# Microbial production of hydroxy fatty acids utilizing crude glycerol

Naomi Murakawa[1], Takaiku Sakamoto[1,2], Mizuho Kanoh[1], Si-Bum Park[3], Shigenobu Kishino[3], Jun Ogawa[3], Eiji Sakuradani[1,2,*]

[1] Graduate School of Advanced Technology and Science, Tokushima University, 2-1 Minamijosanjima-cho, Tokushima 770-8513, Japan

[2] Graduate School of Technology, Industrial and Social Sciences, Tokushima University, 2-1 Minamijosanjima-cho, Tokushima 770-8513, Japan

[3] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

* To whom correspondence should be addressed. E-mail: sakuradani.eiji@tokushima-u.ac.jp, Tel: +81-88-656-7528, Fax: +81-88-656-9074

**Abstract**

Strain D2 was isolated from a natural sample as a crude glycerol-assimilating microorganism. The ITS-5.8S rDNA sequence of strain D2 was most similar to that of *Fusarium solani* deposited in the NCBI database. Strain D2 accumulated 10-hydroxy-*cis*-12-octadecenoic acid (HYA), 10-hydroxyoctadecanoic acid (HYB), and 10-oxooctadecanoic acid (KetoB) in a medium containing crude glycerol, as a carbon source, and yeast extract, named CG medium. The growth and HYB production of strain D2 depended on the crude glycerol concentration in the medium. Strain D2 produced 2.20 g/L (40% of total fatty acids) of HYB on cultivation in CG medium containing 8% crude glycerol. When strain D2 was cultivated in CG medium containing 6% crude glycerol, the yield of HYB on cultivation under 4 days-shaking and 3 days-static conditions reached 1.19 g/L, which was 2.2 times higher than that under 7 days-shaking conditions and accounted for 53% of total fatty acids. The fungus was found not only to efficiently produce fatty acids utilizing crude glycerol, but also to be the first filamentous fungus to produce hydroxy and oxo fatty acids such as HYB, HYA, and KetoB.

**Keywords**

*Fusarium solani*; 10-hydroxyoctadecanoic acid; 10-hydroxy-*cis*-12-octadecenoic acid; 10-oxo-octadecanoic acid; fatty acid hydration; crude glycerol.

## 1. Introduction

Fossil fuels such as coal and petroleum provide energy and chemical products that are very important for our daily life, but they also produce greenhouse gases such as $CO_2$ and NOx, which cause global warming. On the other hand, the limits of fossil fuel reserves are hastening the development of sustainable energy resources to replace them. Biodiesel, produced by the esterification of plant- and animal-derived lipids with alcohols such as methanol and ethanol in the presence of an alkaline catalyst, is one of the sustainable energy sources that are already in practical use (Felizardo et al., 2006). In the European Union, the Renewable Energy Directive II classifies palm oil-based biodiesel into the high Indirect Land Use Change risk category, resulting in a decrease of biodiesel consumption. In the United States, biofuel demand is expected to be sustained by the Renewable Fuel Standard. Due to these factors, the annual global supply of biodiesel is expected to stabilize at about 50 billion liters during 2019-30 (OECD/FAO, 2021). However, about 10% (w/v) of crude glycerol, 5 billion liters per year, is generated as a by-product of biodiesel production. In addition, the soap and alcoholic beverage industries consistently produce crude glycerol (Tan et al., 2013). Effective utilization of this crude glycerol may help to reduce manufacturing costs and produce value-added products.

The chemical composition of crude glycerol varies depending on the type of catalyst used for saponification, the efficiency of ester exchange, and the impurities in the raw material, where the glycerol content ranges between 14 and 87% (Kumar et al., 2019). Filtration and partial vacuum distillation processes to remove impurities such as soaps/lipids, methanol, water, salts, and metals from crude glycerol require a lot of labor and expense (Ma and Hanna, 1999; Talebian-Kiakalaieh et al., 2018). The following studies have been reported

on the use of crude glycerol as a culture medium component for microorganisms to produce useful compounds. Single cell oils, as an alternative renewable biofuel, are produced using crude glycerol containing 42-87% glycerol by *Yarrowia lipolytica* (Dobrowolski et al., 2016). *Schizochytrium* sp., a marine microalga, produces docosahexaenoic acid using crude glycerol containing 8.5% glycerol (Chi et al., 2007). The lipid productivity of *Chlorella protothecoides* was improved by using crude glycerol containing 62% glycerol (Chen and Walker, 2011). *Aspergillus niger* (Iyyappan et al., 2019), *Klebsiella* sp. (Oh et al., 2011), and *Gluconacetobacter sacchari* (Carreira et al., 2011) have been bred for production of malic acid, ethanol, and bacterial cellulose by using crude glycerol containing 35-80% glycerol, respectively.

Methanolysis catalysts, free fatty acids, and organic matter included in crude glycerol inhibit the growth of most microorganisms. To realize microbial production of useful compounds using crude glycerol, it is most important for microorganisms to grow well in a medium containing high concentrations of crude glycerol. In the process of screening microorganisms that grow in medium containing crude glycerol, we succeeded in obtaining strain D2. This strain not only grew well, but also exhibited relatively high lipid productivity. This study reports microbial lipid production using crude glycerol as the carbon source and a characteristic fatty acid composition of strain D2.

## 2. Materials and Methods

### 2.1 Materials

The crude glycerol, which contained approximately 45% (w/w) glycerol, 13% (w/w) methanol, 13% (w/w) lipid, 16% potassium, 0.02% (w/w) nitrogen component, and 0.01%

(w/w) sulfur and other components, was provided by the Kyoto City Waste Cooking Oil Fuel Conversion Facility, Kyoto, Japan. The lipids in the crude glycerol consisted of 10.2% palmitic acid (C16:0), 2.7% stearic acid (C18:0), 53.8% oleic acid (C18:1ω9), 29.4% linoleic acid (C18:2ω6), 3.1% α-linolenic acid (C18:3ω3), and 0.6% eicosanoic acid (C20:0) in total fatty acids. Pure glycerol and yeast extract were purchased from Nacalai Tesque Inc. (Kyoto, Japan) and Oriental Yeast Co., Ltd. (Tokyo, Japan), respectively. 10-Hydroxyoctadecanoic acid (HYB) and 10-hydroxy-*cis*-12-octadecenoic acid (HYA) (supplementary Fig. S1), as authentic standards, were prepared from C18:1ω9 and C18:2ω6, respectively, by washed cells of recombinant *Escherichia coli* having oleate hydratase (*cla-hy*) gene (Accession No. **AB671229**) from *Lactobacillus plantarum* AKU 1009a (Takeuchi et al., 2015, Takeuchi et al., 2016). Most of the products were formed in the reaction mixture outside bacterial cells. The products were purified with Isolera One (Biotage, Uppsala, Sweden) equipped with SNAP Ultra 10g silica cartridge (Biotage) at room temperature. The products were mixed with silica gel, dried in a vacuum desiccator, and then applied to the empty disposable cartridge (Biotage) after loading the mixture of activated clay, sodium sulfate anhydrate, and silica gel (1:2:4, w/w/w) with the thickness of 5 mm at the bottom of the cartridge. The products were eluted with *n*-hexane and diethyl ether at a flow rate of 36 ml $min^{-1}$ with following gradients: 5 column volume (CV, 17ml) of 20 % (v/v), 2 CV of 20–40 % (v/v), and 10 CV of 40 % (v/v) of diethyl ether. A UV detector was used for detection of fatty acids at the wavelength of 200 nm and 225 nm.

10-Oxo octadecanoic acid (KetoB), and 10-oxo-*cis*-12-octadecenoic acid (KetoA) (supplementary Fig. S1) were obtained by Jones oxidation, in which the hydroxyl groups of

purified HYB and HYA are oxidized with $CrO_3$ (Curtis et al., 1953). One hundred mg of hydroxy fatty acids was solved in 2 ml of acetone on ice. Jones solution which contains 2.67 % (w/v) $CrO_3$, 2.3 % (v/v) $H_2SO_4$, and 80 % (v/v) acetone in water was added to the hydroxy fatty acid solution on ice until orange color of Jones solution did not disappear. After that, isopropanol was added until the color of the solution changed to deep green color. The solution was extracted with diethyl ether. The diethyl ether layer was washed twice with water and evaporated. The oxo fatty acids were purified by the chromatographic methods described above. *Fusarium solani* fsp. *pisi* NBRC 9975 was obtained from the National Institute of Technology and Evaluation of Japan.

**2.2 Identification of isolated strain**

The isolated strain was identified by sequencing of the internal transcribed spacer and 5.8S ribosomal DNA region (ITS-5.8S rDNA) amplified with a pair of primers, ITS1: 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3' (White et al., 1990). The genome of the isolated strain used for PCR was extracted according to the protocol described by Izumitsu et al. (2012). The amplified PCR product was sequenced (Macrogen Inc., Kyoto, Japan) and analyzed using the BLASTn algorithm of the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using the UPGMA method, using the CLC sequence viewer in the shareware provided by CLC bio (http://www.clcbio.com/index.php).

**2.3 Culture conditions**

A minimal medium consisting of 0.2 g/L of glucose, 0.2 g/L of sucrose, 0.5 g/L of $MgSO_4 \cdot 7H_2O$, 0.5 g/L of KCl, 1 g/L of $KH_2PO_4$, 1 g/L of $KNO_3$, and 20 g/L of agar, adjusted to pH 5.7 with NaOH, was used for preparation of culture stocks of the isolated strains. The CG medium containing 2-16% (w/v) crude glycerol and 1% (w/v) yeast extract was used for microbial fatty acid production. The strains were cultivated at 28°C and 300 rpm for 7 days.

**2.4 Lipid analysis**

The mycelial cells were harvested by suction filtration and washed with distilled water. Mycelial total lipids were extracted with the chloroform/methanol/water system described by Bligh and Dyer (1959). For preparation of fatty acid methyl esters (FAMEs), the mycelial cells were dried at 110°C for 3 h and then their total lipids were transmethylated in 10% methanolic HCl at 55°C for 3 h. Trimethylsilyl (TMS) derivatives were prepared by incubation of the FAMEs with a TMS agent mixture (pyridine-hexamethyldisilazane-trimethylchlorosilane, 9:3:1, by volume) in screw-cap tubes for 30 min at 60°C, followed by extraction with chloroform.

The FAMEs were analyzed by gas chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-2025 gas chromatograph equipped with a flame ionization detector, a split injection system, and a capillary column (SPB-1, 30 m X 0.25 mm I.D.; SUPELCO, PA, USA). The initial column temperature, 200°C for 14 min, was subsequently increased to 300°C at a rate of 50°C /min, and then maintained at 300°C for 4 min. The injector and detector were operated at 300°C. Helium was used as the carrier gas at a flow rate of 2.32 mL/min. Tricosanoic acid (23:0) was used as the internal standard. The fatty acid peaks were identified by comparing their retention times with those of authentic standards, and their

chemical structures were confirmed by gas chromatography-mass spectrometry (GC/MS) analysis of FAMEs and TMS derivatives. GC/MS was carried out using a GC-2030 Nextis (Shimadzu) interfaced with a GCMS-QP2020 NX (Shimadzu) using a mass range of *m/z* 50–500. The GC conditions for GC/MS were the same as those described for GC in this section. Hydroxy and oxo fatty acids were identified according to characteristic molecular weights and fragment ions in mass spectra published by the LipidWeb (https://www.lipidmaps.org/resources/lipidweb/index.php?page=ms/masspec.html; Christie, 2021).

## 3. Results and Discussion

### 3.1. Screening of crude glycerol-assimilating microorganisms

Since the crude glycerol contained substances that inhibit the growth of microorganisms, such as methanol, most microorganisms grew slowly in CG medium. Various soil and sewage samples were collected in Tokushima prefecture, Japan. These samples were streaked onto CG agar medium containing 2% (w/v) crude glycerol, 1% (w/v) yeast extract and 2% (w/v) agar, and then incubated at 28°C. More than 100 strains of yeasts and fungi that appeared on CG agar medium were isolated and their fatty acid productivities were evaluated. A filamentous fungus, strain D2, isolated from natural sources grew well in CG medium. The dry cell weight (DCW) of strain D2 cultured in CG medium containing 4% crude glycerol reached 10.2 g/L, which was about 1.7 and 2.0 times higher than that by cultivation in pure glycerol-containing and glucose-containing media, respectively (Table 1). When strain D2 was cultivated in the medium containing pure glycerol as a carbon source, C16:0, C18:0, C18:2ω6, and C18:3ω3 were the main fatty acid components (Table 1). Unidentified fatty

174 acids, named UK1 and UK2, were detected in the lipids of strain D2. They were not detected
175 on cultivation in a glucose-containing medium (Table 1). UK1 and UK2 accounted for 2.2%
176 and 46.0% of the total fatty acids derived from strain D2 cultured in CG medium, respectively.
177 As these fatty acids were not included in the crude glycerol, it was considered that they were
178 produced from fatty acids in the crude glycerol. Crude glycerol contains 53.8% C18:1ω9 and
179 29.4% C18:2ω6 per total fatty acids. After cultivation in a crude glycerol-containing medium,
180 C18:1ω9 and C18:2ω6 were 4.2% and 11.2% of the total fatty acids in strain D2, respectively,
181 suggesting that these fatty acids in crude glycerol were converted to UK1 and UK2 (Table 1).
182 On cultivation in the pure glycerol medium, small amounts of UK1 and UK2 were detected,
183 which might have been generated through the conversion of endogenous fatty acids (Table
184 1).
185 The ITS-5.8S rDNA sequence of strain D2 was most similar to that of *Fusarium solani*
186 in the NCBI database (Fig. 1). The 564 bp-DNA sequence in the ITS-5.8S rDNA region of
187 strain D2 showed 98%, 97%, 84%, and 84% identities with those of *F. solani* 7227, *F.*
188 *striatum* CBS 101573, *F. tenuicristatum* IMI 277708, and *F. oxysporum* WPII21-2,
189 respectively.
190
191 **3.2. Identification of fatty acids from strain D2**
192 On GC and GC/MS analyses of FAMEs from strain D2 cultivated in CG medium,
193 saturated and unsaturated FAMEs such as C16:0, C18:1ω9, C18:2ω6, and C18:3ω3 as well
194 as UK1 and UK2 were detected (Fig. 2A(c)). The ion fragmentation pattern of UK2 by
195 GC/MS analysis was consistent with that of HYB stored in a GC/MS compound library, and

196 the retention time of UK2 was also consistent with that of the HYB authentic standard (Fig.
197 2A(b), (c)). The GC/MS analysis suggested that UK1 is a mixture of hydroxy and oxo fatty
198 acids based on data from the GC/MS compound library. Since HYA and KetoA were reported
199 to be converted from C18:2ω6, and HYB and KetoB from C18:1ω9 in a lactic acid bacterium
200 (Kishino et al. 2013), the generation of HYA, KetoA, and KetoB as well as HYB was
201 considered in this study. The retention time of UK1 was consistent with those of KetoB and
202 HYA standards (Figure 2A(a)-(c)), suggesting that UK1 contains KetoB and HYA. TMS
203 treatment was used to derivatize the hydroxy fatty acids in the total fatty acids into TMS
204 derivatives, and furthermore, to separate the compounds that appeared in overlap on GC/MS
205 analysis. After conversion of total FAMEs to TMS derivatives, new peaks of UK1-TMS and
206 UK2-TMS emerged, and the two peaks of UK1 and UK2 became smaller and were named
207 UK1' and UK2', respectively (Figure 2A(c) and (d)). The fragment ions at *m/z* 156 and 214
208 of UK1' showed β cleavage between single bonds 8-9 and 11-12 at the carboxyl end of the
209 molecule, respectively (Fig. 2B(e)). The fragment ion peak pattern of UK1' was consistent
210 with that of KetoB methyl ester that showed the same retention time as UK1' on GC
211 chromatograms (Fig. 2A(b), (d), and 2B(e)). The ion at *m/z* 201 of UK2' represented the
212 carboxyl end of the molecule with α cleavage between the single bonds 10-11 (Fig. 2B(f)).
213 The ion at *m/z* 169 of UK2' revealed that a methanol molecule of ion *m/z* 201 was lost on
214 further fragmentation (Christie, 2021). The fragment ion peak pattern of UK2' was consistent
215 with that of an HYB methyl ester that showed the same retention time as UK2' on GC
216 chromatograms (Fig. 2A(b), (d), and 2B(f)). The ions at *m/z* 169 and 273 were detected for
217 both UK1-TMS and UK2-TMS (Fig. 2B(g) and (h)). The ion at *m/z* 273 is the carboxymethyl

terminus due to α cleavage of the TMS group at position 10, and the ion at *m/z* 169 indicates that the TMS methyl molecule was lost in further fragmentation (Fig. 2B(g) and (h)) (Christie, 2021). The ion at *m/z* 215 of UK2-TMS and the ion at *m/z* 213 of UK1-TMS represented a terminal end molecule containing a TMS group (Fig. 2B(g) and (h)). The ion at *m/z* 213 on the methyl terminal side of UK1-TMS suggested the presence of one double bond. These results suggested that UK1-TMS and UK2-TMS were TMS derivatives of HYA and HYB, respectively, and that a mixture of KetoB methyl ester and HYA methyl ester was contained in the UK1 peak on the GC chromatogram (Fig. 2A(a)-(c), supplementary Fig. S1). The UK2' contained HYB methyl esters because it was not completely converted to the TMS derivative (Fig. 2A(d) and 2B(f)).

In anaerobic bacteria such as *Elizabethkingia* sp. and *Lactobacillus* sp., fatty acid hydratases are known to convert C18:1ω9 and C18:2ω6 to HYB and HYA, respectively (Kishino et al., 2013; Todea et al., 2015). These hydroxy fatty acids are converted to the corresponding oxo fatty acids such as KetoA and KetoB by a dehydrogenase (Kishino et al., 2013). The detection of KetoB, HYA, and HYB in fatty acids from strain D2 suggested that strain D2 has a fatty acid hydratase and a subsequent dehydrogenase. In this study, KetoA, that is produced from HYA through oxidation, was not detected. Further research is needed to determine whether KetoA might be unstable under these analytical conditions or could be enzymatically metabolized in strain D2.

### 3.3. Hydroxy fatty acid production by strain D2 using high concentrations of crude glycerol

240 Strain D2 and *F. solani* fsp. *pisi* NBRC 9975 as a reference strain, grew well in CG
241 medium. The highest growth of strains D2 and NBRC 9975 was observed in CG medium
242 containing 10% and 8% crude glycerol, respectively (Fig. 3). The HYB production by strains
243 D2 and NBRC 9975 reached 2.20 g/L (40% of total fatty acids) and 0.47 g/L (16% of total
244 fatty acids), respectively, on cultivation in CG medium containing 8% crude glycerol. Both
245 strains produced more HYB than HYA. The crude glycerol contained 53.8% C18:1ω9 and
246 29.4% C18:2ω6 in the total fatty acids (Table 2). It was considered that strain D2 efficiently
247 converted C18:1ω9 to HYB and the ratio of C18:1ω9 was accordingly decreased in the total
248 fatty acids. Strain NBRC 9975 also converted C18:1ω9 to HYB as well as strain D2. It was
249 also possible to consider that fatty acid hydratases of these filamentous fungi preferred
250 C18:1ω9 to C18:2ω6 as a substrate. The hydroxy fatty acid productivity of both strains
251 suggested that *F. solani* may possess fatty acid hydration activity. Strain D2 showed
252 resistance to high concentrations of crude glycerol and high productivity of HYB on
253 cultivation in CG medium.
254
255 **3.4. Hydroxy fatty acid production under microaerobic conditions**
256 Strain D2 was cultured in a test tube containing 10 mL of CG medium including 6%
257 crude glycerol for 7 days, and then its DCW and fatty acid content were measured (Fig. 4).
258 The notation X+Y days means shaking cultivation for X days and then static cultivation for
259 Y days. The highest growth rate was observed when strain D2 was cultured with 7 days-
260 shaking (7+0 days), as shown in Fig. 4. The growth rate decreased as the period of static
261 incubation increased. Although hydroxy fatty acid production was detected under all

conditions, it was highest under the 4 days-shaking and 3 days-static conditions. The yield of HYB with 4+3 days reached 1.19 g/L, which was 2.2 times higher than that with 7+0 days and accounted for 53% of total fatty acids. These results confirmed the importance of microaerobic conditions for hydroxy fatty acid production by strain D2.

After the cultivation with shaking for more than 4 days, the fluidity of the medium decreased due to the increased bulk of mycelial cells. This situation was expected to cause insufficient aeration, which lowers the amount of dissolved oxygen in the medium. After the mycelial bulk is sufficiently high in a medium, static cultivation is thought to make a microaerobic condition. After 4 days-shaking and then 3 days-static cultivation, strain D2 formed mycelial pellets in media and mycelial mats on the surface of the media (supplementary Fig. S2). In such an environment, microaerobic conditions were formed in the liquid medium. The fatty acid hydration proceeded efficiently under anaerobic conditions in the previous studies (Joo et al., 2012; Takeuchi et al., 2015). This led us to assume that the hydroxy fatty acid productivity was improved by static cultivation of strain D2. *Fusarium* spp. perform nitrate respiration under microaerobic conditions, unlike bacterial denitrifiers that perform nitrate respiration only under anoxic conditions (Kobayashi et al., 1996; Cheng et al., 2020). The microaerobic conditions were assumed to allow both the slight growth of strain D2 and the proceeding of fatty acid hydration.

Since crude glycerol is produced as a byproduct of the biodiesel production process, most of fatty acids in crude glycerol were methyl esterified. As the fatty acid composition of canola oil is similar to that of crude glycerol, we cultured the strain D2 in a medium containing 2.7% pure glycerol and 1.5% methyl esterified canola oil to mimic components of 6% crude glycerol (Table 2). The composition ratio of UK2 that is HYB in total fatty acids

285 was 21.0% when cultured in a canola oil-containing medium, but it reached 56.3% when
286 cultured in a crude glycerol-containing medium (Table 2). It is assumed that substances
287 contained in the crude glycerol activated the generation of UK2. It is necessary to identify
288 such substances in the crude glycerol in the future. The production of functional fatty acids
289 by oleaginous microorganisms such as *Mortierella alpina* (Okuda et al., 2015; Kikukawa et
290 al., 2018; Mo et al., 2021), *Yarrowia lipolytica* (Liu et al., 2017; Gemperlein et al., 2019),
291 *Lipomyces starkeyi* (Takaku et al., 2020), and Thraustochytrids (Burja et al., 2006; Patel et
292 al., 2020) has been performed under aerobic conditions in a nutrient rich medium containing
293 glucose as a carbon source. These studies were based on the production of triacylglycerols
294 containing unsaturated fatty acids by eukaryotic microorganisms such as filamentous fungi
295 and yeasts. Regarding production of hydroxy fatty acids (HYA, HYB, etc.), enzymatic
296 conversion studies on recombinant *E. coli* have been reported (Joo et al., 2012; Takeuchi et
297 al., 2015), but no studies on fermentation production using living cells have been reported.
298 This is the first report of the production of hydroxy fatty acids by fermentation with
299 filamentous fungi using inexpensive crude glycerol as a carbon source.
300
301 **4. Conclusion**
302 The utilization of crude glycerol, which is emitted in large quantities during the
303 production of biodiesel and soap, is a serious challenge. By screening crude glycerol-
304 assimilating microorganisms, we succeeded in isolating strain D2, which is closely related to
305 *Fusarium solani*. The fungus was found not only to efficiently produce fatty acids utilizing
306 crude glycerol, but also to be the first filamentous fungus to produce hydroxy and oxo fatty
307 acids such as HYB, HYA, and KetoB. Although the growth of many microorganisms was

inhibited in a medium containing a high concentration of crude glycerol, strain D2 grew well in a medium containing 8% crude glycerol and accumulated a large amount of HYB. In addition, the production of HYB by strain D2 was higher in a shaking culture followed by static culture. Although many studies on the production of HYB and HYA by bacteria have been reported, this is the first report on the production of HYB and HYA by filamentous fungi. It is expected that the mechanism of hydroxy fatty acid accumulation will be elucidated and the characterization of fatty acid hydratase derived from filamentous fungi will be performed in the future.

**Funding**

This work was supported partly by the Advanced Low Carbon Technology Research and Development Program (ALCA) of the Japan Science and Technology Agency (JST), to E.S. (JPMJAL1607), by the New Energy and Industrial Technology Development Organization (NEDO) Project Code (P20011), and by JSPS KAKENHI Grant Numbers 20K15436 (to T.S.), 18H04623 (to E.S.), and 20H04779 (to E.S.).

**Authors' contributions**

The authors' responsibilities were as follows: TS and ES conceived and designed the overall research. NM, MK, SBP, and SK carried out the experimental work, and analyzed and interpreted data. TS, JO, and ES recommended and edited the paper. All authors contributed to the article and approved the submitted version.

**Declaration of competing interest**

The authors declare that there is no conflict of interest.

## References


Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911-917. https://doi.org/10.1139/o59-099.

Burja, A.M., Radianingtyas, H., Windust, A., Barrow, C.J., 2006. Isolation and characterization of polyunsaturated fatty acid producing *Thraustochytrium* species: Screening of strains and optimization of omega-3 production. Appl. Microbiol. Biotechnol. 72, 1161–1169. https://doi.org/10.1007/s00253-006-0419-1.

Carreira, P., Mendes, J.A.S., Trovatti, E., Serafim, L.S., Freire, C.S.R., Silvestre, A.J.D., Neto, C.P., 2011. Utilization of residues from agro-forest industries in the production of high value bacterial cellulose. Bioresour. Technol. 102, 7354–7360. https://doi.org/10.1016/j.biortech.2011.04.081.

Chen, Y.H., Walker, T.H., 2011. Biomass and lipid production of heterotrophic microalgae *Chlorella protothecoides* by using biodiesel-derived crude glycerol. Biotechnol. Lett. 33, 1973–1983. https://doi.org/10.1007/s10529-011-0672-y.

Cheng, H.Y., Xu, A.A., Awasthi, M.K., Kong, D.D., Chen, J.S., Wang, Y.F., Xu, P., 2020. Aerobic denitrification performance and nitrate removal pathway analysis of a novel fungus *Fusarium solani* RADF-77. Bioresour. Technol. 295, 122250. https://doi.org/10.1016/j.biortech.2019.122250.

Chi, Z., Pyle, D., Wen, Z., Frear, C., Chen, S., 2007. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. Process Biochem. 42, 1537–1545. https://doi.org/10.1016/j.procbio.2007.08.008.

354 Curtis, R.G., Heilbron, S.I., Jones, E.R.H., Woods, G.F., 1953. The chemistry of the
355 triterpenes. Part XIII. The further characterization of polyporenic acid. A. J. Chem. Soc.
356 457–464. https://doi.org/10.1039/JR9530000457.
357 Christie, W.W., 2021. The LipidWeb. Mass spectrometry of methyl ester derivatives of fatty
358 acids.
359 https://www.lipidmaps.org/resources/lipidweb/index.php?page=info/llcredit.htm
360 (accessed 15 September 2021).
361 Dobrowolski, A., Mituła, P., Rymowicz, W., Mirończuk, A.M., 2016. Efficient conversion of
362 crude glycerol from various industrial wastes into single cell oil by yeast *Yarrowia*
363 *lipolytica*. Bioresour. Technol. 207, 237–243.
364 https://doi.org/10.1016/j.biortech.2016.02.039.
365 Felizardo, P., Neiva Correia, M.J., Raposo, I., Mendes, J.F., Berkemeier, R., Bordado, J.M.,
366 2006. Production of biodiesel from waste frying oils. Waste Manage. 26, 487-494.
367 https://doi.org/10.1016/j.wasman.2005.02.025.
368 Gemperlein, K., Dietrich, D., Kohlstedt, M., Zipf, G., Bernauer, H.S., Wittmann, C., Wenzel,
369 S.C., Müller, R., 2019. Polyunsaturated fatty acid production by *Yarrowia lipolytica*
370 employing designed myxobacterial PUFA synthases. Nat. Commun. 10, 4055.
371 https://doi.org/10.1038/s41467-019-12025-8.
372 Iyyappan, J., Bharathiraja, B., Baskar, G., Kamalanaban, E., 2019. Process optimization and
373 kinetic analysis of malic acid production from crude glycerol using *Aspergillus niger*.
374 Bioresour. Technol. 281, 18–25. https://doi.org/10.1016/j.biortech.2019.02.067.
375 Izumitsu, K., Hatoh, K., Sumita, T., Kitade, Y., Morita, A., Tanaka, C., Gafur, A., Ohta, A.,
376 Kawai, M., Yamanaka, T., Neda, H., Ota, Y., 2012. Rapid and simple preparation of

mushroom DNA directly from colonies and fruiting bodies for PCR. Mycoscience. 53, 396-401. https://doi.org/10.1007/S10267-012-0182-3.

Joo, Y.C., Seo, E.S., Kim, Y.S., Kim, K.R., Park, J.B., Oh, D.K., 2012. Production of 10-hydroxystearic acid from oleic acid by whole cells of recombinant *Escherichia coli* containing oleate hydratase from *Stenotrophomonas maltophilia*. J. Biotechnol. 158, 17–23. https://doi.org/10.1016/j.jbiotec.2012.01.002.

Kikukawa, H., Sakuradani, E., Ando, A., Shimizu, S., Ogawa, J., 2018. Arachidonic acid production by the oleaginous fungus *Mortierella alpina* 1S-4: A review. J. Adv. Res. 11, 15–22. https://doi.org/10.1016/j.jare.2018.02.003.

Kishino, S., Takeuchi, M., Park, S.B., Hirata, A., Kitamura, N., Kunisawa, J., Kiyono, H., Iwamoto, R., Isobe, Y., Arita, M., Arai, H., Ueda, K., Shima, J., Takahashi, S., Yokozeki, K., Shimizu, S., Ogawa. J., 2013. Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. Proc. Natl. Acad. Sci. USA. 110, 17808-17813. https://doi.org/10.1073/pnas.1312937110.

Kobayashi, M., Matsuo, Y., Takimoto, A., Suzuki, S., Maruo, F., Shoun, H., 1996. Denitrification, a novel type of respiratory metabolism in fungal mitochondrion. J. Biol. Chem. 271, 16263-16267. https://doi.org/10.1074/jbc.271.27.16263.

Kumar, L.R., Yellapu, S.K., Tyagi, R.D., Zhang, X., 2019. A review on variation in crude glycerol composition, bio-valorization of crude and purified glycerol as carbon source for lipid production. Bioresour. Technol. 293, 122155. https://doi.org/10.1016/j.biortech.2019.122155.

Liu, H.H., Zeng, S.Y., Shi, T.Q., Ding, Y., Ren, L.J., Song, P., Huang, H., Madzak, C., Ji, X.J., 2017. A *Yarrowia lipolytica* strain engineered for arachidonic acid production

counteracts metabolic burden by redirecting carbon flux towards intracellular fatty acid accumulation at the expense of organic acids secretion. Biochem. Eng. J. 128, 201-209. https://doi.org/10.1016/j.bej.2017.10.007.

Ma, F., Hanna, M.A., 1999. Biodiesel production: a review. Bioresour. Technol. 70, 1-15. https://doi.org/10.1016/S0960-8524(99)00025-5.

Mo, B.K.H., Ando, A., Nakatsuji, R., Okuda, T., Takemoto, Y., Ikemoto, H., Kikukawa, H., Sakamoto, T., Sakuradani, E., Ogawa, J., 2021. Characterization of 3 fatty acid omega-3 fatty acid desaturases from oomycetes and their application toward eicosapentaenoic acid production in *Mortierella alpina*. Biosci. Biotechnol. Biochem. 85, 1252-1265. https://doi.org/10.1093/bbb/zbaa123.

OECD/FAO, 2021. Biofuels. In: Agricultural Outlook 2021-2030, Paris. https://doi.org/10.1787/19428846-en. (accessed 15 September 2021).

Oh, B.R., Seo, J.W., Heo, S.Y., Hong, W.K., Luo, L.H., Joe, M.H., Park, D.H., Kim, C.H., 2011. Efficient production of ethanol from crude glycerol by a *Klebsiella pneumoniae* mutant strain. Bioresour. Technol. 102, 3918–3922. https://doi.org/10.1016/j.biortech.2010.12.007.

Okuda, T., Ando, A., Negoro, H., Muratsubaki, T., Kikukawa, H., Sakamoto, T., Sakuradani, E., Shimizu, S., Ogawa, J., 2015. Eicosapentaenoic acid (EPA) production by an oleaginous fungus *Mortierella alpina* expressing heterologous the Δ17-desaturase gene under ordinary temperature. Eur. J. Lipid Sci. Technol. 117, 1919–1927. https://doi.org/10.1002/ejlt.201400657.

Patel, A., Rova, U., Christakopoulos, P., Matsakas, L., 2020. Assessment of fatty acids profile and omega-3 polyunsaturated fatty acid production by the oleaginous marine

thraustochytrid *Aurantiochytrium* sp. T66 cultivated on volatile fatty acids. Biomolecules. 10, 694. https://doi.org/10.3390/biom10050694.

Takaku, H., Matsuzawa, T., Yaoi, K., Yamazaki, H., 2020. Lipid metabolism of the oleaginous yeast *Lipomyces starkeyi*. Appl. Microbiol. Biotechnol. 104, 6141–6148. https://doi.org/10.1007/s00253-020-10695-9/Published.

Takeuchi, M., Kishino, S., Hirata, A., Park, S.B., Kitamura, N., Ogawa, J., 2015. Characterization of the linoleic acid Δ9 hydratase catalyzing the first step of polyunsaturated fatty acid saturation metabolism in *Lactobacillus plantarum* AKU 1009a. J. Biosci. Bioeng. 119, 636–641. https://doi.org/10.1016/j.jbiosc.2014.10.022.

Takeuchi, M., Kishino, S., Park, S.B., Hirata, A., Kitamura, N., Saika, A., Ogawa, J., 2016. Efficient enzymatic production of hydroxy fatty acids by linoleic acid Δ9 hydratase from *Lactobacillus plantarum* AKU 1009a. J. Appl. Microbiol. 120, 1282-1288. https://doi.org/10.1111/jam.13088.

Talebian-Kiakalaieh, A., Amin, N.A.S., Rajaei, K., Tarighi, S., 2018. Oxidation of bio-renewable glycerol to value-added chemicals through catalytic and electro-chemical processes. Appl. Energy. 230, 1347–1379. https://doi.org/10.1016/j.apenergy.2018.09.006.

Tan, H.W., Abdul Aziz, A.R., Aroua, M.K., 2013. Glycerol production and its applications as a raw material: A review. Renew. Sustain. Energy Rev. 27, 118–127. https://doi.org/10.1016/j.rser.2013.06.035.

Todea, A., Hiseni, A., Otten, L.G., Arends, I.W.C.E., Peter, F., Boeriu, C.G., 2015. Increase of stability of oleate hydratase by appropriate immobilization technique and conditions. J. Mol. Catal. B Enzym. 119, 40–47. https://doi.org/10.1016/j.molcatb.2015.05.012.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand D.H., Sninsky J.J., White T.J. (eds.), PCR Meth Appl. San Diego: Academic Press, 315-322.

**Figure captions**

**Fig. 1. A phylogenetic tree of the ITS-5.8S rDNA sequences of strain D2 and *Fusarium* spp.**

A phylogenetic tree was constructed by combining the ITS-5.8S rDNA sequences of strain D2 and *Fusarium* spp. including *Fusarium tenuicristatum* IMI 277708 (Accession No. **NR_169922**), *F. graminearum* CPFGWY137D51 (No. **KR047057**), *F. longifundum* NRRL 36372 (No. **NR_171059**), *F. oxysporum* FJAT-31099 (No. **KU931550**), *F. oxysporum* WPII21-1 (No. **MK163440**), *F. fujikuroi* CBS 262.54 (No. **MH857321**), *F. citricola* CPC 27805 (No. **NR_172265**), *F. vanettenii* MAFF 840047 (No. **AB513852**), *F. proliferatum* strain 144 (No. **MK828121**), *F. redolens* T1ST190421511 (No. **MN486568**), *F. striatum* CBS 101573 (No. **KM231798**), *F. solani* H4470 (No. **GU595038**), *F. solani* C219 (No. **KU377470**), *F. solani* PCO.30 (No. **HQ248197**), and *F. solani* 7227 (No. **MN922526**). The UPGMA method was used as the algorithm, and the bootstrap test was repeated 1,000 times.

**Fig. 2. GC (A) and GC/MS (B) analyses of fatty acid methyl esters from strain D2 cultured in CG medium.**

(a) Methyl-esterified KetoA and HYA authentic standards; (b) methyl-esterified KetoB and HYB authentic standards; (c) methyl-esterified lipids derived from strain D2; (d) TMS derivatives of methyl-esterified lipids; (e) UK1'; (f) UK2'; (g) UK1-TMS; (h) UK2-TMS. Abbreviations: TMS, trimethylsilyl derivative; KetoA, 10-oxo-*cis*-12-octadecenoic acid; KetoB, 10-oxo octadecanoic acid; HYA, 10-hydroxy-*cis*-12-octadecenoic acid; HYB, 10-hydroxy octadecanoic acid.

**Fig. 3. Effect of crude glycerol concentration on growth and fatty acid production in (A) strain D2 and (B) *F. solani* fsp. *pisi* NBRC 9975.**

Both strains were cultured in 4 mL of CG medium for 7 days with shaking. “Others” include C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3, and C20:0. DCW, dry cell weight. Data represent means ± standard deviations (n = 3).

**Fig. 4. Effect of shaking and static culture conditions on hydroxy fatty acid production by strain D2.**

Strain D2 was cultured in 10 mL of CG medium containing 6% crude glycerol. (X+Y) means that strain D2 was cultured under shaking culture conditions for X days and then under static culture conditions for Y days. “Others” include C16:0, C18:0, C18:1ω9, C18:2ω6, C18:3ω3, and C20:0. DCW, dry cell weight. Data represent means ± standard deviations (n = 3).

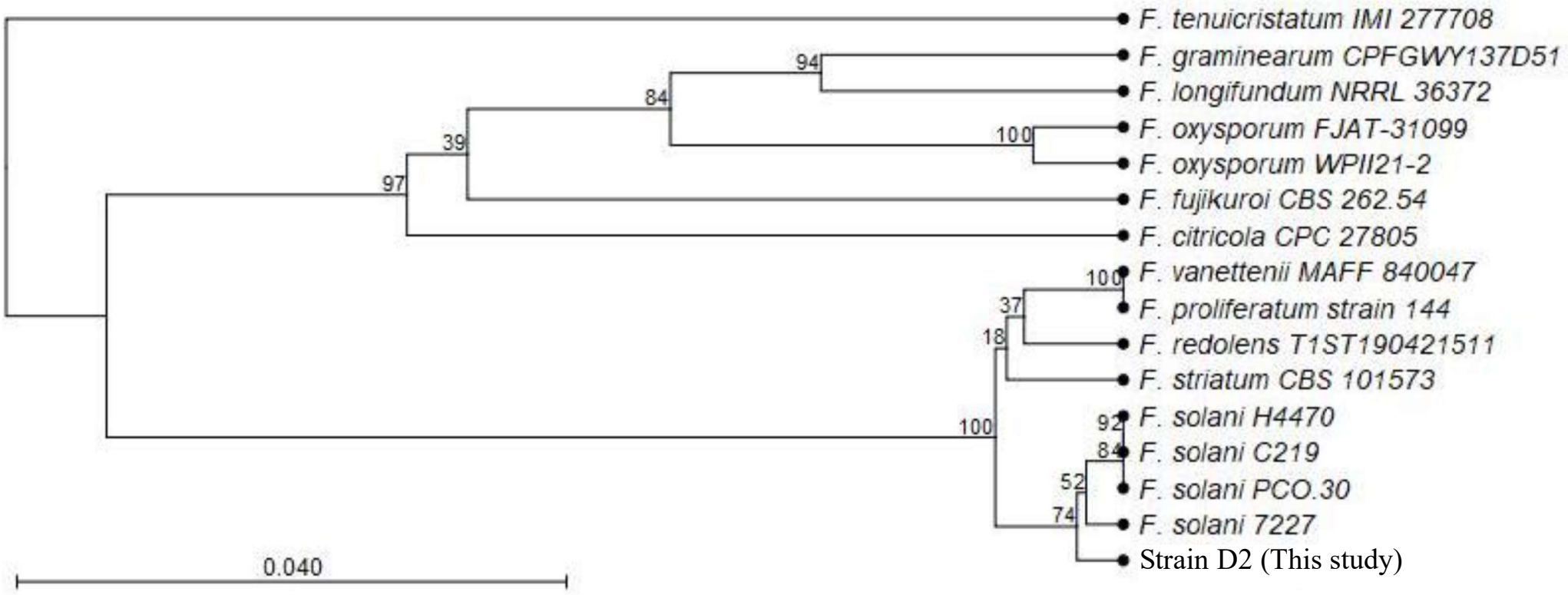

F. tenuicristatum IMI 277708
F. graminearum CPFGWY137D51
F. longifundum NRRL 36372
F. oxysporum FJAT-31099
F. oxysporum WPII21-2
F. fujikuroi CBS 262.54
F. citricola CPC 27805
F. vanettenii MAFF 840047
F. proliferatum strain 144
F. redolens T1ST190421511
F. striatum CBS 101573
F. solani H4470
F. solani C219
F. solani PCO.30
F. solani 7227
Strain D2 (This study)
94
84
100
39
97
100
37
18
100
92
84
52
74
0.040

Fig. 1

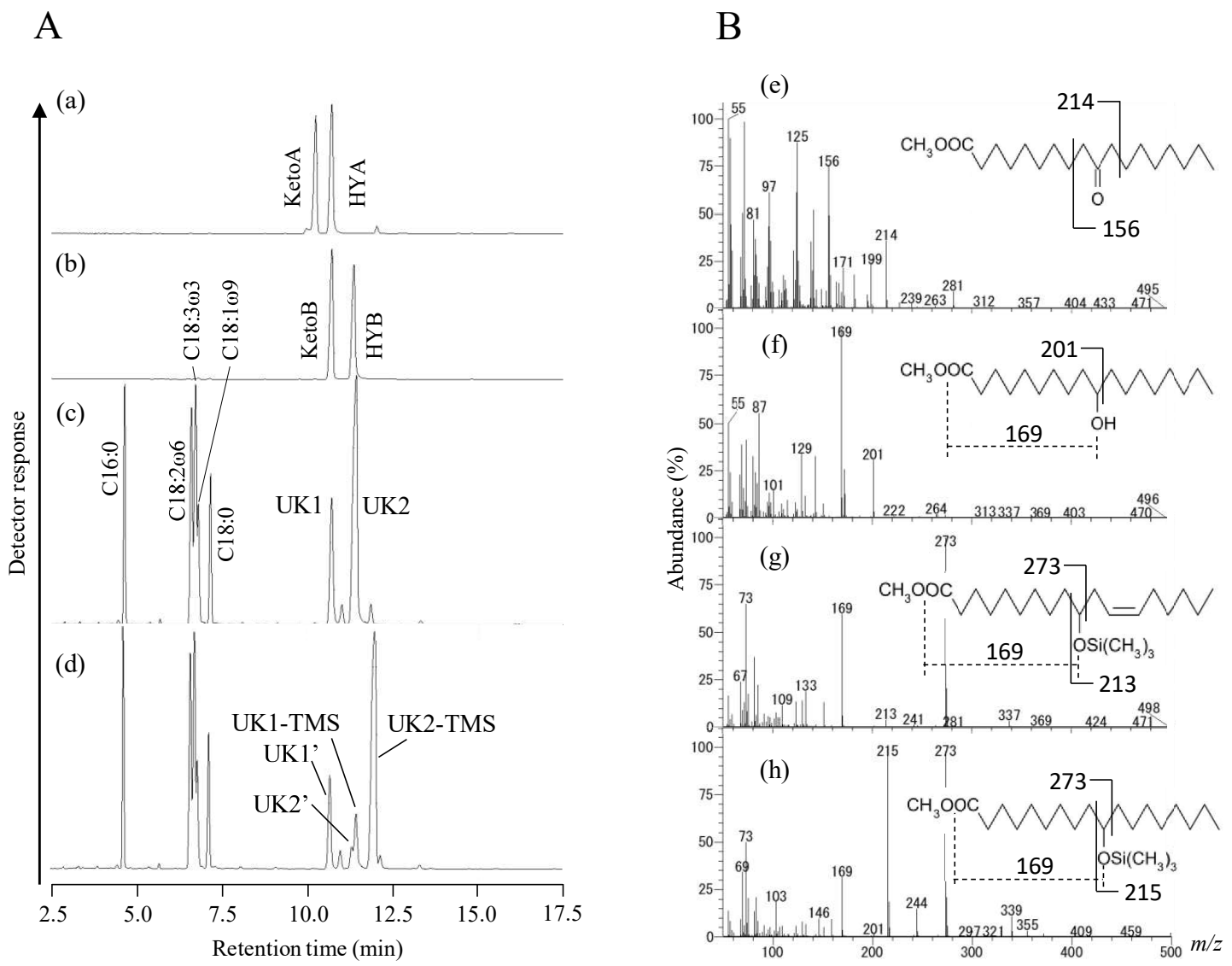

A
B
(a)
KetoA
HYA
(b)
C18:3ω3
C18:1ω9
KetoB
HYB
(c)
C16:0
C18:2ω6
C18:0
UK1
UK2
(d)
UK1-TMS
UK1'
UK2'
UK2-TMS
Detector response
2.5
5.0
7.5
10.0
12.5
15.0
17.5
Retention time (min)
(e)
214
156
(f)
201
169
(g)
273
169
213
(h)
273
169
215
Abundance (%)
m/z

Fig. 2

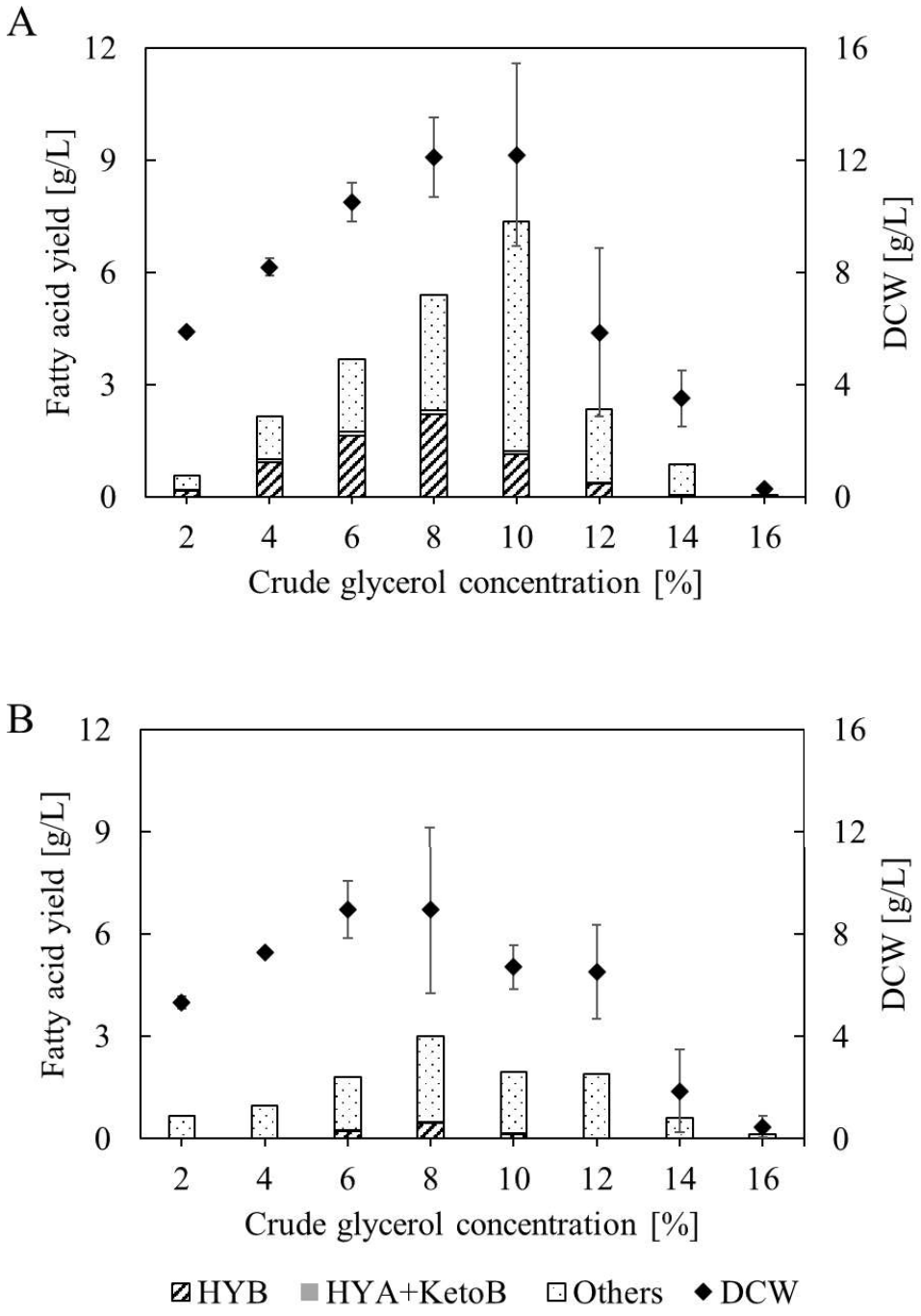

A
Fatty acid yield [g/L]
DCW [g/L]
Crude glycerol concentration [%]
B
Fatty acid yield [g/L]
DCW [g/L]
Crude glycerol concentration [%]
HYB
HYA+KetoB
Others
DCW

Fig. 3

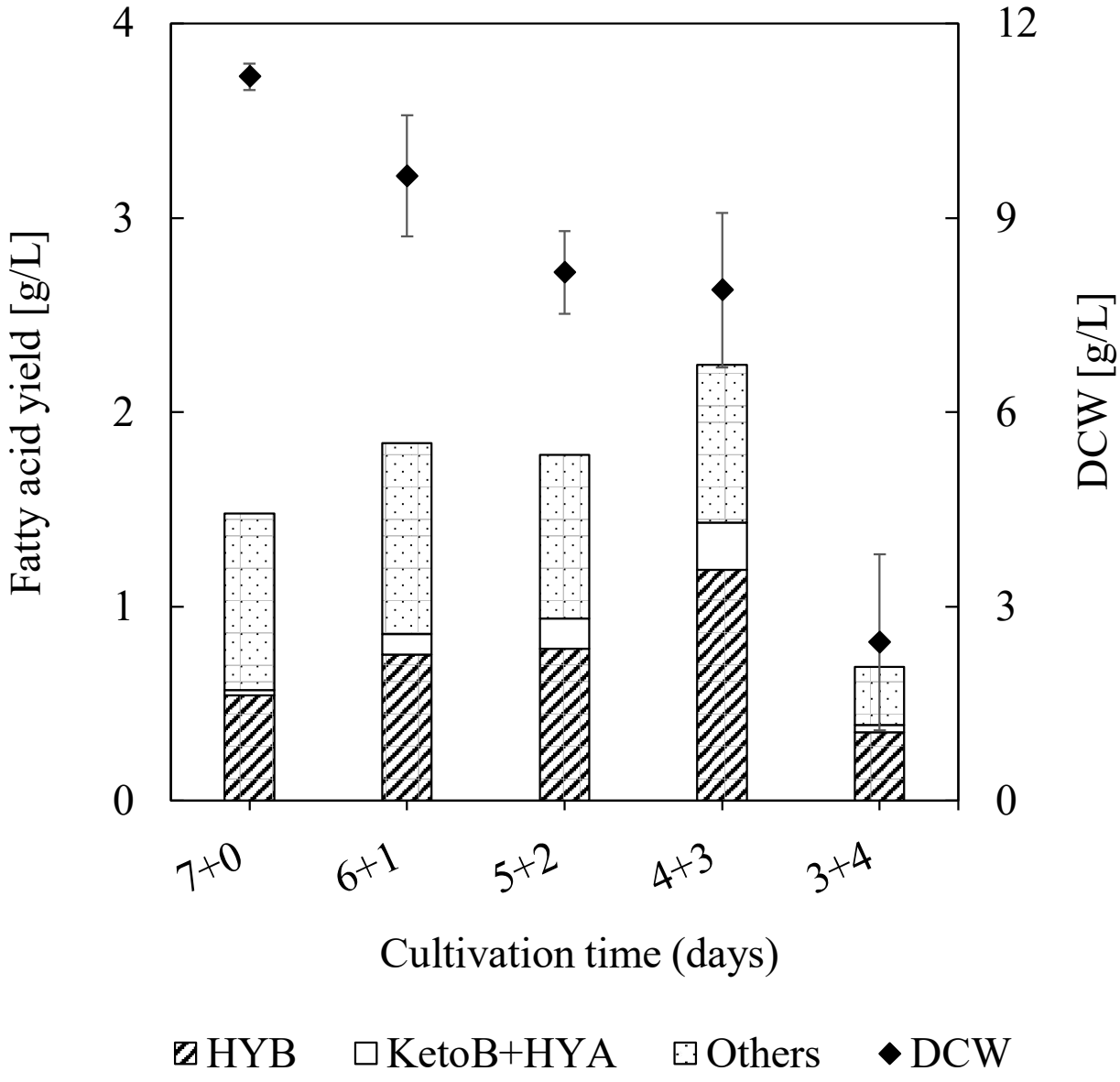

4
3
2
1
0
12
9
6
3
0
Fatty acid yield [g/L]
DCW [g/L]
7+0
6+1
5+2
4+3
3+4
Cultivation time (days)
HYB
KetoB+HYA
Others
DCW

Fig. 4

**Table 1. Dry cell weights and fatty acid compositions of strain D2 cultured in the medium containing commercial pure glycerol or crude glycerol.[a]**

| Carbon source | DCW[b] (g/L) | Total fatty acid (g/L) | Fatty acid composition (%)[c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | C16:0 | C18:0 | C18:1ω9 | C18:2ω6 | C18:3ω3 | C20:0 | UK1 | UK2 |
| Pure glycerol | 6.2±0.3 | 0.29±0.07 | 19.8±0.4 | 15.4±0.9 | 0.9±0.02 | 48.2±0.9 | 15.1±0.5 | ND[d] | 0.4±0.2 | 0.2±0.1 |
| Crude glycerol | 10.2±0.1 | 2.18±0.12 | 18.4±0.3 | 12.2±0.7 | 4.2±0.4 | 11.1±1.2 | 5.3±0.3 | 0.7±0.1 | 2.2±0.4 | 46.0±0.2 |
| Glucose | 5.2±0.5 | 0.18±0.02 | 16.0±0.3 | 3.5±0.8 | 12.4±0.4 | 61.9±0.8 | 6.1±0.3 | ND | ND | ND |

[a]Strain D2 was cultured in the medium containing 2% pure glycerol, 4% crude glycerol (approximately 1.8% glycerol), or 2% glucose, and 1% yeast extract for 7 days. All data are the means ± standard deviation (SD) for triplicate samples.

[b]DCW, dry cell weight.

[c]C16:0, palmitic acid; C18:0, stearic acid; C18:1ω9, oleic acid; C18:2ω6, linoleic acid; C18:3ω3, α-linolenic acid; C20:0, eicosanoic acid.

[d]ND, not detected.

**Table 2. Fatty acid compositions of strain D2 cultured in the medium containing canola oil methyl esters or crude glycerol as a carbon source.**

| | DCW[a] (g/L) | Total fatty acid (g/L) | Fatty acid composition (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | C16:0 | C18:0 | C18:1ω9 | C18:2ω6 | C18:3ω3 | C20:0 | UK1 | UK2 |
| D2 Mycelium-derived fatty acids[b] | | | | | | | | | | |
| Canola oil-ME[c] | 8.3±1.2 | 3.04±0.60 | 6.5±0.02 | 4.1±0.2 | 4.7±0.2 | 14.7±2.2 | 39.7±6.2 | 1.2±0.03 | 8.0±1.9 | 21.0±6.6 |
| Crude glycerol | 7.7±2.0 | 3.58±0.47 | 11.4±0.6 | 5.0±0.3 | 3.2±0.2 | 8.8±0.8 | 5.2±0.7 | 0.6±0.04 | 9.6±0.8 | 56.3±2.6 |
| Lipid as a carbon source[e] | | | | | | | | | | |
| Canola oill | | | 3.8 | 1.6 | 66.5 | 19.2 | 7.7 | 1.1 | ND[d] | ND |
| Crude glycerol | | | 10.2 | 2.7 | 53.8 | 29.4 | 3.1 | 0.6 | ND | ND |

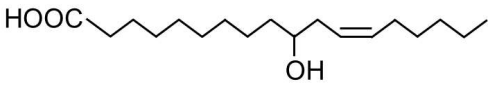
[a]DCW, dry cell weight.

[b]Strain D2 was cultured in a medium containing 2.7% pure glycerol and 1.5% lipid or 6% crude glycerol (about 2.7% glycerol) as a carbon source, and 1% yeast extract as a nitrogen source for 4-days-shaking and 3-days-static conditions. All data are the means ± standard deviation (SD) for triplicate samples.

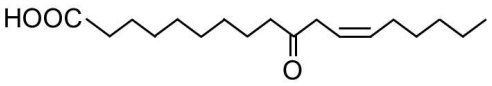
[c]ME, methyl ester.

[d]ND, not detected.

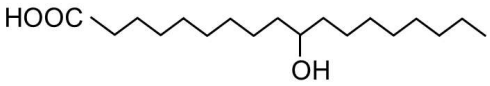
[e]Fatty acid compositions of canola oil and crude glycerol used as carbon sources.



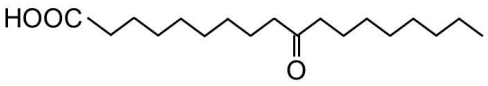

**10-Hydroxy-*cis*-12-octadecenoic acid**
**(HYA)**




**10-Oxo-*cis*-12-octadecenoic acid**
**(KetoA)**

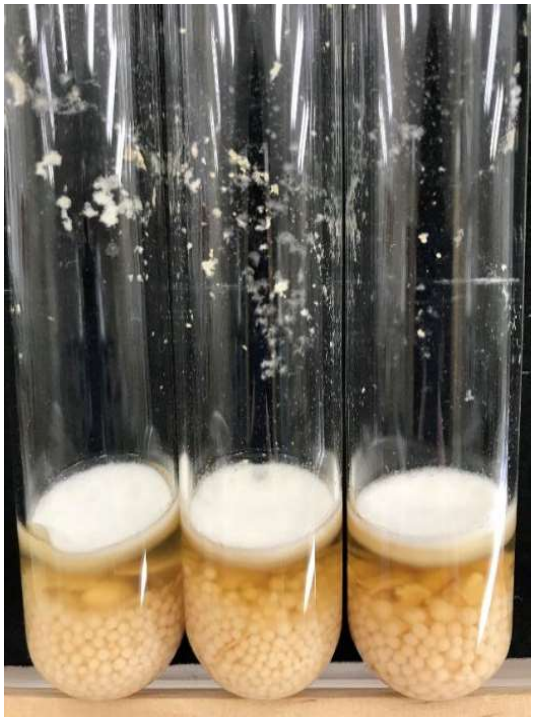



**10-Hydroxyoctadecanoic acid**
**(HYB)**




**10-Oxo-octadecanoic acid**
**(KetoB)**

**Supplementary Fig. S1. Structural formulas of HYA, HYB, KetoA and KetoB.**



**Supplementary Fig. S2. Liquid culture of strain D2 in test tubes.**

After 4 days-shaking and then 3 days-static cultivation, strain D2 formed mycelial pellets in media and mycelial mats on the surface of the media.