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Producing Docosahexaenoic Acid (DHA)-Rich Algae from Biodiesel-Derived Crude Glycerol: Effects of Impurities on DHA Production and Algal Biomass Composition

DENVER J. PYLE,[†] RAFAEL A. GARCIA,[§] AND ZHIYOU WEN*[,†,#]

Department of Biological Systems Engineering and Institute for Critical Technology and Applied Science, 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 the primary byproduct of the biodiesel industry. Producing docosahexaenoic acid (DHA, 22:6 n-3) through fermentation of the alga *Schizochytrium limacinum* on crude glycerol provides a unique opportunity to utilize a large quantity of this byproduct. The objective of this work is to investigate the effects of impurities contained in the crude glycerol on DHA production and algal biomass composition. Crude glycerol streams were obtained from different biodiesel refineries. All of the glycerol samples contained methanol, soaps, and various elements including calcium, phosphorus, potassium, silicon, sodium, and zinc. Both methanol and soap were found to negatively influence algal DHA production; these two impurities can be removed from culture medium by evaporation through autoclaving (for methanol) and by precipitation through pH adjustment (for soap). The glycerol-derived algal biomass contained 45−50% lipid, 14−20% protein, and 25% carbohydrate, with 8−13% ash content. Palmitic acid (C16:0) and DHA were the two major fatty acids in the algal lipid. The algal biomass was rich in lysine and cysteine, relative to many common feedstuffs. Elemental analysis by inductively coupled plasma showed that boron, calcium, copper, iron, magnesium, phosphorus, potassium, silicon, sodium, and sulfur were present in the biomass, whereas no heavy metals (such as mercury) were detected in the algal biomass. Overall, the results show that crude glycerol was a suitable carbon source for algal fermentation. The crude glycerol-derived algal biomass had a high level of DHA and a nutritional profile similar to that of commercial algal biomass, suggesting a great potential for using crude glycerol-derived algae in omega-3-fortified food or feed.

KEYWORDS: Biodiesel; crude glyercol; soap; methanol; docosahexaenoic acid (DHA); algal biomass; proximate analysis; fatty acids profile; amino acid composition; elemental composition; heavy metal contamination

INTRODUCTION

In recent years, biodiesel has become an important source of alternative fuel for the United States. The National Biodiesel Board has projected an annual production of biodiesel in 2007 of 450 million gallons, a sharp increase from <100 million gallons prior to 2005 (*1*). The major byproduct of biodiesel production is crude glycerol. In general, for every gallon of biodiesel produced, 0.3 kg of glycerol is generated (*2*). As biodiesel production skyrockets, the market is being flooded with crude glycerol. Indeed, crude glycerol prices have dropped from $0.25/lb in 2004 to $0.025−0.05/lb in 2006 (*3, 4*) because the current U.S. demand for glycerol is not large enough for all of this excess glycerol. It is clear that new uses for biodiesel-derived crude glycerol are needed.

Several avenues for utilizing this crude glycerol have been investigated. For example, glycerol can be thermochemically converted into propylene glycol (*5*) or acetol (*6*). It can also be used in fermentation processes to produce 1,3-propanediol (*7*), lipids (*8–10*), pigments (*9*), and a mixture of succinic acid, butanol, ethanol, and hydrogen (*11*). Recently, research conducted in our laboratory has shown that crude glycerol can be used to produce docosahexaenoic acid (DHA, 22:5 n-3) through fermentation of the alga *Schizochytrium limacinum* (*12*).

* Address correspondence to this author at the Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 [e-mail wenz@vt.edu; fax (540) 231-3199].

[†] Department of Biological Systems Engineering, Virginia Polytechnic Institute and State University.

[§] U.S. Department of Agriculture.

[#] Institute for Critical Technology and Applied Science, Virginia Polytechnic Institute and State University.

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DHA is an important omega-3 polyunsaturated fatty acid (n-3 PUFA) that has been shown to have beneficial effects on preventing human cardiovascular diseases, cancer, schizophrenia, and Alzheimer's disease (*13*). DHA also plays an important role in infant brain and retinal development (*14*). In the aquaculture industry, n-3 PUFAs are essential nutrients for cultured marine fish. The major commercial source of n-3 PUFAs is fish oil, which faces challenges such as odor/taste problems, heavy metal contamination, and limited supply (*15*). Currently, the aquaculture industry is experiencing rapid increases in fish oil price due to flat supply and increased global demand for this commodity. In fact, the Food and Agriculture Organization (FAO) of the United Nations predicts that fish oil demand in 2015 will be 145% of historical global production capacity (*16*). The inability to expand fish oil production makes development of fish oil alternatives imperative.

Developing DHA-containing microalgae from biodiesel waste glycerol is an excellent opportunity to provide alternative omega-3 sources. The fatty acids can be extracted from algae and used in fortified foods, or the biomass can be used directly as a feed additive in various animal industries such as aquaculture (*17*) or poultry (*18*). In addition, potential environmental impacts associated with crude glycerol disposal could be minimized.

Although it has been shown that crude glycerol from the biodiesel industry can support the growth and DHA production of *S. limacinum* (*12*), a thorough chemical characterization of the algal biomass is needed before the alga-derived omega-3 fatty acids are used as food or feed additives. In general, the composition of crude glycerol varies from plant to plant; it contains methanol and soap as the two major impurities, with various elements such as calcium, potassium, phosphorus, magnesium, sulfur, and sodium present. Although the biodiesel manufacturing process does not involve any heavy metals, the oil feedstock may contain trace amounts of heavy metals, which may eventually end up in algal cells. The objective of this work is to investigate the effects of these impurities on algal DHA production and the "quality" of algal biomass in terms of its elemental composition and nutritional levels. Ensuring that algal biomass can be easily produced and safely utilized will open new markets for biodiesel-derived crude glycerol.

MATERIALS AND METHODS

Algal Strain, Medium, and Culture Conditions. *S. limacinum* SR-21 (ATCC MYA-1381) was used. The cells were maintained in a medium containing 10 g/L glucose, 1.0 g/L yeast extract, and 1.0 g/L peptone in artificial seawater. The artificial seawater contained (per liter) 18 g of NaCl, 2.44 g of $MgSO_4 \cdot 7H_2O$, 0.6 g of KCl, 1.0 g of $NaNO_3$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.05 g of KH_2PO_4, 1.0 g of Tris buffer (Sigma Co.), 0.027 g of NH_4Cl, 15.0×10^{-8} g of vitamin B_{12}, 3 mL of chelated iron solution, and 10 mL of trace element solution including boron, cobalt, manganese, zinc, and molybdenum (*19*). The pH was adjusted to 7.5−8.0 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 20 °C in an orbital shaker set to 170 rpm. Subcultured cells were used as inoculum. The inoculum size was 10% of the total liquid volume in each flask. In the study of DHA production from crude glycerol, all of the medium components were the same as those used in the subculture except that different concentrations of crude glycerol were used to replace glucose.

Crude Glycerol Sources and Soap Removal Procedures. Different crude glycerol streams were tested, including (G1) glycerol derived from soybean oil by Virginia Biodiesel Refinery (West Point, VA), (G2) glycerol from a 50:50 (w/w) chicken fat and soybean oil mixture by Virginia Biodiesel, and (G3) glycerol from canola oil by Seattle Biodiesel LLC (Seattle, WA). Both of the biodiesel plants used alkali-catalyzed transesterification of oil with methanol to produce the biodiesel. Virginia Biodiesel used KOH as catalyst, whereas Seattle Biodiesel used NaOH as catalyst.

Table 1. Detectable Limits of Various Elements with ICP Analysis

element	detection limit (ppm)	element	detection limit (ppm)
aluminum	10	nickel	5
antimony	20	niobium	50
arsenic	50	osmium	10
barium	5	palladium	50
berylium	2	phosphorus	50
bismuth	10	platinum	50
boron	10	potassium	100
cadmium	2	praseodymium	50
calcium	50	rhenium	50
cerium	50	rhodium	100
chromium	2	ruthenium	50
cobalt	5	samarium	50
copper	2	scandium	10
dysprosium	10	selenium	50
erbium	10	silicon	10
europium	10	silver	2
gadolinium	20	sodium	50
gallium	20	strontium	10
germanium	50	sulfur	100
gold	20	tantalum	50
hafnium	50	tellurium	100
holminum	50	terbium	50
indium	50	thallium	50
iodine	200	thorium	50
iridium	50	thulium	50
iron	10	tin	10
lanthanum	50	titanium	2
lead	20	tungsten	50
lithium	10	uranium	100
lutetium	10	vanadium	5
magnesium	50	ytterbium	10
manganese	2	ytrtrium	2
mercury[a]	50	zinc	2
molybdenum	10	zirconium	10
neodymium	20		

[a] EPA method SW-846 7471A was also used to detect trace amounts of mercury with a detection limit of 0.025 ppm.

All of the crude glycerol samples contained soaps, which were formed from a side reaction. The soaps can be split into free fatty acids (FFAs) and salt by adding a strong acid to the glycerol to neutralize the catalyst, that is

(1)

Depending on the experimental conditions, soaps either remained in or were removed from the algal culture medium. To prepare soap-free medium, the following procedures were used: (i) the glycerol was mixed with distilled water at a ratio of 1:4 (v/v) to reduce the viscosity of the fluid, (ii) the pH of the fluid was adjusted to 3 with hydrochloric acid to convert soap into free fatty acids that precipitated from the liquid, (iii) precipitated free fatty acids were separated from the crude glycerol solution by centrifugation at 5000 rpm, and (iv) other nutrients (mineral salts, nitrogen source, etc.) were added to the glycerol solution to adjust to the desired levels.

Analysis. *Cell Dry Weight and Glycerol and Methanol Concentrations.* Cell dry weight was determined as previously reported (*12*). Glycerol and methanol concentrations were determined by a Shimadzu Prominence HPLC System (Shimadzu Scientific Instruments, Inc., Columbia, MD). The detailed procedures were described previously (*12*).

Proximate Analysis. Algal biomass was freeze-dried prior to proximate analysis. The lipids from the algal biomass were extracted

Table 2. Composition of Methanol and Soap Residues in Crude Glycerol Streams[a]

		crude glycerol stream		
composition	unit	G1	G2	G3
methanol	%, w/w	12.79 ± 0.75	14.41 ± 2.70	28.27 ± 1.99
soap	%, w/w	25.17 ± 1.70	23.24 ± 4.49	15.28 ± 3.27
glycerol	%, w/w	62.04 ± 1.26	62.35 ± 3.89	56.45 ± 2.98

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

and quantified according to the Bligh and Dyer method (*20*). The crude protein content was estimated by measuring the Kjeldahl nitrogen content and multiplying the result by 6.25. The ash content was determined by heating the sample at 550 °C overnight and then weighing the remaining matter. The carbohydrate was then calculated by subtraction.

Fatty Acid Analysis. Fatty acid compositions of the lyophilized algal biomass and of free fatty acid from the crude glycerol were determined. Fatty acid methyl esters (FAME) were prepared by direct methylation with 5% methanolic HCl (*21–23*) and determined by a Shimadzu 2010 gas chromatograph (Shimadzu Scientific Instruments) equipped with a flame ionization detector and a SGE SolGel-Wax capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used carrier gas. The temperature settings for injector, column, and detector were described previously (*12*). The fatty acids were identified by comparing the retention times with those of standard fatty acids (Nu-Chek Prep Inc., Elysian, MN) and quantified by comparing their peak area with that of the internal standard (C17:0) (*12*).

Amino Acid Analysis. Freeze-dried algal biomass (~25 mg) was hydrolyzed in triplicate using a PicoTag workstation (Waters Corp., Milford, MA) according to the manufacturer's directions. Hydrolyzed samples were filtered, dried under vacuum, and derivatized with AccQFluor reagent (Waters) following the manufacturer's directions. Chromatography was performed using procedures described as "mixture 1" by van Wandelen and Cohen (*24*), with α-aminobutyric acid as an internal standard. Separate analyses were performed for cyst(e)ine, using the method described by Finley (*25*) to quantitatively oxidize cysteine and cystine to cysteic acid prior to hydrolysis; these samples were then analyzed in the same manner as the other samples.

Elemental Analysis. Elemental composition of the crude glycerol and algal biomass was determined by an inductively coupled plasma semi-quantitative scan of 69 elements according to EPA method SW-846 6010B (SuperScan 69 performed by Prochem Analytical Inc., Elliston, VA). The elements and their detection limits are listed in **Table 1**. In addition, EPA method SW-846 7471A was further used to detect any trace amount of mercury possibly contained in the algal biomass; the detection limit for this measurement was 0.025 ppm (25 ppb).

RESULTS AND DISCUSSION

Characterization of Crude Glycerol. The crude glycerol used in this work had a dark brown color with a high pH level (*11, 12*). Because the biodiesel refineries used excess methanol to drive the transesterification toward a maximum biodiesel yield, the crude glycerol contained methanol. Soap was also found in the crude glycerol due to a side reaction. Considering the difficulty to determine the exact amount of soap dissolved in the crude glycerol solution, we used free fatty acid precipitated from the crude glycerol (eq 1) to roughly estimate the amount of the soap residue.

As shown in **Table 2**, glycerols G1 and G2 contained 12−15% methanol and 23−25% soap, wheras crude glycerol from Seattle Biodiesel (G3) had less soap and more methanol, indicating a more complete transesterification but more methanol being used by this refinery. The glycerol contents in these crude streams were around 55−62%. Glycerol purity in crude glycerol streams has been reported in a wide range from 65 (*7*) to 85% (*26*), which probably results from different glycerol purification procedures used by biodiesel plants.

Table 3. Fatty Acid Composition (Percent of Total Fatty Acid, TFA) of the Soaps[a]

		soap source		
fatty acid	unit	G1	G2	G3
16:0	% TFA	11.61 ± 1.05	19.15 ± 2.82	11.14 ± 0.00
16:1	% TFA	ND[b]	2.70 ± 0.24	ND
18:0	% TFA	4.01 ± 0.89	3.07 ± 2.66	3.61 ± 0.04
18:1	% TFA	22.97 ± 0.39	30.87 ± 3.59	23.53 ± 0.08
18:2	% TFA	54.35 ± 1.47	39.58 ± 1.43	54.46 ± 0.16
18:3 n-3	% TFA	7.05 ± 0.11	4.63 ± 0.34	7.25 ± 0.20
TFA	mg/g of DW	866.46 ± 73.53	830.04 ± 59.93	895.94 ± 19.49

[a] Data are means of three replicates ± standard deviations. [b] Not detected.

Table 4. Elemental Composition of Crude Glycerol Streams As Detected by ICP Analysis (Elements Analyzed in **Table 1** But Not Included in This Table Were Not Detected in Any Sample)[a]

		crude glycerol		
element	unit	G1	G2	G3
boron	ppm	BDL[b]	BDL	11.50 ± 0.75
calcium	ppm	BDL	140 ± 36	BDL
phosphorus	ppm	BDL	480 ± 104	BDL
potassium	ppm	31250 ± 212	27300 ± 5515	BDL
silicon	ppm	62 ± 23	29.80 ± 6.36	17.30 ± 2.83
sodium	ppm	214 ± 45	248 ± 45	12550 ± 2757
zinc	ppm	2.50 ± 0.25	2.30 ± 0.15	2.30 ± 0.15

[a] Data are means of three replicates ± standard deviations. [b] Below detection limit.

The fatty acid compositions of the soap residues were also determined. As shown in **Table 3**. for all three soaps residues, oleic acid (18:1) and linoleic acid (18:2) were the two major fatty acids. G1 and G3 had very similar fatty acid profiles with high levels of linoleic acid, whereas G2 had a relatively higher portion of oleic acid (18:1). These fatty acid profiles were an indication of different feedstocks used in the biodiesel production.

Table 4 shows ICP elemental analysis of the crude glycerol. Potassium was present at high levels in G1 and G2, whereas sodium was the major element in G3. This was attributed to the fact that G1 and G2 came from Virginia Biodiesel using KOH as a catalyst, whereas G3 was provided by Seattle Biodiesel utilizing NaOH as a catalyst. Trace amounts of boron, calcium, phosphorus, silicon, and zinc were also found in the crude glycerol samples (**Table 4**). No heavy metals (such as mercury) were found in any of these samples, suggesting crude glycerol can be used as a safe substrate for algal DHA production. **Table 4** also shows that the standard deviations for potassium, silicon, and sodium were relatively high, indicating the fluctuation of these elements in each individual sample.

The above results show that the composition of crude glycerol streams varied depending on the feedstocks and biodiesel production processes employed by the refineries. However, all batches of crude glycerol consisted of methanol and soaps as the major impurities. The effects of these two components on DHA production by *S. limacinum* were further investigated.

Effects of Methanol on Algal DHA Production. It has been reported that ethanol is beneficial for the growth of some microalgae producing DHA (*27*). However, the effect of methanol on algal growth is unknown; this leads us to explore the effects of methanol on DHA production by *S. limacinum*. The algae were grown in a medium containing pure glycerol (70 g/L) with different concentrations of methanol. Considering the fact that methanol evaporates at 65 °C, the autoclave process (at 121 °C for 15 min) resulted in a significant loss of methanol. We did not autoclave the methanol in the medium; instead, the

Table 5. Effects of Methanol Concentration on Growth and DHA Production of *S. limacinum* (Grown in 70 g/L of Pure Glycerol)[a]

		methanol concentration			
parameter	unit	0 g/L	10 g/L	15 g/L	20 g/L
specific growth rate, μ	day^{-1}	0.59 ± 0.01	0.50 ± 0.03	0.52 ± 0.03	0.32 ± 0.01
max cell dry wt	g/L	11.49 ± 0.49	10.31 ± 0.07	8.36 ± 0.31	5.63 ± 0.47
biomass productivity	g/L-day	1.92 ± 0.08	1.72 ± 0.01	1.39 ± 0.05	0.94 ± 0.08
growth yield	g/g	0.23 ± 0.01	0.23 ± 0.01	0.18 ± 0.01	0.13 ± 0.01
DHA content	mg/g of DW	146.4 ± 6.9	158.5 ± 17.7	118.7 ± 14.9	124.6 ± 2.2
DHA yield	g/L	1.68 ± 0.11	1.63 ± 0.18	0.99 ± 0.13	0.70 ± 0.06
DHA productivity	g/L-day	0.28 ± 0.02	0.27 ± 0.03	0.17 ± 0.02	0.12 ± 0.01

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

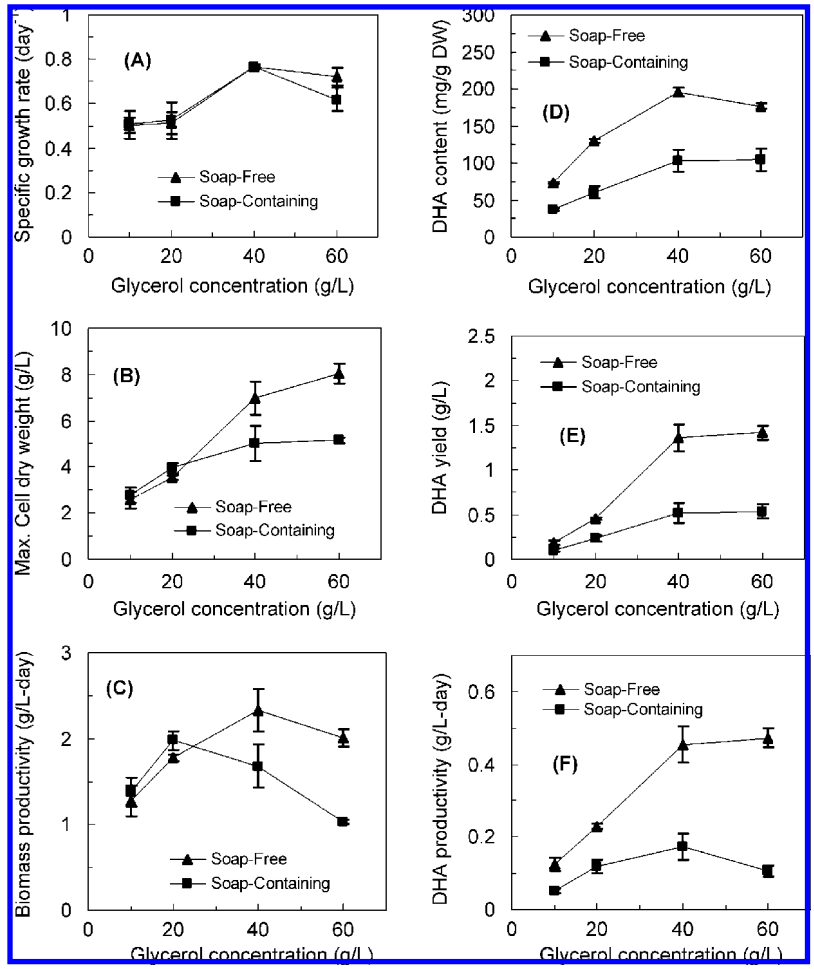

Figure 1. Cell growth performance (**A**−**C**) and DHA production (**D**−**F**) by *S. limacinum* on soap-free and soap-containing media with different crude glycerol concentrations. Data are means of three replicates, and error bars show standard deviations.

autoclaved medium was spiked with various levels of methanol, sterilized by passage through a 0.2 μm filter.

As shown in **Table 5**, the maximum cell dry weight, biomass productivity, and cell yield decreased as methanol concentration increased from 0 to 20 g/L. In terms of fatty acid composition of the algal biomass, it was found that the proportion of DHA in total fatty acids maintained a relatively constant level; however, the total fatty acid content decreased with methanol concentration (data not shown). This fatty acid profile, together with the cell growth performance, resulted in a decreased DHA production level at higher methanol concentrations.

It should be noted that in a previous report (*12*) in which the methanol effect was studied, methanol was added to the medium and then autoclaved (at 121 °C for 15 min). We later found that this protocol resulted in a complete loss of methanol within the range of 0–20 g/L, as confirmed by HPLC analysis (data not shown). In this study, the sterilization protocols were changed to avoid autoclaving the methanol. Therefore, we believe the results presented in this work reflect the true methanol effects on algal DHA production.

The above results clearly indicate the negative effects of methanol on growth and DHA production of *S. limacinum*. Fortunately, under the typical crude glycerol levels used for algal culture (e.g., 60−90 g/L), autoclaving the media can evaporate all the methanol (ca. 4–20 g/L) contained in the original glycerol stream; this allows the algae to thrive on the methanol-free crude glycerol.

Effects of Soap on Algal DHA Production. The effects of soap on algal growth and DHA production were investigated by growing the algal cells in different levels of crude glycerol. The soap residues in the crude glycerol either remained in the medium (soap-containing) or were precipitated into free fatty

Table 6. Fatty Acid Composition (Percent of Total Fatty Acid, TFA) of the Algal Biomass Grown in Soap-Free and Soap-Containing Media with Different Crude Glycerol Concentration Loadings[a]

		glycerol concentration			
fatty acid	unit	10 g/L	20 g/L	40 g/L	60 g/L
		Algae on Soap-Free Medium			
C14:0	% TFA	2.06 ± 0.18	3.32 ± 0.24	4.21 ± 0.65	4.77 ± 0.05
C16:0	% TFA	35.50 ± 1.28	44.11 ± 0.53	49.80 ± 3.76	52.64 ± 0.37
C18:0	% TFA	0.81 ± 0.04	0.93 ± 0.05	0.97 ± 0.05	1.04 ± 0.02
C22:5	% TFA	8.58 ± 0.22	7.35 ± 0.27	6.29 ± 0.77	5.66 ± 0.05
C22:6	% TFA	53.05 ± 1.30	44.30 ± 0.53	38.73 ± 3.69	35.89 ± 0.38
TFA	mg/g of DW	138.1 ± 2.6	292.2 ± 5.9	503.9 ± 7.2	492.3 ± 12.6
		Algae on Soap-Containing Medium			
C14:0	% TFA	2.59 ± 0.98	3.14 ± 0.32	2.68 ± 0.25	2.99 ± 0.09
C16:0	% TFA	40.40 ± 12.73	42.35 ± 4.28	38.35 ± 3.04	39.35 ± 0.84
C18:0	% TFA	2.33 ± 0.28	1.91 ± 0.50	2.79 ± 1.48	1.10 ± 0.08
C18:1	% TFA	9.17 ± 1.72	7.43 ± 1.07	6.65 ± 1.00	3.93 ± 0.53
C18:2	% TFA	21.50 ± 6.11	17.68 ± 3.21	18.77 ± 3.28	19.23 ± 1.89
C18:3	% TFA	2.81 ± 0.91	2.72 ± 0.61	3.37 ± 0.69	4.85 ± 0.35
C22:5	% TFA	2.93 ± 0.66	3.55 ± 0.36	3.88 ± 0.49	4.07 ± 0.34
C22:6	% TFA	18.26 ± 4.94	21.23 ± 2.03	23.50 ± 2.94	24.49 ± 2.04
TFA	mg/g of DW	199.9 ± 62.9	282.9 ± 23.1	446.2 ± 92.9	427.3 ± 71.2

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

acids and then removed from the medium (soap-free). The glycerol G1 was used as representative of all the crude glycerol samples.

The growth kinetics of *S. limacinum* on soap-containing and soap-free medium were compared. As shown in **Figure 1A−C** in the lower range of crude glycerol concentrations (10 and 20 g/L), the algal growth rates on soap-free and soap-containing medium were similar. When the crude glycerol concentration increased to 40 g/L, the specific growth rates of the two cultures were still similar (**Figure 1A**), but the maximum cell dry weight and biomass productivity for soap-containing culture were lower than those for soap-free cultures (**Figure 1B**,**C**). At 60 g/L glycerol concentration, the growth in soap-containing medium was significantly inhibited. It was found that when crude glycerol concentration exceeded 60 g/L, the algal cells in soap-containing medium were not viable after 1 day of culture (data not shown).

The soap also had significant effects on fatty acid composition. As shown in **Table 6**, in soap-free medium, the proportion of C18 fatty acids was negligible (~1% of TFA), whereas soap-containing medium resulted in significant proportions of C18s fatty acids (25−35% of TFA). Considering the fact that C18s were the major fatty acids contained in soaps (**Table 3**), the difference in fatty acid composition between the soap-free and soap-containing algal biomass indicates that *S. limacinum* was capable of absorbing those soap C18s into the cells (**Table 6**). However, the proportion of long-chain fatty acids (C22:5 and C22:6) in the soap-containing algae was lower than that in the soap-free algae (**Table 6**), suggesting that those absorbed C18s were not further elongated by the algal cells. These fatty acid profiles agree with results reported by Hauvermale et al. (*28*). These authors used radiolabeled C16:0, C18:1, or C18:3 fatty acids to feed *Schizochytrium* sp. and found that none of the labels appeared in long-chain PUFA (*28*). **Table 6** also shows that at 10 g/L of crude glycerol, the algae in soap-containing medium had more total fatty acids (TFAs) than algae in soap-free medium; when crude glycerol concentrations exceeded 20 g/L, the TFA content of soap-free algae became more than that of soap-containing algae (**Table 6**). This is probably due to growth inhibition at higher soap levels (**Figure 1A−C**).

By combining the soap effects on both cell growth (**Figure 1A−C**) and fatty acid profile of *S. limacinum* (**Table 6**), it was found that under different crude glycerol levels, DHA content, yield, and productivity from the soap-containing algae were lower than those obtained from the soap-free algae (**Figure 1D−F**). The results clearly indicated a negative effect of soap on algal DHA production. This effect is more pronounced at higher levels.

The inhibitory effect of soap on *S. limacinum* agreed with other papers in which the growth of various algal species was inhibited by surfactants including soap (*29, 30*). This inhibition was caused by the complex interaction between the cell wall/membrane and the surfactants (*30*). To elucidate the mechanism of soap effects on *S. limacinum*, further study of the interaction between soaps and cell membrane of this species is needed.

Table 7. Proximate Analysis of Freeze-Dried Algal Biomass Grown on Glucose and Different Crude Glycerol Streams (Soap-free) (75 g/L of Carbon Source Was Used)[a]

	carbon source			
component	glucose	G1	G2	G3
lipid	44.61 ± 3.91	43.24 ± 1.28	50.57 ± 1.32	46.71 ± 1.01
protein	13.05 ± 0.10	16.22 ± 0.00	13.90 ± 0.00	20.78 ± 0.66
carbohydrate	33.38 ± 3.91	25.69 ± 1.38	23.93 ± 1.34	23.02 ± 1.48
ash	7.99 ± 0.14	13.88 ± 0.52	10.62 ± 0.19	8.51 ± 0.86
moisture	0.97 ± 0.00	0.97 ± 0.01	0.98 ± 0.00	0.97 ± 0.00

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

Characterization of Algal Biomass Composition. To ensure that crude glycerol-derived algal biomass is suitable for use in omega-3-fortified food or animal feed, a thorough chemical characterization of the algal biomass was performed. Because soap residue had shown negative effects on algal growth and DHA production (**Figure 1**), the algae were grown in soap-free medium. Algae derived from all three crude glycerol samples were evaluated. Algae grown on glucose were also evaluated as a control.

Table 7 shows the proximate analysis of the algal biomass. Overall, the algae grown on the three crude glycerol samples had similar nutritional profiles, except that G2 had a relatively high portion of lipid and low portion of protein. The ash content of G1 was higher than those of G2 and G3. Compared with the glucose-derived algae, the glycerol-derived algae had similar lipid and protein contents, but less carbohydrate; the ash content of glycerol algae was higher.

Table 8. Fatty Acid Composition of Algal Biomass Grown on Glucose and Different Crude Glycerol Streams (Soap-free) (75 g/L of Carbon Source Was Used)[a]

	mg/g of dry biomass from carbon source				mass % of total fatty acids from carbon source			
fatty acid	glucose	G1	G2	G3	glucose	G1	G2	G3
C14:0	33.7 ± 1.0	28.8 ± 0.6	29.2 ± 0.3	31.1 ± 2.38	7.7 ± 0.2	6.8 ± 0.1	5.9 ± 0.1	7.0 ± 0.5
C16:0	271.2 ± 17.6	263.0 ± 1.5	287.5 ± 2.2	276.1 ± 2.2	62.2 ± 4.0	62.2 ± 0.4	58.4 ± 0.4	62.1 ± 0.5
C18:0	3.2 ± 0.5	3.3 ± 0.1	4.2 ± 0.1	4.6 ± 1.1	0.7 ± 0.1	0.8 ± 0.0	0.8 ± 0.0	1.0 ± 0.2
C22:5	8.3 ± 2.7	9.8 ± 1.2	14.3 ± 1.2	11.7 ± 0.8	1.9 ± 0.6	2.3 ± 0.2	2.9 ± 0.2	2.6 ± 0.2
C22:6	119.4 ± 13.4	117.7 ± 3.1	156.9 ± 1.2	121.2 ± 2.6	27.4 ± 3.1	27.8 ± 0.7	31.9 ± 0.2	27.3 ± 0.6

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

Table 9. Amino Acid Composition of Algal Biomass Grown on Glucose and Different Crude Glycerol Streams (Soap-free) (75 g/L of Carbon Source Was Used)[a]

	mg/g of dry biomass from carbon source				mass % of protein from carbon source			
amino acid	glucose	G1	G2	G3	glucose	G1	G2	G3
Asx [b]	10.4 ± 0.2	12.1 ± 1.4	10.7 ± 0.4	10.4 ± 0.2	9.6 ± 0.2	9.4 ± 1.1	9.3 ± 0.4	9.3 ± 0.3
Ser	5.3 ± 0.1	6.2 ± 0.7	5.5 ± 0.3	5.3 ± 0.1	4.9 ± 0.1	4.8 ± 0.5	4.7 ± 0.2	4.7 ± 0.2
Glx [b]	17.2 ± 0.3	20.5 ± 2.2	18.8 ± 0.8	17.2 ± 0.3	16.0 ± 0.3	15.9 ± 1.7	16.2 ± 0.7	15.3 ± 0.5
Gly	4.4 ± 0.1	5.3 ± 0.7	4.8 ± 0.2	4.4 ± 0.1	4.1 ± 0.1	4.1 ± 0.5	4.1 ± 0.2	4.1 ± 0.1
His	2.4 ± 0.1	2.9 ± 0.4	2.5 ± 0.1	2.4 ± 0.1	2.2 ± 0.1	2.2 ± 0.3	2.2 ± 0.1	2.2 ± 0.1
NH_3 [c]	2.2 ± 0.0	2.6 ± 0.1	2.3 ± 0.1	2.2 ± 0.0	2.1 ± 0.0	2.0 ± 0.1	2.0 ± 0.1	1.9 ± 0.2
Arg	8.4 ± 0.8	9.4 ± 0.1	7.7 ± 0.3	8.4 ± 0.8	7.8 ± 0.8	7.3 ± 0.1	6.7 ± 0.3	7.5 ± 1.1
Thr	5.4 ± 0.0	6.2 ± 0.7	5.6 ± 0.2	5.4 ± 0.0	5.1 ± 0.0	4.8 ± 0.5	4.8 ± 0.2	4.7 ± 0.2
Ala	6.2 ± 0.1	7.5 ± 0.9	6.7 ± 0.3	6.2 ± 0.1	5.8 ± 0.1	5.8 ± 0.7	5.8 ± 0.3	5.9 ± 0.2
Pro	4.2 ± 0.0	5.0 ± 0.6	4.4 ± 0.2	4.2 ± 0.0	3.9 ± 0.0	3.9 ± 0.5	3.8 ± 0.2	3.9 ± 0.2
Tyr	4.3 ± 0.3	5.1 ± 0.9	5.4 ± 0.2	4.3 ± 0.3	4.0 ± 0.3	4.0 ± 0.7	4.7 ± 0.2	3.9 ± 0.1
Val	5.8 ± 0.2	7.0 ± 0.8	6.3 ± 0.3	5.8 ± 0.2	5.4 ± 0.1	5.5 ± 0.6	5.5 ± 0.2	5.5 ± 0.2
Cys	5.2 ± 0.1	4.6 ± 1.6	5.1 ± 0.5	5.2 ± 0.1	4.8 ± 0.1	3.6 ± 1.2	4.4 ± 0.4	4.0 ± 0.1
Met	1.3 ± 1.4	3.9 ± 0.2	3.2 ± 0.3	1.3 ± 1.4	1.2 ± 1.3	3.0 ± 0.1	2.7 ± 0.2	3.4 ± 1.3
Ile	4.6 ± 0.1	5.9 ± 0.7	5.3 ± 0.2	4.6 ± 0.1	4.3 ± 0.1	4.6 ± 0.5	4.6 ± 0.2	4.6 ± 0.2
Leu	8.1 ± 0.1	9.8 ± 1.2	8.7 ± 0.4	8.1 ± 0.1	7.5 ± 0.1	7.6 ± 0.9	7.5 ± 0.3	7.8 ± 0.3
Lys	7.1 ± 0.2	8.5 ± 1.1	7.5 ± 0.4	7.1 ± 0.2	6.6 ± 0.1	6.6 ± 0.8	6.5 ± 0.3	6.6 ± 0.2
Phe	5.0 ± 0.1	6.1 ± 0.7	5.4 ± 0.2	5.0 ± 0.1	4.6 ± 0.1	4.7 ± 0.6	4.7 ± 0.2	4.8 ± 0.2

[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.

Table 8 shows the fatty acid profiles of the algae. All of the carbon sources yield similar fatty acid compositions. C16:0 and C22:6 (DHA) accounted for about 90% of the total fatty acid (TFA). The alga also contained small amounts of C14:0, C18:0, and C22:5 (DPA).

The amino acid composition of algal biomass (**Table 9**, left-hand side) is rich in cysteine (4.6−5.2 mg/g) and lysine (7.1−8.5 mg/g) relative to many common feedstuffs; lysine and cysteine are frequently the first or second limiting amino acid in feedstuffs for a given species (*31*–*33*). Although there are significant differences in the concentrations of many amino acids between the algae grown on different carbon sources, this is largely due to differences in overall concentration of protein (**Table 7**). Comparison of the amino acid composition in terms of percent of total protein (**Table 9**, right-hand side) reveals that the compositions of the protein in each alga culture are very similar.

Table 10 shows the results of ICP elemental analysis of algal biomass. All samples have essentially the same elemental profile. The biomass from G1 and G2 had higher potassium content than the biomass from G3 and glucose, probably due to the high potassium contained in the original G1 and G2 streams (**Table 4**). Although the stream G3 had a higher sodium content than G1 and G2 (**Table 4**), the algal biomass from these glycerol samples was not significantly different (**Table 10**), due to the fact that the artificial seawater medium contained a high level of sodium. All of the other elements contained in the algal biomass were considered to be resultant from the seawater medium solution, instead of from the crude glycerol. It should be noted that no heavy metals were detected by the ICP analysis.

Table 10. ICP Elemental Analysis of Algal Biomass Grown on Glucose and Different Crude Glycerol Streams (Soap-free) (75 g/L of Carbon Source Was Used; Elements Analyzed in **Table 1** But Not Included in This Table Were Not Detected in Any Sample)[a]

		carbon source			
element	unit	glucose	G1	G2	G3
boron	ppm	17.2 ± 5.3	23.7 ± 4.5	15.4 ± 1.6	14.7 ± 0.95
calcium	ppm	506 ± 19	603 ± 55	647 ± 43	592 ± 102
copper	ppm	6.5 ± 0.9	BDL[b]	4.9 ± 1.8	BDL
iron	ppm	69.0 ± 4.2	102 ± 4.7	95.8 ± 8.2	94.5 ± 5.9
magnesium	ppm	1825 ± 155	2050 ± 335	1715 ± 205	1380 ± 152
phosphorus	ppm	2595 ± 355	3740 ± 63	4290 ± 114	3610 ± 124
potassium	ppm	5503 ± 998	18600 ± 1700	12450 ± 1150	8310 ± 560
silicon	ppm	16.1 ± 3.2	26.5 ± 3.95	19.6 ± 0.4	10.6 ± 1.74
sodium	ppm	156650 ± 950	26400 ± 1250	14750 ± 1750	17900 ± 1932
sulfur	ppm	6145 ± 747	6530 ± 605	7900 ± 600	5320 ± 386

[a] Data are means of three replicates ± standard deviations. [b] Below detection limit.

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. Concerns about heavy metal contamination have limited the usage of fish oil products in food or animal feed, but these results alleviate any concerns about heavy metal contamination of algal biomass.

In summary, the above compositional analysis indicates that the algal biomass grown in crude glycerol had a similar composition to the algae grown in glucose. Currently, *Schizochytrium* algae are available from commercial producers such as Aquafauna Bio-Marine Inc. (Hawthorne, CA; http://www.aquafauna.com/Profiles-AlgaMac-3000.htm) and Advanced

BioNutrition Corp. (Columbia, MD; http://www.abn-corp.com/html/abn_dha.pdf). The algae produced from biodiesel-derived glycerol had a composition similar to the commercial algae. Different sources of crude glycerol did not cause significant variation of algal biomass composition. The results indicate that producing omega-3 algae from crude glycerol is a feasible and durable option for utilizing this glycerol. The algal biomass is a safe option for use as an ingredient in omega-3-fortified food or animal feed.

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