
C16:2	nd	2.4	2.2	9.6	8.1	nd	27.0	29.9	nd	5.8	7.1
C16:3	nd	0.2	nd	2.9	1.6	nd	1.5	nd	nd	11.7	13.1
C18:0	2.0	1.2	1.9	nd	0.6	2.6	0.2	2.1	3.6	0.4	0.7
C18:1	62.4	55.1	50.0	23.8	31.5	13.7	4.2	5.7	20.5	7.3	10.8
C18:2	20.0	18.6	14.7	15.1	12.1	76.7	49.6	43.0	15.8	12.5	10.8
C18:3	8.2	4.2	2.9	6.7	4.5	nd	1.2	nd	54.5	46.4	36.5

nd Not detected (<0.5 wt%)

[a] Fatty acid composition of vegetable oil

[b] Fatty acid percentage in total fatty acids obtained from the wax esters

[c] Fatty alcohol percentage in total fatty alcohols obtained from the wax esters

Determination of Productivity of Wax Esters

The isolated microorganisms were inoculated into 5 mL NB medium, and then incubated with reciprocal shaking (400 rpm) at 28 °C for 1 day. Then 250 mg rapeseed oil was added to the culture broth and the cultivation was continued for 3 days. The culture broth was then mixed vigorously by hand (30 s) with 10 mL chloroform/methanol (2:1, vol/vol) and 0.1 mL of 10 M HCl. After centrifugation (8,000×*g*, 10 min), the organic solvent phase was recovered. The remaining aqueous phase was extracted twice with 20 mL *n*-hexane. All the organic phases were combined for analysis. Cellular and extracellular oil materials were recovered by evaporating the organic solvents, and the concentration of wax esters was measured by gas chromatography (GC).

Determination of Fatty Acid and Fatty Alcohol Composition of the Wax Esters

The wax esters were first purified by silica gel chromatography. The oil materials extracted from the 5 mL culture broth were dissolved in 2 mL *n*-hexane and applied to a silica gel column (5 g, Merck) equilibrated with *n*-hexane/ethyl acetate (98:2, vol/vol). The wax esters were eluted with the same solvents, and the organic solvents in the wax ester fractions were removed using an evaporator. Then the free fatty acids (FFAs) and fatty alcohols were fractionated by chemical hydrolysis of the wax esters, which was followed by an extraction with *n*-hexane. The hydrolysis was conducted as follows: The wax esters (10 mg) were dissolved in 1.5 mL ethanol solution containing 0.1 mL water and 12 mg NaOH. The mixture was heated at 65 °C for 10 min with occasional shaking. Subsequent to hydrolysis, 6 mL water was added, and the fatty alcohols were extracted twice with 10 mL *n*-hexane. This was followed by an evaporation step. The aqueous phase from the extraction was acidified to pH 2 with 1.0 M HCl. The FFAs were then extracted twice with 10 mL *n*-hexane followed by evaporation. Finally, the fatty acid and fatty alcohol composition was determined by GC analysis.

GC Analyses

The wax ester content in the oil materials extracted from the culture broth was analyzed with a GC-18A gas chromatograph (Shimadzu, Kyoto, Japan) connected to a DB-1ht capillary column (0.25 mm × 5 m; Agilent Technologies, Palo Alto, CA) using *n*-tricaproine as an internal standard. The column temperature was raised from 120 to 280 °C at 15 °C/min and from 280 to 370 °C at 10 °C/min. This final temperature was maintained for 1 min. The injector and detector (FID) temperatures were set at 370 and 390 °C, respectively.

The chemical conversion of the FFAs to fatty acid methyl esters (FAMEs) was performed at 75 °C for 10 min in 3 mL methanol solution containing 3.9% BF_3. FAMEs and fatty alcohols were analyzed with a gas chromatograph (6890N, Agilent Technologies) connected to a DB-23 capillary column (0.25 mm × 30 m, Agilent Technologies). The column temperature was maintained at 150 °C for 0.5 min, increased to 170 °C at 4 °C/min, then raised to 195 °C at 5 °C/min, and further increased to 215 °C at 10 °C/min. The temperature was maintained at 215 °C for 11 min. The injector and FID temperatures were 245 and 250 °C, respectively.

Preparation of Picolinyl Ester Derivatives from Fatty Acids and Fatty Alcohols

The FFAs (25 mg) were converted to fatty acid chlorides by a reaction with 0.1 mL thionyl chloride and 0.05 mL *N*,*N*-dimethylformamide at 25 °C for 10 min. After the excess reagents were removed by evaporation, 0.072 mL pyridine and 0.02 mL 3-pyridinemethanol were added to the fatty acid chlorides, and the mixture was stored at 25 °C for 10 min. Then 2 mL water was added and the resulting picolinyl esters produced by the fatty acids were extracted with 3 mL *n*-hexane/ethyl acetate (1:2, vol/vol).

The fatty alcohols (50 mg) were dissolved in 2 mL toluene containing 33 mg 3-pyridylacetic acid hydrochloride, 59 mg *N*,*N*′-dicyclohexylcarbodiimide, and 58 mg 4-dimethylaminopyridine. The reaction mixture was kept at 30 °C for 18 h with stirring at 500 rpm. Then 9 mL toluene and 5 mL 0.5 M HCl were added to the mixture. The toluene layer containing the resulting picolinyl esters produced by the fatty alcohols was washed again with 5 mL of 0.5 M HCl.

GC–Mass Spectrometry (GC–MS) Analysis

A GC–MS QP2010 with a GC-2010 (Shimadzu) was used for mass spectral analyses. The GC separation of the wax and picolinyl esters was performed on a DB-5 capillary column (0.25 mm × 10 m, Agilent Technologies). To analyze the wax esters, the column temperature was raised from 230 to 325 °C at 5 °C/min, and then maintained at 325 °C for 10 min. To analyze picolinyl esters, the column temperature was raised from 230 to 280 °C at 2 °C/min, and then maintained at 280 °C for 1 min. For both analyses, the injector temperature was set at 280 °C, and MS was performed in the electron impact mode at 70 eV with a source temperature of 200 °C.

Results

Screening of Microorganisms Capable of Converting Rapeseed Oil to New Oil Materials

We used 60 soil samples for initial screening. Because the solid medium used to grow microorganisms contained rapeseed oil, microorganisms able to metabolize triacylglycerol (TAG) grew on the culture plates. Five hundred colonies were then selected for a second round of screening. By the thin layer chromatography analysis of the cellular and extracellular oil materials, we found that 11 isolates produced unknown compounds, which were more hydrophobic than TAG (data not shown). We determined that these 11 isolates were bacteria, and they were named nos. 6, 15, 144, 115, 212, 308, 310, 325, 340, 476-1, and 476-2.

GC–MS Identification of the Novel Compounds

Figure 1a shows GC analysis of the cellular and extracellular oil materials extracted from strain no. 6. At least four unknown peaks (peaks 1–4) were detected. The cellular and extracellular oil materials also contained FFAs, diacylglycerols (DAGs), and TAGs. These new compounds (peaks 1–4) were chemically hydrolyzed following their purification by silica gel chromatography. By this analysis, we detected compounds that we believed to be fatty acids and fatty alcohols. Therefore, we hypothesized that the novel substances were wax esters. This idea was confirmed by GC–MS analysis. Figure 1b shows a representative GC–MS spectrum of peak 3. In general, the wax ester fragment ions (RCO_2R'; R and R' indicated the alkyl chains in the fatty acid and fatty alcohol, respectively) were observed at m/z $[RCO_2H_2]^+$, $[RCO]^+$, $[RCO\text{-}1]^+$, and $[R'\text{-}1]^+$ [12]. The m/z 236, 237, and 255 fragments were presumed to be the derivatives of C16:1 fatty acid, while the m/z 250 fragment was believed to be a derivative of C18:1 fatty alcohol. These were all clearly detected in the GC–MS profile. A major molecular ion ($[M]^+$, m/z 504) coincided with the molecular weight of a wax ester composed of C16:1 fatty acid and C18:1 fatty alcohol. These results indicated that peak 3 shown in Fig. 1a was a wax ester composed mainly of C16:1 fatty acid and C18:1 fatty alcohol (with a total carbon number of 34). Several minor fragments and molecular ions believed to be other minor wax esters also possessing a total carbon number of 34 were present in Fig. 1b. The other unknown peaks (peaks 1, 2, and 4) in Fig. 1a were also identified as wax esters [with total carbon numbers of 30 (peak 1), 32 (peak 2), and 36 (peak 4)] by GC–MS in a similar manner (data not shown).

Productivity of Wax Esters and the Composition of Fatty Acids and Fatty Alcohols

The cellular and extracellular oil materials contained not only wax esters but also FFAs, DAGs, and TAGs. The fatty alcohols were not detected in a free form. Thus, the wax esters from strain no. 6 were purified by silica gel chromatography and analyzed by GC to determine the concentration of wax esters relative to an internal standard (*n*-tricaproine). The

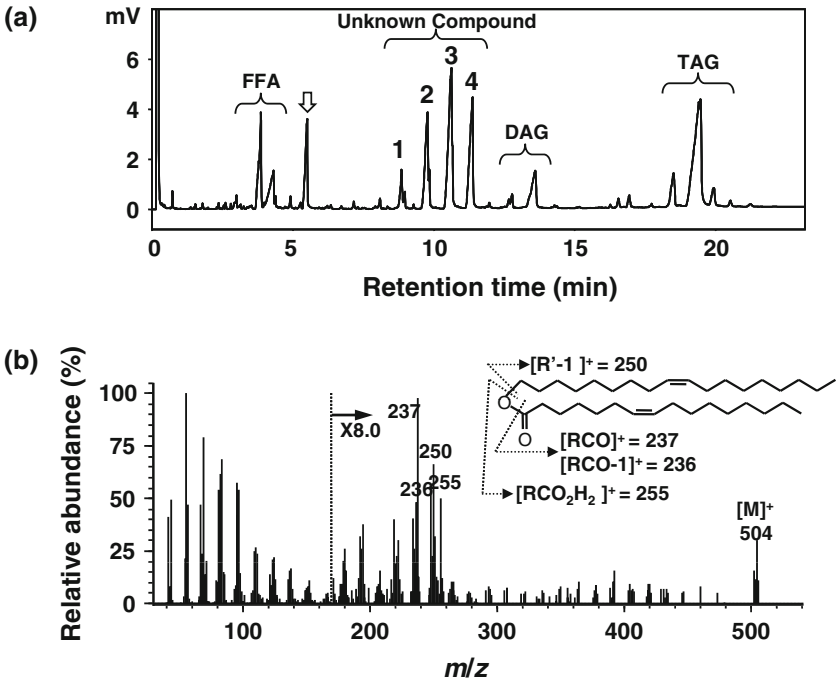

Fig. 1 **a** A GC chromatogram of the oil materials extracted from the no. 6 strain. The *open arrow* indicates the internal standard *n*-tricaproine. **b** GC–MS spectra of an unknown compound, designated as peak 3, produced by the no. 6 strain. *FFA* Free fatty acid, *DAG* diacylglycerol, *TAG* triacylglycerol

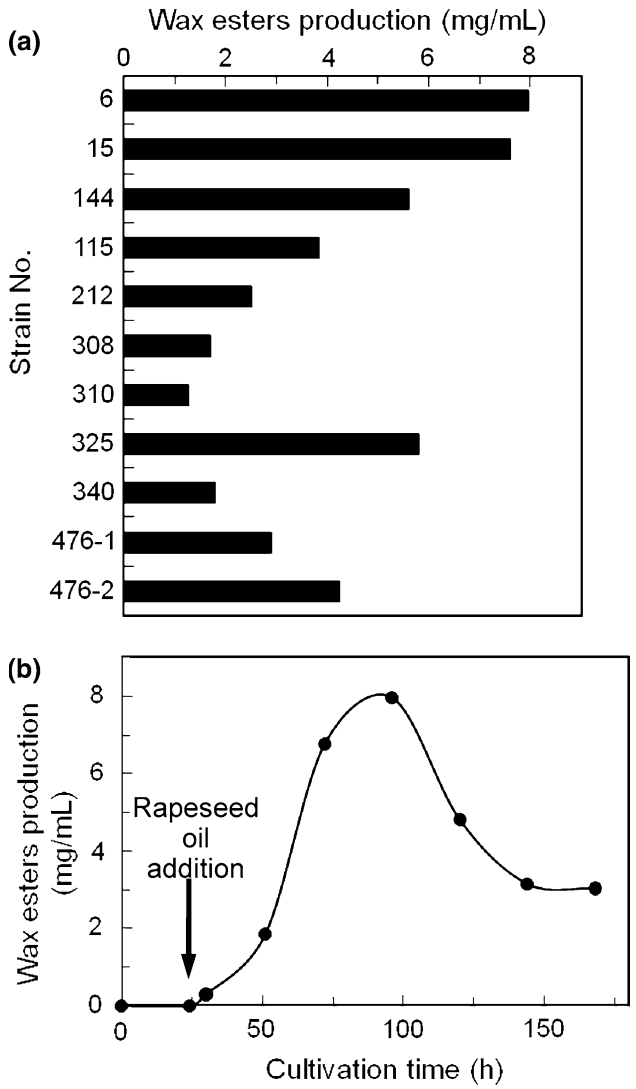

Fig. 2 **a** The wax ester production of the 11 isolated strains. After the oil materials were extracted from 5 mL cultures of each strain, the amount of the wax ester was calculated from GC analysis using an internal standard, *n*-tricaproine. The wax ester production is expressed in terms of milligrams per culture volume (mL). **b** The time course of the wax esters production by strain no. 6. After the strain was inoculated into 5 mL NB medium, rapeseed oil was added to the culture broth at 24 h (indicated by *arrow*)

obtained relativity factor at GC analysis using FID detector was 1:0.67 (*n*-tricaproine to wax esters). This value was used to determine the wax ester productivity by GC analysis of cellular and extracellular oil materials (Fig. 2a). By this means, we determined that the 11 strains produced 1.3–7.9 mg wax esters/mL culture; the greatest concentration was achieved by strain no. 6. Figure 2b shows the time course of the wax ester production by strain no. 6. The maximum productivity was attained at 4 days (the cultivation times included preculture). Since 250 mg rapeseed oil was added to 5 mL culture, the yield of the wax ester produced by strain no. 6 was 16% of the initial weight of the starting material.

The wax esters produced by the 11 strains were purified, and esters were chemically hydrolyzed followed by fractionation into FFAs and fatty alcohols. Figure 3 shows the fatty acid and fatty alcohol composition of the wax esters obtained. The results are expressed in terms of carbon chain length (e.g., total content of C18:0, C18:1, C18:2, and C18:3). Compared to rapeseed oil, all the strains decreased the content of C18 fatty acids and fatty alcohols, and increased the content of the C16 fatty acids and fatty alcohols. Strain nos. 6 and 15 produced exceptional amounts of C16 fatty acids and fatty alcohols. Table 1 shows the composition of fatty acids and fatty alcohols in the wax esters produced by strain no. 6 in detail. The values are expressed in terms of fatty acid percentage in total fatty acids obtained from the wax esters or fatty alcohol percentage in total fatty alcohols obtained from the wax esters, but not percentages of fatty acid or fatty alcohol in total lipids obtained.

More importantly, rare fatty acids and rare fatty alcohols were also detected. These rare species included 28.1 wt% C16:1 fatty acid, 9.6 wt% C16:2 fatty acid, 8.0 wt% C14:1 fatty acid, 12.1 wt% C18:2 fatty alcohol, 32.4 wt% C16:1 fatty alcohol, 8.1 wt% C16:2 fatty alcohol, and 4.6 wt% C14:1 fatty alcohol. In contrast to these results, the other nine strains, nos. 144 to 476-2, produced lower amounts of C16:1 fatty acid, C16:2 fatty acid, C16:1 fatty alcohols, and so on (Table 1 shows the results of strain no. 308). These findings indicated that strain nos. 6 and 15 produced a number of unique unsaturated fatty acids and fatty alcohols. Due to the facts that the degree of wax ester production (Fig. 2), the composition of the fatty acids and fatty alcohols produced (Fig. 3), and the morphological characteristics of strains no. 6 and 15 were similar, only strain no. 6 was selected for further investigation.

To determine whether the wax esters were produced intracellularly or extracellularly, the cells and culture supernatant were separated by centrifugation. The wax esters were only detected in the cell fraction. Furthermore, when the purified wax esters were mixed with the medium, the wax esters did not precipitate by centrifugation. These results indicating that they accumulated inside the cells.

To check whether wax esters and fatty alcohols were spontaneously generated by reciprocal shaking with NB medium, or by the subsequent extraction method, NB medium containing rapeseed oil without the strain was shaken at 28 °C for 3 days. After the incubation, the oil materials were extracted from the medium, but the wax esters and fatty alcohols were not generated. Next, NB medium containing oleic acid/oleyl alcohol mixture (1:1, wt/wt) without the strain was shaken at 28 °C for 3 days. The oil materials were extracted from the medium, but the wax esters were not generated. Furthermore, the ratio of oleic acid and oleyl alcohol was not changed. Thus, wax esters and fatty alcohols were not spontaneously generated

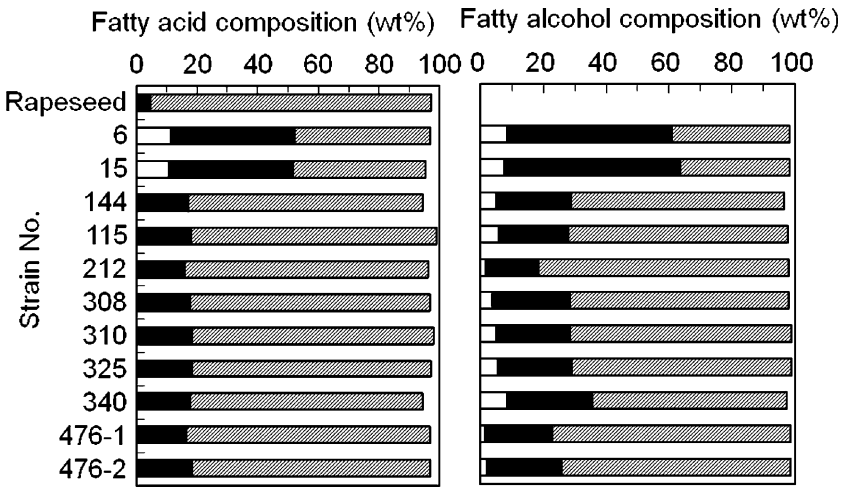

Fig. 3 The fatty acid and fatty alcohol composition of the wax esters produced by the 11 strains. The *diagonal box* represents the total contents of C18:0, C18:1, C18:2, and C18:3. The *closed box* represents the total contents of C16:0, C16:1, C16:2, and C16:3. The *open box* indicates the total contents of C14:0, C14:1, C14:2, and C14:3

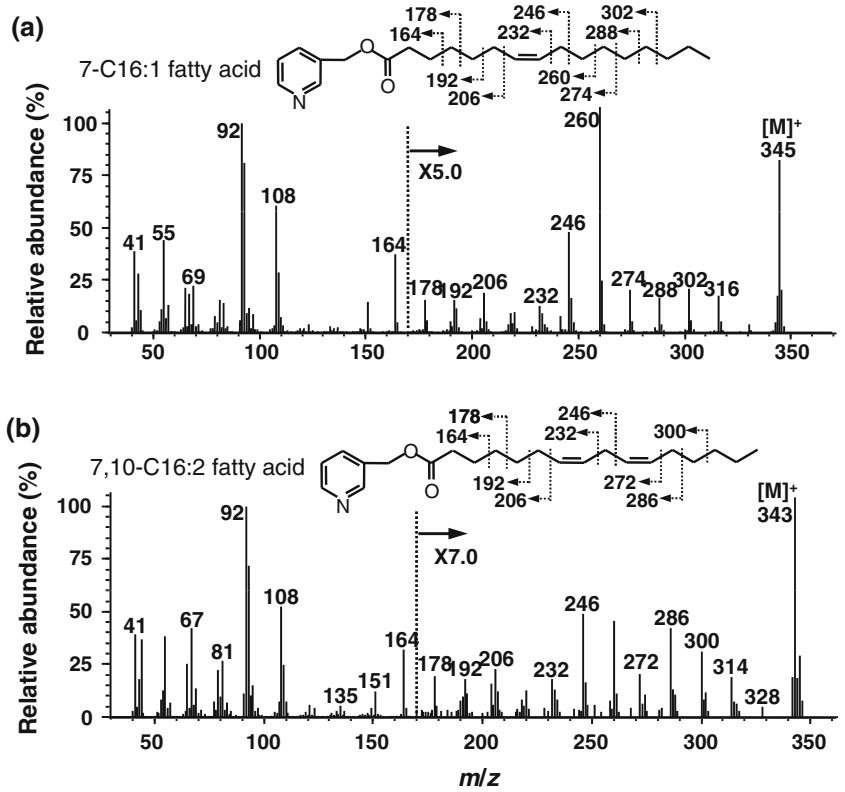

Fig. 4 GC–MS spectra of the picolinyl ester derivatives of the C16:1 and C16:2 fatty acids produced by strain no. 6

by the reciprocal shaking with NB medium without the strain, or the subsequent extraction method.

GC–MS Analysis for Identification of the Fatty Acid and Fatty Alcohol Structure

The fatty acid composition (Table 1) was estimated using a standard FAME mixture (Supelco 37 Component FAME Mix, Supelco, Bellefonte, PA). However, FAME standards for C16:2, C16:3, C14:2, and standard fatty alcohols [except for oleyl alcohol (C18:1) and saturated fatty alcohols] were not commercially available. Furthermore, we were unable to determine the position of the double bonds in the structures using only GC analysis. Therefore, we examined the structures of the fatty acids and fatty alcohols by conducting GC–MS analysis of their picolinyl ester derivatives [13, 14].

The picolinyl ester derivatives of the C16:1 fatty acids had a molecular weight of 345 (Fig. 4a), which corresponded with the molecular mass of C16 fatty acid containing one double bond. An interval of 26 atomic mass units, caused by

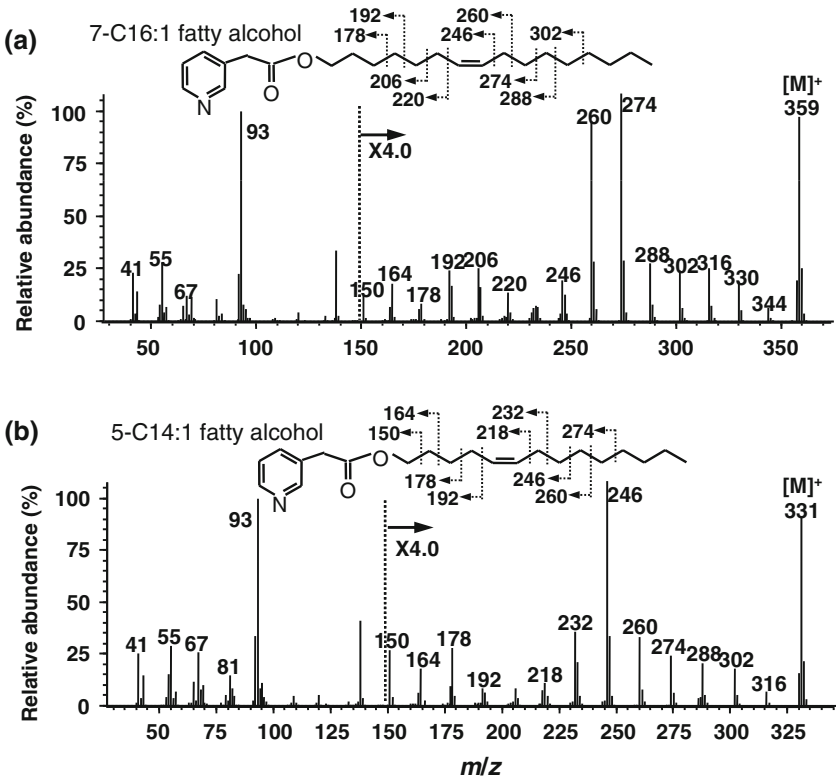

Fig. 5 GC–MS spectra of the picolinyl ester derivatives of the C16:1 and C14:1 fatty alcohols produced by strain no. 6

a double bond, was found between *m/z* 206 and *m/z* 232 fragments, indicating the possible presence of one double bond at the Δ7 position. Thus, the C16:1 fatty acid was identified as 7-hexadecenoic acid (7-C16:1). In a similar manner, the C14:1 and C16:2 fatty acids were identified as 5-tetradecenoic acid (5-C14:1, data not shown) and 7,10-hexadecadienoic acid (7,10-C16:2, Fig. 4b), respectively.

The *cis* or *trans* configurations of these double bonds could not be precisely defined in this study. However, the retention times of 7-C16:1 and 5-C14:1 fatty acids during our GC analyses on the DB-23 column were identical to *cis*-9-C16:1 and *cis*-9-C14:1 obtained from the standard FAME mixture. Because the DB-23 column can separate *cis* and *trans* fatty acids, the double bonds for the 7-C16:1 and 5-C14:1 fatty acids could provisionally be assigned a *cis* configuration. Further analyses, however, will be necessary to determine their precise configurations.

The structures of the fatty alcohols, whose double-bond positions were not assigned using only GC analysis, were also determined by conducting a GC–MS analysis of their picolinyl ester derivatives. We clearly detected *m/z* 220 and *m/z* 246 fragments, and thus, the C16:1 fatty alcohol was identified as 7-hexadecenol (7-C16:1; Fig. 5a). In a similar manner, C14:1 fatty alcohol was identified as 5-tetradecenol (5-C14:1; Fig. 5b).

Fatty Acid and Fatty Alcohol Composition of Wax Esters Produced from Other Vegetable Oils

We observed that strain no. 6 was useful for the microbial conversion of vegetable oil to rare unsaturated fatty acids and fatty alcohols. Therefore, we were curious to discover other rare fatty acids and fatty alcohols that the strain could generate using safflower and linseed oils, which are rich in C18:2 and C18:3 fatty acids, respectively (Table 1). Strain no. 6 produced 27.0 wt% C16:2 fatty acid, 4.8 wt% C14:2 fatty acid, 43.0 wt% C18:2 fatty alcohol, 29.9 wt% C16:2 fatty alcohol, and 2.6 wt% C14:2 fatty alcohol from safflower oil. Furthermore, 11.7 wt% C16:3 fatty acid, 36.5 wt% C18:3 fatty alcohol, and 13.1 wt% C16:3 fatty alcohol were detected in the mixture of wax esters produced when the strain metabolized linseed oil. These fatty acids and fatty alcohols are also rare compounds. Our subsequent GC–MS analysis determined that the C14:2 and C16:3 fatty acids were 5,8-tetradecadienoic acid and 7,10,13-hexadecatrienoic acid, respectively. We also used GC–MS to determine that the C18:2, C16:2, C14:2, C18:3, and C16:3 fatty alcohols were 9,12-octadecadienol, 7,10-hexadecadienol, 5,8-tetradecadienol, 9,12,15-octadecatrienol, and 7,10,13-hexadecatrienol, respectively (data not shown).

Strain Identification

Strain no. 6 was determined to be a mobile aerobic Gram-negative rod (0.6–0.7 × 1.2–1.5 μm) and was also observed to score positive for both oxidase and catalase activities. From these characteristics and the 16S rDNA nucleotide sequence obtained (data not shown), strain no. 6 was assigned to *Aeromonas hydrophila* by TechnoSuruga Laboratory (Shizuoka, Japan). Therefore, we named strain no. 6 *A. hydrophila* N-6. Strain no. 15, which differed from the strain no. 6 by only two base pairs (bps) of 16S rDNA nucleotide sequence, was assigned to the same genus and species.

The other nine strains, nos. 144 to 476-2, produced wax esters from vegetable oil. However, the carbon chain lengths of the fatty acids and fatty alcohols were not shortened to the same degree as was observed using strain nos. 6 and 15 (Fig. 3, Table 1). Among these other nine strains, we selected four strains (nos. 144, 308, 310, and 340) and determined that all four strains belonged to the genus *Acinetobacter*.

Discussion

Hypothesized Pathway Used for the Microbial Conversion of Vegetable Oil to Wax Esters

Several strains in the genus *Acinetobacter* are known to metabolize long chain *n*-alkanes. These strains accumulate wax esters from *n*-alkane as storage material [15, 16], and the pathway for the production of wax esters by these strains has already been clarified [17–19]. We thus presumed that the pathway for the production of wax esters from vegetable oils by *A. hydrophila* N-6 (Fig. 6) would be similar. Further analysis, of course, will be required to confirm this hypothesis.

In brief, we suggest that after TAG was hydrolyzed, the resulting FFA was converted to acyl-CoA by an acyl-CoA synthetase. The acyl-CoA was introduced into the β-oxidation pathway, and two methyl groups (–CH_2–CH_2–) from the carboxyl end were metabolized by the four enzymes involved in β-oxidation pathway. In general, all methyl groups are metabolized once FFA is introduced into the β-oxidation pathway. However, *A. hydrophila* N-6 accumulated acyl-CoA corresponding to the rare fatty acids (e.g., 7-C16:1 and 5-C14:1) when rapeseed oil was used as the substrate. This implied that the β-oxidation pathway was stopped after two or four methyl groups (i.e., two or four carbon chain lengths) were metabolized from the carboxyl end. The accumulated acyl-CoA was continuously reduced by an acyl-CoA reductase to its corresponding fatty aldehyde. This fatty aldehyde was then further reduced to its corresponding fatty alcohol by a fatty aldehyde reductase. Finally, an acyl-CoA fatty alcohol transferase condensed the fatty alcohol with the acyl-CoA to produce the final wax ester.

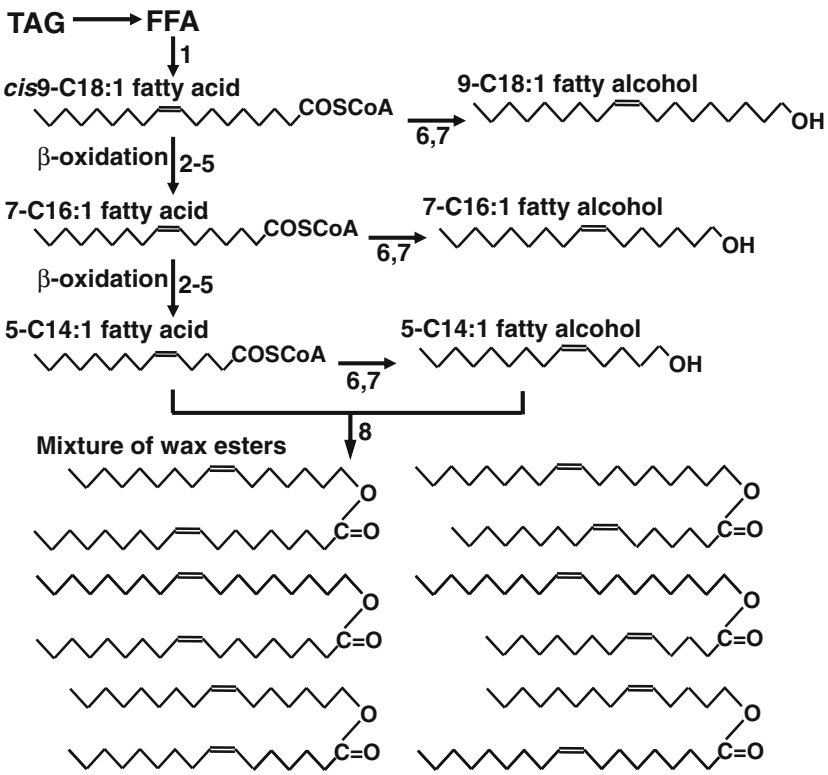

Fig. 6 Proposed microbial pathway for the conversion of vegetable oil to wax esters by *A. hydrophila* N-6. The enzymes involved in the pathway include the following: *1* acyl-CoA synthetase, *2* acyl-CoA dehydrogenase, *3* enoyl-CoA hydratase, *4* 3-hydroyxacyl-CoA dehydrogenase, *5* 3-oxoacyl-CoA thiolase, *6* acyl-CoA reductase, *7* fatty aldehyde reductase, *8* acyl-CoA fatty alcohol transferase

Advantages of Using *A. hydrophila* for Bioprocessing

It has been reported that a number of *Acinetobacter* strains transform *n*-alkane to wax esters, but the carbon chain lengths of the produced fatty acids and fatty alcohols are usually the same as in the starting material [16]. Furthermore, previous research did not focus on the use of TAG as a substrate, although four strains (nos. 144, 308, 310, and 340) belonging to the genus *Acinetobacter* produced wax esters from vegetable oil in our study. In contrast, our newly isolated strain, *A. hydrophila* N-6, produced wax esters from TAG having decreased carbon chain lengths, and the resulting products contained large amounts of rare fatty acids and rare fatty alcohols. Thus, we suggest that new bioprocesses using this strain can be developed to generate these unique fatty acids and fatty alcohols.

The technology would have two major advantages: (1) Many fatty alcohols are industrially produced chemically, but these are limited to oleyl alcohol (C18:1) and several saturated fatty alcohols. This limitation is caused by the inevitable breakdown of the fatty acid double bonds due to the extreme conditions, including high temperatures (240–330 °C) and high pressures (20–70 MPa), necessary for the chemical processing to take place. In contrast, bioprocessing using *A. hydrophila* N-6 would make it possible to reduce unsaturated fatty acids to unsaturated fatty alcohols, especially those having more than two double bonds. In addition, the process can be performed using benign conditions (30 °C, 0.1 MPa). (2) The chemical reduction of the fatty acid carbon chain length from the carboxyl end is difficult to accomplish. In contrast, bioprocessing using the β-oxidation pathway is relatively straightforward.

Even though the physiological activity and commercial application of the rare fatty acids and rare fatty alcohols produced by *A. hydrophila* N-6 have yet to be fully realized, the positive implications of our work for designing new, useful, and trouble-free industrial processes using these bacteria cannot be underestimated. Our future work will focus on laying a framework for these potentially important applications.

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