JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Use of Biodiesel-Derived Crude Glycerol for Producing Eicosapentaenoic Acid (EPA) by the Fungus *Pythium irregulare*

SNEHA K. ATHALYE,[†] RAFAEL A. GARCIA,[§] AND ZHIYOU WEN*,[†]

Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, and Fats, Oils and Animal Coproducts Research Unit, Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038

Crude glycerol is a major byproduct for the biodiesel industry. Producing value-added products through microbial fermentation on crude glycerol provides opportunities to utilize a large quantity of this byproduct. The objective of this study is to explore the potential of using crude glycerol for producing eicosapentaenoic acid (EPA, 20:5 n-3) by the fungus *Pythium irregulare*. When *P. irregulare* was grown in medium containing 30 g/L crude glycerol and 10 g/L yeast extract, EPA yield and productivity reached 90 mg/L and 14.9 mg/L·day, respectively. Adding pure vegetable oils (flaxseed oil and soybean oil) to the culture greatly enhanced the biomass and the EPA production. This enhancement was due to the oil absorption by the fungal cells and elongation of shorter chain fatty acids (e.g., linoleic acid and α-linolenic acid) into longer chain fatty acid (e.g., EPA). The major impurities contained in crude glycerol, soap and methanol, were inhibitory to fungal growth. Soap can be precipitated from the liquid medium through pH adjustment, whereas methanol can be evaporated from the medium during autoclaving. The glycerol-derived fungal biomass contained about 15% lipid, 36% protein, and 40% carbohydrate, with 9% ash. In addition to EPA, the fungal biomass was also rich in the essential amino acids lysine, arginine, and leucine, relative to many common feedstuffs. Elemental analysis by inductively coupled plasma showed that aluminum, calcium, copper, iron, magnesium, manganese, phosphorus, potassium, silicon, sodium, sulfur, and zinc were present in the biomass, whereas no heavy metals (such as mercury and lead) were detected. The results show that it is feasible to use crude glycerol for producing fungal biomass that can serve as EPA-fortified food or feed.

KEYWORDS: Biodiesel; crude glycerol; soap; methanol; eicosapentaenoic acid (EPA); proximate analysis; fatty acids profile; amino acid composition; elemental composition; heavy metal contamination

INTRODUCTION

Biodiesel as an alternative fuel has attracted increasing attention in recent years. In the United States, for example, the annual biodiesel production has increased sharply from <100 million gallons prior to 2005 to 450 million gallons in 2007 (*1*). During the biodiesel production process, crude glycerol is created as a byproduct. In general, for every gallon of biodiesel produced, 0.3 kg of glycerol is produced (*2*). With biodiesel production growing exponentially, the market is being flooded with crude glycerol. Because it is prohibitively expensive to convert and purify the crude glycerol into material that can be used for food, cosmetics, or pharmaceutical industries, biodiesel producers must seek new uses.

Crude glycerol can be utilized through a variety of methods such as combustion (*3*), composting (*4*), anaerobic digestion (*5*), or feeding for various animals such as pigs (*6*), broiler chickens (*7*), and laying hens (*8*). Converting crude glycerol into valued-added products through thermochemical methods (*3, 9*−*11*) or biological methods (*12*−*15*) is another alternative for utilizing this waste stream.

Our laboratory has been studying the production of docosahexaenoic acid (DHA)-rich algae by using crude glycerol as a substrate (*16*). DHA (C22:6 n-3) is one important omega-3 polyunsaturated fatty acid that has various beneficial effects on human health (*17*). Algae grown in crude glycerol-containing medium can produce DHA at a level comparable to that obtained with a glucose-based culture (*16*). The glycerol-derived biomass was confirmed to contain no heavy metals and had a nutritional quality similar to that of commercial algae (*18*).

In addition to DHA, eicosapentaenoic acid (EPA, C20:5 n-3) is another important fatty acid in the omega-3 family on the

* Corresponding author [e-mail wenz@vt.edu; telephone (540) 231-9356; fax (540) 231-3199].
[†] Virginia Polytechnic Institute and State University.
[§] U.S. Department of Agriculture.

10.1021/jf803922w CCC: $40.75
Published on Web 03/06/2009

basis of its medically established therapeutic capabilities against cardiovascular diseases, cancers, schizophrenia, and Alzheimer's disease (*19*, *20*). Currently, DHA-containing algae have been developed as a replacement of fish oil for omega-3 fatty acids. However, a microbial-based EPA source has not been commercially available. Among various EPA-containing microorganisms, *Pythium irregulare* has shown great potential as an EPA producer. It has been reported that *P. irregulare* is capable of utilizing a variety of organic waste streams for producing EPA (*21*). A recent study also shows this species can use crude glycerol as substrate to produce EPA (*22*).

In general, the crude glycerol contains methanol and soap as the two major impurities, with various elements such as calcium, potassium, phosphorus, magnesium, sulfur, and sodium present. Little work has been done to understand the effects of these impurities on EPA production by *P. irregulare* and to evaluate the composition of *P. irregulare* biomass as a potential EPA fortified food or feed additives. The objective of this work is to address these issues by investigating the effects of crude glycerol impurities on EPA production and biomass composition. The potential benefit of adding vegetable oil to the glycerol-based culture on EPA production enhancement was also investigated.

MATERIALS AND METHODS

Crude Glycerol Source. Crude glycerol used in this study was a dark brown stream obtained from Virginia Biodiesel Refinery (West Point, VA). The refinery used alkali (KOH)-catalyzed transesterification of soybean oil with methanol for producing biodiesel. The crude glycerol resulting from this process (pH ~12) contains approximately 20% (w/w) soap residues. When this glycerol is pH-adjusted to 6.0−6.5, which is more suitable for fungus growth (*21*), soaps are converted into free fatty acids (*18*).

Fungal Species and Culture Conditions. *P. irregulare* (ATCC 10951) was used in this experiment. The organism used to be classified as a fungus; recent taxonomic definitions of *Pythium* have been changed to the Kingdom Chromista (algae) (*23*). In this study, we still use the term fungus to define *Pythium* in order to keep our terminology consistent with other papers (*21*, *24*). The fungus was grown on agar plates that were prepared by dissolving 20 g/L glucose and 10 g/L yeast extract (Acros Organics, Fail Lawn, NJ) in water with the addition of 1.5% (w/w) of agar (Fluka, Sigma-Aldrich, MO). The pH of this agar medium was 5.8−5.9. After 5 days of incubation at a temperature of 25 °C, the agar plates were washed with distilled water containing glass beads to dislodge the spores. This spore suspension was used as inoculum for further trials. The inoculum size was 10% (v/v) of the total culture medium.

In the study of EPA production from crude glycerol, the fungal cells were grown in medium containing 30 g/L crude glycerol and 10 g/L yeast extract. The pH was adjusted to 6.0−6.5 before the medium was autoclaved at 121 °C for 15 min. The cells were grown in 250 mL Erlenmeyer flasks, each containing 50 mL of medium, and incubated at 25 °C in an orbital shaker set to 170 rpm. For each experimental condition, three replicates were used, and the standard deviation was calculated.

Depending on the experimental conditions, soaps were either removed from or remained in the culture medium. The preparation of soap-free medium was the same as described previously (*18*). In brief, the crude glycerol was mixed with distilled water to reduce the fluid viscosity and then pH-adjusted to 3 with hydrochloric acid; the free fatty acids that precipitated from the liquid were separated by centrifugation at 5000 rpm. Then, yeast extract was added to the glycerol solution at the desired level and the medium pH was adjusted to 6.0−6.5. To prepare soap-containing medium, the soap that was originally precipitated from crude glycerol was spiked back into the culture medium at the desired levels.

Analysis. *Cell Dry Weight.* The fungal biomass from each flask was harvested, vacuum-filtered through Whatman no. 1 filter paper, and washed with 25 mL of distilled water. The biomass was then transferred to a preweighed tube and freeze-dried (model 12SL freeze drier, The Virtis Co., Gardiner, NY). After the dry weight had been measured, the freeze-dried biomass was used for proximate, fatty acid, amino acid, and elemental analyses.

Glycerol Concentration. Glycerol concentrations were determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc., Columbia, MD) with a refractive index detector. An Aminex HPX-87H (Bio-Rad, Sunnyvale, CA) column was used with 0.1% (v/v) H_2SO_4 solution as mobile phase. The flow rate was controlled at 0.6 mL/min, and the column temperature was 65 °C.

Proximate Analysis. The lipids from the freeze-dried biomass were extracted and quantified according to the Bligh and Dyer method (*25*). The protein content was estimated by summation of each amino acid. The nonprotein nitrogen (NPN) content was determined by subtracting protein nitrogen (PN) from the total nitrogen (TN) of the biomass. Here, TN was determined according to the Kjehldahl method; PN was estimated by dividing the protein content by 6.25. The ash content was determined by heating the sample at 550 °C overnight and weighing the remaining matter. The carbohydrate was then calculated by subtraction.

Fatty Acid, Amino Acid, and Elemental Analysis. Fungal biomass, vegetable oils, and the soap were analyzed for their fatty acid compositions. The protocols for fatty acid methyl esters (FAME) preparation and the gas chromatography analyses were the same as previously reported (*18*). To analyze amino acid composition, the fungal biomass was first defatted with hexane to reduce interference. The defatted biomass was then analyzed chromatographically for its amino acid composition using the methods described previously (*18*). The elemental composition of the fungal biomass was determined by an inductively coupled plasma semiquantitative scan of 69 elements according to EPA method SW-846 6010B (SuperScan 69 performed by Prochem Analytical Inc., Elliston, VA). EPA method SW-846 7471A was further used to detect any trace amount of mercury possibly contained in the biomass; the detection limit for this measurement was 0.025 ppm (25 ppb).

RESULTS

Growth Characteristics and EPA Production. *P. irregulare* was grown in medium containing 30 g/L crude glycerol and 10 g/L yeast extract. When the impurities in crude glycerol were accounted for, the "real" glycerol concentration in the medium was 22 g/L. As shown in **Figure 1A**, maximum biomass value of 6.31 g/L was reached at day 6 when glycerol was almost completely consumed. **Figure 1B** shows that both the total fatty acids (TFA) and EPA contents increased through the first 3−4 days and leveled off at a later stage of culture. The growth kinetics and EPA production parameters are summarized in **Table 1**. The specific growth rate (0.512 day^{-1}) and the growth yield coefficient based on glycerol (0.33 g/g) were comparable to those of other omega-3 fatty acid-producing species grown in crude glycerol (*18*).

Fungal Culture Added with Vegetable Oils. Pure vegetable oils (soybean oil and flaxseed oil) were added to the culture of *P. irregulare* to investigate if this addition would enhance the fungal growth and EPA production. We ensured the shaking was strong enough to disperse the oil into small droplets so the fungal biomass could access it. As shown in **Figure 2**, addition of vegetable oils appreciably increased the biomass and EPA yield (units = mg/L) compared with the control (oil-free) culture. Soybean oil and flaxseed oil resulted in similar enhancements. However, due to the different fatty acid profiles contained in these two types of vegetable oils, the fatty acid compositions of the resulting biomasses were quite different. As shown in **Table 2**, flaxseed oil had an appreciable amount of α-linolenic acid (C18:3), whereas soybean oil was rich in linoleic acid (C18:2). The fungal biomasses derived from these two types of oil culture had fatty acid distributions similar to

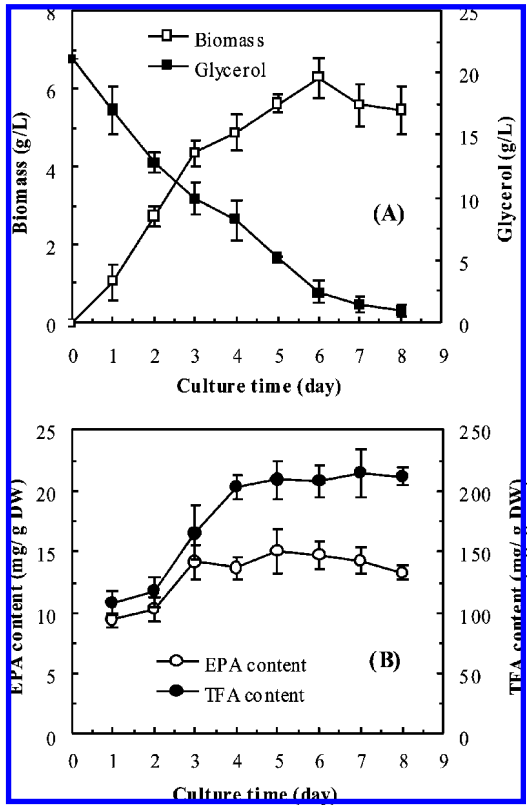

Figure 1. Time course of biomass, residual glycerol concentration (**A**); EPA content and total fatty acids (TFA) content (**B**) of *P. irregulare* grown in medium containing 30 g/L crude glycerol and 10 g/L yeast extract. Data are means of three replicates, and error bars show standard deviations.

Table 1. Cell Growth and EPA Production Parameters of *P. irregulare*[a]

parameter	unit	value
max cell dry wt, X_{max}	g/L	6.31 ± 0.43
specific growth rate	day^{-1}	0.512 ± 0.023
biomass productivity	g/L·day	1.05 ± 0.09
growth yield, $Y_{X/S}$	g/g	0.33 ± 0.02
EPA content	mg/g of DW	14.71 ± 1.01
EPA yield	mg/L	89.76 ± 7.25
EPA productivity	mg/L·day	14.96 ± 1.21

[a] Data are means of three replicates ± standard deviations.

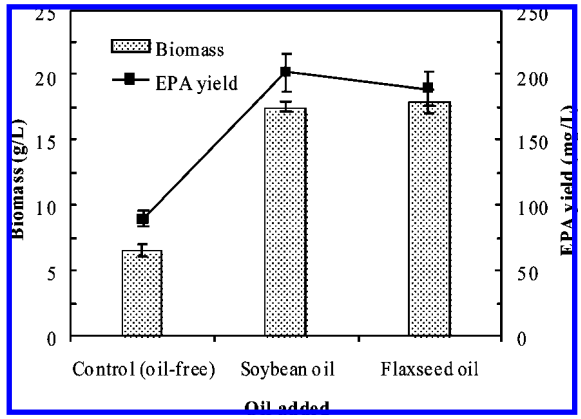

Figure 2. Biomass and EPA yield of *P. irregulare* grown in crude glycerol medium with different vegetable oils added at 1%. Data are means of three replicates, and error bars show standard deviations.

that of the feedstock oil. **Table 2** also shows that the TFA content of the oil-added biomass was much higher than that of the control. However, both EPA percentage (%TFA) and content (mg/g of DW) of the oil-derived biomass were reduced compared with those of the control (**Table 2**). Therefore, the increase of EPA yield was due to the increase in biomass (**Figure 2**).

Effects of Soap. The effects of soap on fungal growth and EPA production were first studied by inoculating fungal spores to the soap-containing medium. However, no cell growth was observed in the soap-containing medium, which may be due to the soap enveloping the fungal spores and cutting off the nutrient and oxygen supplies. To alleviate this inhibition, we grew the spores in soap-free medium for a period of time (3 days) until a clump of fungal mycelium was observed; this mycelium was then inoculated into medium with added soap. With the crude glycerol concentration (30 g/L) and soap content (~20%) commonly used, the soap concentration in the medium would be 6 g/L if not removed. Therefore, we adjusted the soap addition level at 1, 2, 4, and 6 g/L.

As shown in **Figure 3**, the fungal biomass in medium with added soap was lower than that in soap-free medium. The EPA production was also inhibited with the addition of soap, as evidenced by the sharp decrease of EPA yield even with a soap addition as low as 1 g/L (**Figure 3**). **Table 3** shows the fatty acid composition of the soap and the fungal biomass growing in media with different levels of soap. The soap mainly contained C18:1 and C18:2 fatty acids, which accounted for 80% of the TFA. When this soap was added to the medium, independent of the soap amount, the resulting biomass had about 18−19% of C16:0, 31−32% of C18:1, and 35−38% of C18:2. The EPA (C20:5) percentage in TFA of the control (soap-free) biomass was about 12%, whereas this percentage was reduced (0.85−2.31%) when the fungus was grown in soap-containing medium (**Table 3**). It was also found that the soap addition appreciably increased the TFA content of the biomass (**Table 3**). However, this increase could not compensate for the loss of EPA percentage in TFA; as a result, EPA content per unit of biomass was decreased with the addition of soap to the crude glycerol medium.

Effects of Methanol. The crude glycerol used in this work contained ~12% (w/w) methanol. When 30 g/L crude glycerol concentration was used in this study, the methanol concentration was about 3.6 g/L. It was found that this amount of methanol was completely evaporated when the medium was autoclaved at 121 °C for 15 min. To study the "true" effects of methanol on fungal growth and EPA production, we prepared a medium containing 20 g/L pure glycerol spiked with 2, 4, and 8 g/L methanol. The medium was then passed through a 0.2 μm filter before fungal spores were inoculatied. However, we did not observe any growth in the methanol-containing medium (data not shown). The result clearly indicated the negative effects of methanol on growth and EPA production of *P. irregulare*.

Fungal Biomass Composition. The above results indicate that soap and methanol, two major impurities in crude glycerol, were inhibitory to the growth and EPA production of *P. irregulare*. Fortunately, these two impurities can be removed from the culture medium in relatively easy ways. Soap can be precipitated from the solution by pH adjustment and then removed through phase separation; methanol can be evaporated during autoclaving of the medium. In this study, the fungal biomass obtained from this soap-and methanol-free medium was characterized in terms of its nutritional values and elemental composition.

Table 4 shows the proximate analysis of the biomass. Lipid, protein, and carbohydrate were the three major components of

Table 2. Fatty Acid Compositions of the Vegetable Oils and the Fungal Biomass Derived from Oil Addition Cultures[a]

		control (oil free)	flaxseed oil addition		soybean oil addition	
fatty acid	unit	fungal biomass	flaxseed oil	fungal biomass	soybean oil	fungal biomass
C14:0	%TFA[b]	7.76 ± 0.74	nd[c]	0.36 ± 0.01	nd	0.17 ± 0.01
C16:0	%TFA	25.25 ± 3.24	5.51 ± 0.01	11.26 ± 0.01	10.48 ± 0.44	10.75 ± 0.08
C16:1	%TFA	13.58 ± 0.15	nd	1.44 ± 0.09	nd	1.52 ± 0.01
C18:0	%TFA	3.19 ± 0.02	2.87 ± 0.02	4.03 ± 0.09	4.36 ± 0.17	4.67 ± 0.02
C18:1	%TFA	16.74 ± 0.02	16.45 ± 0.03	21.55 ± 0.64	24.83 ± 0.99	26.19 ± 0.08
C18:2 (n-6)	%TFA	18.37 ± 2.32	21.01 ± 0.02	14.28 ± 0.01	56.09 ± 2.48	54.05 ± 0.28
C18:3 (n-3)	%TFA	nd	54.15 ± 0.04	49.09 ± 0.06	6.77 ± 0.33	nd
C20:4 (n-6)	%TFA	6.79 ± 0.77	nd	0.75 ± 0.07	nd	0.89 ± 0.01
C20:5 (n-3)	%TFA	12.55 ± 1.05	nd	2.21 ± 0.16	nd	2.64 ± 0.23
EPA content	mg/g of DW	14.93 ± 0.68	na	10.67 ± 0.72	na	11.42 ± 0.87
TFA content	mg/g of DW	119.01 ± 9.94	na	496.42 ± 14.59	na	427.34 ± 18.89

[a] Data are means of three replicates ± standard deviations. [b] TFA, total fatty acids. [c] nd, not detected; na, not applicable.

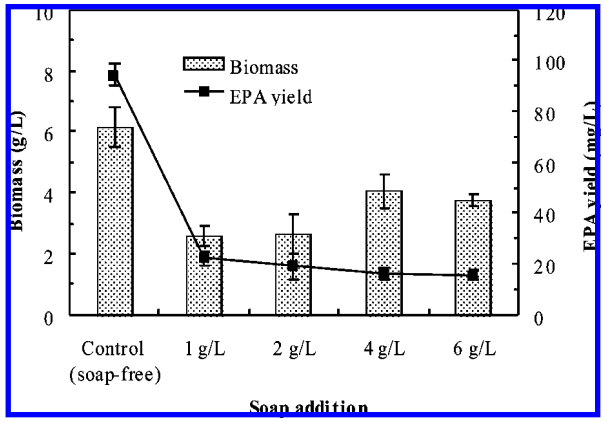

Figure 3. Biomass and EPA yield of *P. irregulare* grown in crude glycerol medium with different levels of soap residues added. Data are means of three replicates, and error bars show standard deviations.

the biomass. The nonprotein nitrogen content of the biomass was 2.31% (**Table 4**). The fatty acid profile of the fungal biomass is presented in **Tables 2** and **3** (the "control"); the major fatty acids were C16:0 and C18 with EPA accounting for 12% of TFA. In addition to EPA, the fungus also contained small amounts of C14:0 and C20:4 (arachidonic acid, AA). The amino acid composition shows that the fungal biomass was rich in asparagine plus aspartic acid and glutamine plus glutamic acid (>10% of total protein each) followed by leucine and lysine (**Table 5**). **Table 6** shows the results of ICP elemental analysis of the fungal biomass. The biomass had a high potassium content, probably due to the high amount of potassium contained in the original crude glycerol solution as the biodiesel producer used a potassium hydroxide based transesterification process. No heavy metals such as mercury or lead were detected by the ICP analysis. Even specialized testing, using EPA method SW-846 7471A with a reporting limit of 0.025 ppm, did not detect any mercury in the algal biomass.

DISCUSSION

P. irregulare has shown to be capable of using a variety of substrates for its growth and EPA production, such as soymeal waste, crude soybean oil, sucrose waste stream (*21*), or sweet whey permeate (*24*). Our previous study also showed the feasibility of growing *P. irregulare* using biodiesel-derived crude glycerol (*22*). The EPA production level obtained from crude glycerol culture was comparable to those reported using glucose or other organic waste materials (*21, 22, 24, 26*).

It has shown that adding soybean oil to glucose-based culture of *P. irregulare* enhanced fungal growth and EPA accumulation (*21*). In this study, we observed a similar phenomenon with the crude glycerol-based culture with added flaxseed oil or soybean oil (**Figure 2**). The increase of biomass was due to the storage of excess oil in the fungal cells. As shown in **Table 2**, flaxseed oil contained an appreciable amount of α-linolenic acid (C18:3 n-3), whereas soybean oil was rich in linoleic acid (C18:2 n-6); when the two oils were added to the culture medium, their resultant biomass contained a high proportion of these two fatty acids. The TFA contents in oil-added biomass were also much higher than that of the control (oil-free). All of these results indicate the storage of excess oil in the fungal cells (**Table 2**).

The storage of linoleic acid (C18:2 n-6) and α-linolenic acid (C18:3 n-3) in the fungal cells can also serve as precursors for the synthesis of longer chained fatty acid such as EPA. In general, EPA can be synthesized through either the n-6 route (i.e., from linoleic acid to arachidonic acid and subsequently to EPA) or the n-3 route (i.e., from α-linolenic acid to EPA) (*27, 28*). In the oil-free culture, *P. irregulare* synthesizes EPA mainly through the n-6 route rather than the n-3 route, because most fungi exhibit high activity of Δ12 desaturase that converts oleic acid into linoleic acid and Δ17 desaturase that converts arachidonic acid into EPA (*27, 29*). The fatty acid distribution profile in the oil-free culture also confirms this hypothesis, as an appreciable amount of linoleic acid (C18:2) and arachidonic acid (C20:4) was in the fungal cells, whereas no α-linolenic acid (C18:3) existed (**Table 2**). When vegetable oils were added to the culture, flaxseed oil mainly provided α-linolenic acid (C18:3), whereas soybean oil mainly provided linoleic acid (C18:2), to the cells. The fungal cells can use these external precursors for *P. irregulare* to synthesize EPA. As a result, the overall EPA yield was increased in the oil addition cultures compared with oil-free culture (**Figure 2**).

It should be noted that the proportions of EPA among total fatty acids (%TFA) and cellular content of EPA (mg/g of DW) were decreased in the oil-added cultures (**Table 2**). These decreases were caused by the "dilution" effect as more fat and biomass were accumulated in these cultures, and not all of this accumulated fat was converted into EPA.

The beneficial effects of adding vegetable oil on the culture of *P. irregulare* led us to explore the possible enhancement of fungal growth and EPA production when soap is included in the medium. Indeed, soap also contained high levels of C18 fatty acids (**Table 3**), which may serve as additional precursors for fungi to synthesize EPA (**Figure 3**). However, both the fungal growth and the fatty acid composition from the soap addition culture (**Figure 3** and **Table 3**) suggest that instead of being beneficial, soap strongly inhibited the fungal growth and EPA production. This inhibitory effect may be caused by the

Table 3. Fatty Acid Compositions of the Soap and Fungal Biomass Growing in Crude Glycerol Medium with Added Soap[a]

			fungal biomass grown at different soap levels				
fatty acid	unit	soap	control (soap-free)	1 g/L	2 g/L	4 g/L	6 g/L
C14:0	%TFA[b]	nd[c]	7.43 ± 0.34	1.46 ± 0.26	1.03 ± 0.00	0.81 ± 0.03	0.84 ± 0.07
C16:0	%TFA	10.89 ± 0.14	21.61 ± 0.40	19.06 ± 0.15	18.92 ± 0.80	18.98 ± 0.46	19.3 ± 0.57
C16:1	%TFA	nd	12.46 ± 1.14	4.59 ± 0.19	4.07 ± 0.22	3.76 ± 0.02	3.88 ± 0.19
C18:0	%TFA	5.42 ± 0.23	1.51 ± 0.06	4.37 ± 0.19	4.59 ± 0.01	4.82 ± 0.03	4.72 ± 0.03
C18:1	%TFA	24.38 ± 1.47	16.57 ± 0.42	31.51 ± 0.16	31.93 ± 1.08	32.23 ± 0.22	32.48 ± 0.24
C18:2 (n-6)	%TFA	56.77 ± 2.88	18.17 ± 0.4	35.42 ± 0.72	37.07 ± 0.42	38.05 ± 0.39	37.31 ± 1.03
C18:3 (n-3)	%TFA	4.83 ± 0.21	nd	nd	nd	nd	nd
C20:4 (n-6)	%TFA	nd	7.07 ± 0.78	1.29 ± 0.21	0.92 ± 0.01	0.63 ± 0.04	0.57 ± 0.02
C20:5 (n-3)	%TFA	nd	11.89 ± 0.56	2.31 ± 0.17	1.48 ± 0.09	0.85 ± 0.05	0.89 ± 0.12
EPA content	mg/g of DW	na	15.31 ± 0.71	8.63 ± 0.41	7.12 ± 0.62	3.97 ± 0.26	4.14 ± 0.55
TFA content	mg/g of DW	na	128.79 ± 11.90	373.87 ± 17.56	481.49 ± 41.92	525.89 ± 22.39	465.94 ± 32.35

[a] Data are means of three replicates ± standard deviations. [b] TFA, total fatty acids. [c] nd, not detected; na, not applicable.

Table 4. Proximate Analysis of Freeze-Dried Fungal Biomass Grown on Crude Glycerol[a]

composition	mass % of dry biomass
lipid	15.29 ± 0.59
protein	35.64 ± 1.41
carbohydrate	40.09 ± 0.74
total nitrogen	8.02 ± 0.04
nonprotein nitrogen	2.31 ± 0.07
ash	8.97 ± 0.63

[a] Data are means of three replicates ± standard deviations.

Table 5. Amino Acid Composition of Fungal Biomass Grown on Crude Glycerol[a]

amino acid	mass % of protein	mg/g of dry biomass
Asx[b]	10.18 ± 0.07	36.28 ± 1.27
Ser	4.34 ± 0.03	15.47 ± 0.56
Glx[b]	15.60 ± 0.16	55.62 ± 2.20
Gly	4.21 ± 0.01	15.03 ± 0.61
His	2.60 ± 0.01	9.29 ± 0.41
NH_3[c]	2.84 ± 0.02	10.12 ± 0.43
Arg	6.54 ± 0.07	23.33 ± 1.00
Thr	4.89 ± 0.01	17.43 ± 0.70
Ala	5.61 ± 0.01	20.01 ± 0.75
Pro	3.61 ± 0.04	12.89 ± 0.57
Tyr	4.11 ± 0.04	14.66 ± 0.65
Val	5.87 ± 0.09	20.94 ± 0.99
Ile	4.88 ± 0.08	17.41 ± 0.83
Leu	7.71 ± 0.10	27.48 ± 1.24
Lys	7.67 ± 0.08	27.36 ± 1.18
Phe	4.90 ± 0.07	17.48 ± 0.82
Cys[d]	1.93 ± 0.15	6.88 ± 0.48
Met[d]	2.44 ± 0.17	8.71 ± 0.64

[a] Data are means of three replicates ± standard deviations. [b] Glx = Glu + Gln; Asx = Asp + Asn. [c] NH_3 resulted from the deamination of asparagine and glutamine during the analysis process. [d] Cys and Met were oxidized quatitatively and measured as cysteic acid and methionine sulfone, respectively.

Table 6. Elemental Composition of Fungal Biomass As Detected by ICP Analysis[a]

element	detection limit (ppm)	content (mg/kg)
aluminum	10	12.10 ± 1.68
calcium	50	528 ± 31
copper	5	12.55 ± 0.21
iron	10	56.75 ± 3.89
magnesium	50	310 ± 2
manganese	2	2.95 ± 0.64
phosphorus	50	7795 ± 134
potassium	100	19700 ± 1131
silicon	10	72.8 ± 5.5
sodium	50	1380 ± 127
sulfur	100	3875 ± 191
zinc	2	122 ± 8

[a] Data are means of three replicates ± standard deviations. A total of 69 elements as described in Table 1 of ref *18* were analyzed; the elements listed in this table were detected in the fungal biomass.

complex interaction between the cell wall/membrane and the soap (*30*). As a matter of fact, we observed physical attachment of the soap on the biomass when the fungus was grown in soap-containing medium. After 3−4 days of incubation of the fungus in soap-containing medium, the initial "cloudy" medium solution became clear, whereas the surface of the fungal mycelium was covered by greasy soaps.

In addition to soap, methanol also exhibited inhibitory effects on fungal growth, although the mechanism of this inhibition was unclear. In a similar study of producing DHA from crude glycerol by the alga *Schizochytrium limacinum*, a similar negative effect of methanol on algal growth was observed.

Currently, there have been no reports on the chemical composition analyses of EPA-producing fungi, particularly *P. irregulare*. We therefore use the composition of DHA-producing *S. limacinum* derived from crude glycerol (*18*) as "baseline" data to evaluate the quality of *P. irregulare* biomass. The chemical analysis of the biomass obtained from crude glycerol culture (soap- and methanol-free) shows that carbohydrate accounted for the biggest portion of the dried biomass followed by protein and the lipid (**Table 4**). Compared with *S. limacinum*, *P. irregulare* has fewer lipids but more proteins. The biomass is rich in the essential amino acids lysine, arginine, and leucine, which will increase its value as a feed ingredient. Overall, this amino acid distribution was similar to that of *S. limacinum* (*18*). Elemental analysis shows that the fungal biomass did not contain any toxic heavy metals such as mercury or lead (**Table 6**). However, modest amounts of aluminum and zinc need to be considered when the fungal biomass is developed as food or animal feed, as excessive levels of these metals can be damaging.

In summary, the above results indicate the great potential of producing EPA from biodiesel-derived crude glycerol by fungal fermentation. However, compared with microalgae (e.g., diatom) for EPA production, particularly at heterotrophic conditions (*31*−*33*), the biomass, EPA content, and EPA yield obtained from this work were still low. This may be due to the use of crude carbon substrate (crude glycerol) rather than pure glucose, which was used for heterotrophic algal cultivation. To fully realize the potential benefits of fungal EPA production from crude glycerol, future work is needed to thoroughly optimize culture conditions and develop high cell density culture techniques.

LITERATURE CITED

(1) http://www.biodiesel.org/pdf_files/fuelfactsheets/Production_Graph_Slide.pdf; National Biodiesel Board, 2008.

(2) Thompson, J. C.; He, B. B. Characterization of crude glycerol from biodiesel production from multiple feedstocks. *Appl. Eng. Agric.* **2006**, *22*, 261–265.

(3) Johnson, D. T.; Taconi, K. A. The glycerin glut: options for the value-added conversion of crude glycerol resulting from biodiesel production. *Environ. Prog.* **2007**, *26*, 338–348.

(4) Brown, R. http://www.icastusa.org/images/projects/biodiesel/biodiesel_coproduct_markets.pdf; Biodiesel Co-Product Markets in Wyoming, Wyoming Department of Agriculture, and International Center for Appropriate and Sustainable Technology, Lakewood, CO, 2007.

(5) Holm-Nielsen, J. B.; Lomborg, C. J.; Oleskowicz-Popiel, P.; Esbensen, K. H. On-line near infrared monitoring of glycerol-boosted anaerobic digestion processes: evaluation of process analytical technologies. *Biotechnol. Bioeng.* **2008**, *99*, 302–313.

(6) Lammers, P. J.; Kerr, B. J.; Weber, T. E.; Dozier, W. A.; Kidd, M. T.; Bregendahl, K.; Honeyman, M. S. Digestible and metabolizable energy of crude glycerol for growing pigs. *J. Anim. Sci.* **2008**, *86*, 602–608.

(7) Cerrate, S.; Yan, F.; Wang, Z.; Coto, C.; Sacakli, P.; Waldroupand, P. W. Evaluation of glycerine from biodiesel production as a feed ingredient for broilers. *Int. J. Poult. Sci.* **2006**, *5*, 1001–1007.

(8) Lammers, P. J.; Kerr, B. J.; Honeyman, M. S.; Stalder, K.; Dozier, W. A.; Weber, T. E.; Kidd, M. T.; Bregendahl, K. Nitrogen-corrected apparent metabolizable energy value of crude glycerol for laying hens. *Poult. Sci.* **2008**, *87*, 104–107.

(9) Alhanash, A.; Kozhevnikova, E. F.; Kozhevnikov, I. V. Hydrogenolysis of glycerol to propanediol over Ru: polyoxometalate bifunctional catalyst. *Catal. Lett.* **2008**, *120*, 307–311.

(10) Dasari, M. A.; Kiatsimkul, P. P.; Sutterlin, W. R.; Suppes, G. J. Low-pressure hydrogenolysis of glycerol to propylene glycol. *Appl. Catal. A: Gen.* **2005**, *281*, 225–231.

(11) Chiu, C. W.; Dasari, M. A.; Sutterlin, W. R.; Suppes, G. J. Removal of residual catalyst from simulated biodiesel's crude glycerol for glycerol hydrogenolysis to propylene glycol. *Ind. Eng. Chem. Res.* **2006**, *45*, 791–795.

(12) Lee, P. C.; Lee, W. G.; Lee, S. Y.; Chang, H. N. Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon source. *Biotechnol. Bioeng.* **2001**, *72*, 41–48.

(13) Papanikolaou, S.; Muniglia, L.; Chevalot, I.; Aggelis, G.; Marc, I. Yarrowia lipolytica as a potential producer of citric acid from raw glycerol. *J. Appl. Microbiol.* **2002**, *92*, 737–744.

(14) Rymowicz, W.; Rywinska, A.; Zarowska, B.; Juszczyk, P. Citric acid production from raw glycerol by acetate mutants of *Yarrowia lipolytica*. *Chem. Pap.* **2006**, *60*, 391–394.

(15) Papanikolaou, S.; Fick, M.; Aggelis, G. The effect of raw glycerol concentration on the production of 1,3-propanediol by *Clostridium butyricum*. *J. Chem. Technol. Biotechnol.* **2004**, *79*, 1189–1196.

(16) Chi, Z.; Pyle, D.; Wen, Z.; Frear, C.; Chen, S. A laboratory study of producing docosahexaenoic acid from biodiesel-waste glycerol by microalgal fermentation. *Process Biochem.* **2007**, *42*, 1537–1545.

(17) Nettleton, J. A. *Omega-3 Fatty Acids and Health*; Chapman and Hall: New York, 1995.

(18) Pyle, D. J.; Garcia, R. A.; Wen, Z. Y. Producing docosahexaenoic acid (DHA)-rich algae from blodiesel-derived crude glycerol: effects of impurities on DHA production and algal biomass composition. *J. Agric. Food Chem.* **2008**, *56*, 3933–3939.

(19) Simopoulos, A. P. Essential fatty acids in health and chronic disease. *Am. J. Clin. Nutr.* **1999**, *70*, 560S–569S.

(20) Ursin, V. M. Modification of plant lipids for human health: development of functional land-based omega-3 fatty acids. *J. Nutr.* **2003**, *133*, 4271–4274.

(21) Cheng, M. H.; Walker, T. H.; Hulbert, G. J.; Raman, D. R. Fungal production of eicosapentaenoic and arachidonic acids from industrial waste streams and crude soybean oil. *Bioresour. Technol.* **1999**, *67*, 101–110.

(22) Wen, Z.; Pyle, D. J.; Athalye, S. K. Chapter 5: Production of omega-3 polyunsaturated fatty acids from biodiesel-derived crude glycerol by microalgal and fungal fermentation. In *Microbial Conversions of Raw Glycerol*; Aggelis G., Ed.; Nova Science Publisher: Hauppauge, NY, 2009 (in press).

(23) GBIF. http://data.gbif.org/species/Global; Biodiversity Information Facility, 2008.

(24) Obrien, D. J.; Kurantz, M. J.; Kwoczak, R. Production of eicosapentaenoic acid by the filamentous fungus *Pythium irregulare*. *Appl. Microbiol. Biotechnol.* **1993**, *40*, 211–214.

(25) Bligh, E. G.; Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol* **1959**, *37*, 911–917.

(26) Stinson, E. E.; Kwoczak, R.; Kurantz, M. J. Effect of cultural conditions on production of eicosapentaenoic acid by *Pythium irregulare*. *J. Ind. Microbiol.* **1991**, *8*, 171–178.

(27) Shimizu, S.; Kawashima, H.; Akimoto, K.; Shinmen, Y.; Yamada, H. Microbial conversion of an oil containing α-linolenic acid to an oil containing eicosapentaenoic acid. *J. Am. Oil Chem. Soc.* **1989**, *66*, 342–347.

(28) Wen, Z. Y.; Chen, F. Heterotrophic production of eicosapentaenoic acid by microalgae. *Biotechnol. Adv.* **2003**, *21*, 273–294.

(29) Jareonkitmongkol, S.; Shimizu, S.; Yamada, H. Production of an eicosapentaenoic acid-containing oil by a delta-12 desaturase-defective mutant of *Mortierella alpina* 1s-4. *J. Am. Oil Chem. Soc.* **1993**, *70*, 119–123.

(30) Ukeles, R. Inhibition of unicellular algae by synthetic surface-active agents. *J. Phycol.* **1965**, *1*, 102–110.

(31) Lebeau, T.; Robert, J. M. Diatom cultivation and biotechnologically relevant products. Part II: Current and putative products. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 624–632.

(32) Wen, Z. Y.; Chen, F. A perfusion-cell bleeding culture strategy for enhancing the productivity of eicosapentaenoic acid by *Nitzschia laevis*. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 316–322.

(33) Wen, Z. Y.; Chen, F. Application of statistically-based experimental designs for the optimization of eicosapentaenoic acid production by the diatom *Nitzschia laevis*. *Biotechnol. Bioeng.* **2001**, *75*, 159–169.

Received for review December 17, 2008. Revised manuscript received February 10, 2009. Accepted February 10, 2009. We gratefully acknowledge the Virginia Tech Institute for Critical Technology and Applied Science (ICTAS), Fats and Proteins Research Foundation, and Virginia Sea Grant for their financial support of this project. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF803922W