CHEMICAL ENGINEERING TRANSACTIONS
VOL. 45, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Petar Sabev Varbanov, Jiří Jaromír Klemeš, Sharifah Rafidah Wan Alwi, Jun Yow Yong, Xia Liu

ISBN 978-88-95608-36-5; **ISSN** 2283-9216
DOI: 10.3303/CET1545162

Docosahexaenoic Acid Production from Crude Glycerol by Schizochytrium Limacinum SR21

Yun-Tsun Lung[a], Chung Hong Tan[b], Pau Loke Show[b,c*], Hon Loong Lam[b], John Chi-Wei Lan[a]

[a]Biorefinery & Bioprocess Engineering Laboratory, Department of Chemical Engineering and Material Science, Yuan Ze University, No. 135 Yuan-Tung Road, Chungli, Taoyuan, 320, Taiwan
[b]Department of Chemical and Environmental Engineering, Faculty of Engineering, University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia
[c]Manufacturing and Industrial Processes Division, Faculty of Engineering, Centre for Food and Bioproduct Processing University of Nottingham Malaysia Campus, Jalan Broga, 43500 Semenyih, Selangor Darul Ehsan, Malaysia
PauLoke.Show@nottingham.edu.my

The development and promotion of biodiesel, a substitute energy source for fossil fuels, has resulted in the overproduction of crude glycerol, causing undesirable environmental issues. However, the environmental harm can be minimized by converting crude glycerol into useful products. One solution involves using microalga Schizochytrium limacinum SR21 to convert glycerol into docosahexaenoic acid (DHA). DHA is an essential fatty acid, and is necessary to develop and maintain brain functions in infants and adults. The highest DHA productivity of 233.73 mg/g biomass was obtained using 3 % crude glycerol in Medium 2 at 20 °C under mixo/heterotrophic cultivation.

1. Introduction

Since the industrial revolution began in the 18th century, the world has entered an age of modernization. Machines have replaced human labour in many different industrial tasks. Although work has been simplified by the use of machines, the rapid burning of fossil fuels for energy have also caused major global issues such as global warming and depletion of world fossil resource (Ikeda et al., 2006). These problems have sparked the search for alternative green energy sources like wind and solar power etc. Biomass energy, in particular, is a widely-researched area due to the ability to convert any biomass (agricultural wastes or lignocellulosic residues) or organic wastes (from industries, eateries or domestic households) into useful energy sources, which includes biodiesel (Glisic and Orlović, 2014), bioethanol (Antonio Bizzo et al., 2014), biogas (El-Mashad and Zhang, 2010) and syngas (Calvo et al., 2012).
Biodiesel is a popular alternative fuel because 1) it has similar energy density as diesel fuel, 2) it can be burned in current vehicle engines with minor modifications, and 3) its gas emissions are cleaner than diesel fuel (Xue et al., 2011). This prompted the increase in biodiesel production mainly from first generation energy crops such as oil palm and rapeseed. For every 10 kg of biodiesel produced, 1 kg of crude glycerol will be generated (Hu et al., 2012). This resulted in a surplus of crude glycerol in the market, sliding the price of crude glycerol down to approximately 0.1 $/kg. Crude glycerol contains many impurities like methanol, soap and catalyst residues, making it a huge financial and environmental burden for the biodiesel industry (Johnson and Taconi, 2007). As it is very costly to purify crude glycerol into pure glycerol (99.5 % purity), crude glycerol has been used as an alternative carbon source in microorganism culture (Mata et al., 2014). For instance, a microalga *Chlorella protothecoides* was cultured in fed-batch mode with crude glycerol as the main carbon source. The maximum biomass and lipid concentration achieved were 45.2 and 24.6 g/L after a cultivation period of 8.2 d (Chen and Walker, 2011).
According to a report by the Joint Research Center of the European Commission (Christien et al., 2014), the global marine biotechnology market (with microalgae as main component) was valued at € 2.4 billion, with an expected 10 % annual growth (Guedes et al., 2011). In the last decade, more than 75 % of

Please cite this article as: Lung Y.-T., Tan C.H., Show P.L., Lam H.L., Lan J.C.W., 2015, Docosahexaenoic acid production from crude glycerol by schizochytrium limacinum sr21, Chemical Engineering Transactions, 45, 967-972 DOI:10.3303/CET1545162

microalgae commodities were used as dietary supplements (Chacón‐Lee and González‐Mariño, 2010). For instance, microalgae-derived DHA by Martek (now DSM) is added to 99 % of all baby food in the USA (Christien et al., 2014). Therefore, the main objective of this paper is to produce docosahexaenoic acid (DHA) from an indigenous marine microalga Schizochytrium limacinum SR21 using crude glycerol, in the hopes of solving the environmental crisis posed by excess glycerol production. DHA plays a vital role in our health. As an essential fatty acid, DHA is needed by infants for the growth and development of their brains. Apart from that, DHA is also necessary to maintain brain function in adults (Horrocks and Yeo, 1999). The effects of different cultivation modes (mixotrophic, heterotrophic and mixo/heterotrophic cultivation) and concentration of carbon sources (glucose, pure glycerol and crude glycerol) on microalgal growth and lipid accumulation were examined. The effects of temperature and illumination period were also investigated. Finally the best culture conditions for DHA accumulation in *S.* limacinum SR21 were identified.

2. Materials and Methods

2.1 Microalga culture and medium composition

The microalga chosen was Schizochytrium limacinum SR21 (ATCC MYA-1381) provided by the Food Industry Research and Development Institute (FIRDI), Taiwan. The microalga was pre-cultured for 3 d. Then 10 % (v/v) crude pre-culture was transferred to a 500 mL conical flask containing fresh medium. The total working volume was 100 mL. The microalga was grown in an incubator shaker (Model TLT-808050, Cherng Huei, Taiwan) at 25 °C and stirring speed of 150 rpm for 5 d. Fluorescent lights with light intensity of 1700 lx were used and a light/dark cycle of 12/12 h was set.

Two different mediums were used in this experiment. Medium 1 was artificial seawater made from synthetic sea salt (TAAM, U.S.A.) while Medium 2 was artificial seawater medium prepared according to UTEX (UTEX The Culture Collection of Algae). The composition of Medium 1 was (g/L): artificial seawater, 30; glucose, 2; yeast, 4; peptone, 4. The composition of Medium 2 was (g/L): NaCl, 18; $MgSO_4.7H_2O$, 2.6; KCl, 0.6; $NaNO_3$, 0.1; $CaCl_2.2H_2O$, 0.3; KH_2PO_4, 0.05; Tricine, 4.48; $Na_2EDTA.2H_2O$, 0.051; H_3BO_3, 0.00114; $FeCl_3.6H_2O$, 0.004099; $MnSO_4.H_2O$, 0.000164; $ZnSO_4.7H_2O$, 0.000022; $CoCl_2.6H_2O$, 0.0000048; NH_4Cl, 0.27; and 0.1 M HCl, 4.5 mL/L. The microalgal growth rate is related to the concentration of limiting substrate by the following equation:

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad (1)$$

μ = Specific growth rate
X = Microalgae cell concentration
t = Time

2.2 Determination of microalga cell concentration

1 mL of crude medium was extracted every 24 h and the optical density was recorded at a wavelength of 680 nm (OD_{680}) using UV/Vis spectrophotometer (Model TM-32, Tomin, Taiwan). To obtain the relationship between biomass dry weight (g/L) and OD_{680}, 35 mL of crude medium was extracted and centrifuged at 10,000 rpm for 10 min. After washing the biomass thoroughly with deionized water, the biomass was mixed with different amounts of deionized water and their OD_{680} values were recorded. The biomass samples were dried in an oven at 105 °C overnight, after which their dry weights were recorded. Finally, a graph of biomass dry weight vs OD_{680} was plotted and used for subsequent calculations of biomass dry weight.

2.3 Determination of glycerol concentration

After OD_{680} test, the extracted crude medium was centrifuged for 10 min. The resulting supernatant was collected. The analysis method for glycerol concentration was identical to that for glucose, except the analysis time was changed to 30 min.

2.4 Determination of lipid content

After 5 days of culture, the crude medium was centrifuged at 10,000 rpm to collect the microalga biomass. Deionized water was used to wash the biomass twice. The biomass was then put to dry overnight in -20 °C freezer before freeze drying. The freeze-dried biomass underwent lipid extraction and transesterification using Folch method (Folch et al., 1957). The lipid samples were analyzed using gas chromatography (GC) equipped with a flame ionization detector (FID) (Model 6890, Agilent, USA). The oven temperature was set at 190 °C and held to 220 °C for 3 min. The temperature of the injector and detector was set at 190 °C and

250 °C. The flow rate of helium was 0.5 mL/min, whereas the flow rate of hydrogen and air was 45 mL/min and 450 mL/min.

3. Results and Discussions

3.1 Effects of different cultivation mode on microalga growth

The effect of three different cultivation modes (mixotrophic, heterotrophic and mixo/heterotrophic) on microalga growth was investigated. Three different carbon sources (glucose, pure glycerol and crude glycerol) were added to Medium 1 at a concentration of 20 g/L. As shown in Figure 1(a), when glucose was used, the microalgal biomass achieved a maximum value of 3.9 g/L after 3 d of mixotrophic cultivation. The biomass concentration achieved under mixo/heterotrophic and heterotrophic cultivation was 3.19 and 2.84 g/L. The presence of light in mixotrophic cultivation enables photosynthesis in the microalgal cells and increases the uptake of glucose by the cells compared to heterotrophic cultivation. This will also enhance the growth rate of the cells (Wan et al., 2011). Figure 1(b) shows the biomass growth and residual pure glycerol concentration with time. The maximum biomass concentration was 4.23 g/L under mixotrophic cultivation, whereas the biomass concentration under mixo/heterotrophic and heterotrophic cultivation was 4.14 and 3.17 g/L. Compared to glucose, using pure glycerol improved the microalga biomass growth. Figure 1(c) shows the relationship between biomass growth and residual crude glycerol concentration. The highest biomass obtained for all three cultivation modes was 3.67 g/L. The crude glycerol was completely oxidized in all three cases, proving that the microalga could utilize crude glycerol for growth. Compared to glucose, crude glycerol showed a similar growth curve and did not improve microalga growth. But since the cost of crude glycerol is lower than glucose and pure glycerol, crude glycerol was chosen for further optimization.

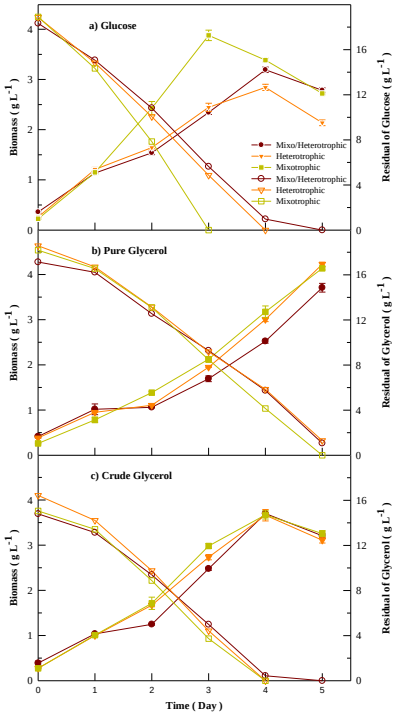

Figure 1: Effect of different cultivation modes on microalgal biomass using: a) Glucose, b) Pure glycerol, c) Crude glycerol. The lines that start near 0 indicate biomass, the lines that start near 4 indicate carbon source.

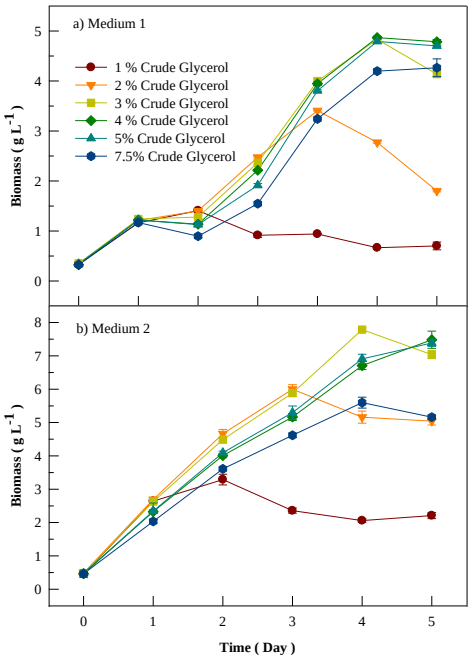

Figure 2: Effect of different concentrations of crude glycerol on microalgal growth in: a) Medium 1, b) Medium 2

3.2 Effects of different concentration of crude glycerol on microalgal growth and lipid accumulation

Six different concentrations of crude glycerol were investigated in Medium 1 and Medium 2 to determine the effect of crude glycerol concentration on microalgal growth and lipid accumulation. The concentrations of crude glycerol were 10 g/L, 20 g/L, 30 g/L, 40 g/L, 50 g/L and 75 g/L, depicted as 1 %, 2 %, 3 %, 4 %, 5 %, 7.5 % in short. Both fluorescent and horticulture grow lights were used in parallel with a combined light intensity of 1,700 lx. When the concentration of crude glycerol was increased to 3 % and 4 % in Medium 1,

the highest specific growth rate obtained were 0.61 d^{-1} and 0.62 d^{-1} (data not shown). From Figure 2(a), 5 % crude glycerol achieved the highest DHA content of 141.89 mg/g, occupying 24.72 % out of 57.40 % total lipid content (Table 1). At 2 % crude glycerol, the biomass obtained was 3.41 g/L at day 4. The microalga had completely metabolized the crude glycerol at day 4, resulting in a decline in growth after day 4. However, at 3 % crude glycerol, the highest biomass of 4.83 g/L was reached. When cultured in Medium 2, the highest specific growth rate and biomass of 0.71 d^{-1} and 7.8 g/L were obtained at 3 % crude glycerol (data not shown). But the highest DHA yield of 157.91 mg/g was obtained at 5 % crude glycerol (Figure 2(b)), occupying 26.41 % out of 59.78 % total lipid content (Table 1). Medium 2 with 3 % crude glycerol as carbon source was chosen for the next experiment.

3.3 Effect of temperature and illumination period on microalgal growth and lipid accumulation

Zhu et al. (2007) investigated the effect of temperature (16 °C, 23 °C, 30 °C, 37 °C) on the growth of S. limacinum OUC88. Upon increasing the temperature from 23 to 30 °C, the biomass decreased slightly from 24.80 to 22.15 g/L. But at 37 °C, the biomass reduced significantly to 8.53 g/L. This shows that temperature is an important factor in cell growth (Zhu et al., 2007). Thus, we investigated the influence of temperature (20 °C, 25 °C, 30 °C) on the three different cultivation modes. Medium 2 was used with either pure or crude glycerol as the carbon source. When pure glycerol was used, 20 °C was too low causing the cells to grow slower, 25 °C was most suitable for cell growth and 30 °C suppressed cell growth. Despite slower growth rate at 20 °C, the microalgal biomass still reached 8.13 g/L after 7 d of cultivation with pure glycerol (Figure 3(a)). When crude glycerol was used at 20 °C, there was a significant difference in biomass concentration under mixotrophic (8.29 g/L) and heterotrophic cultivation (6.63 g/L) (Figure 3(d)). At 25 °C with pure glycerol, a biomass concentration of 7.99 g/L was reached after 5 d (Figure 3(b)), whereas with crude glycerol, the maximum biomass concentration under mixotrophic (9.03 g/L) and heterotrophic (6.34 g/L) cultivation were reached after 4 days (Fig. 3(e)). Therefore at 20 °C and 25 °C, crude glycerol under mixotrophic cultivation was suitable for microalgal cell culture.

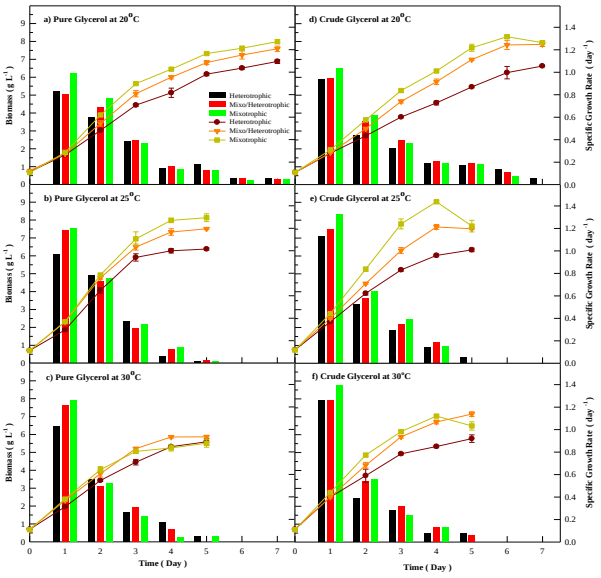

Figure 3: Effect of different cultivation modes on microalgal growth and specific growth rate using pure glycerol at: a) 20 °C, b) 25 °C, c) 30 °C; and crude glycerol at: d) 20 °C, e) 25 °C, f) 30 °C. The lines indicate biomass, the bars indicate specific growth rate.

At 30 °C with pure glycerol, the microalgal cells could not flourish due to the high surrounding heat. The biomass concentration obtained in mixotrophic and heterotrophic cultivation was 5.51 and 5.87 g/L (Figure 3(c)). At 30 °C with crude glycerol, a biomass concentration of 7.04 g/L was obtained under mixotrophic cultivation after 4 d, compared to 5.78 g/L under heterotrophic cultivation (Figure 3(f)). Therefore at 30 °C, S. limacinum SR21 could still utilize crude glycerol under mixotrophic cultivation for growth. The microalga specific growth rates at 25 °C were higher than 20 °C and 30 °C for both pure and crude glycerol. At 20 °C, the microalgal cells required time to adapt to the cooler environment, hence longer growth periods. At 30 °C, the specific growth rates were very low, indicating the suppression of cell growth (Figure 3). The higher

temperature (30 °C) might have reduced the solubility of CO_2 gas in the culture medium, disrupting photosynthesis process and leading to lower cell growth (Pulz, 2001).

As seen from Table 2, when S. limacinum SR21 was cultivated using pure glycerol under all three cultivation modes, temperature did not show a significant effect on total lipid content. Under mixotrophic and mixo/heterotrophic cultivation, the highest DHA content achieved was 211.54 and 228.25 mg/g at 20 °C. However, DHA content decreased with increasing temperature. Under heterotrophic cultivation, the DHA content did not have any measurable improvement at 20 °C compared to 25 °C and 30 °C. When switched to using crude glycerol, the highest DHA content was 233.73 mg/g under mixo/heterotrophic cultivation at 20 °C. The total lipid content increased with increasing temperature, and the highest total lipid content of 71.86 % was reached at 30 °C (Table 2). Under mixotrophic cultivation, the highest DHA content was 217.02 mg/g at 20 °C. The total lipid and DHA content decreased with increasing temperature. Under heterotrophic cultivation, the total lipid content did not change, but the DHA content increased with increasing temperature. The highest DHA content was 201.28 mg/g at 30 °C.

Table 1: Lipid analysis of different concentrations of crude glycerol

	Conc. of Crude Glycerol (%)	Biomass (g/L)	Total Lipid (%)	Content (mg/g)					
				C14	C16	C18	C20:5	C22:5	C22:6
Medium 1	1 %	0.70	13.81	1.28	63.64	5.42	2.91	15.38	44.84
	2 %	1.22	33.14	4.66	142.02	7.63	9.19	30.88	133.92
	3 %	4.14	52.46	10.96	317.68	11.4	7.59	31.64	130.63
	4 %	4.78	56.13	12.66	338.25	11.56	6.97	29.27	138.75
	5 %	4.70	57.40	13.62	357.31	12.10	6.26	29.69	141.89
	7.5 %	4.27	49.80	11.15	276.64	12.49	8.50	31.18	141.48
Medium 2	1 %	2.21	13.17	3.13	43.46	1.59	2.37	14.61	62.63
	2 %	5.04	33.96	12.58	159.90	4.29	4.93	28.62	122.79
	3 %	7.04	38.15	14.58	191.11	5.34	3.95	29.42	127.60
	4 %	7.48	47.32	19.93	255.37	6.85	3.48	30.38	139.82
	5 %	7.39	59.78	27.74	346.66	8.77	4.66	36.23	157.91
	7.5 %	5.16	52.40	28.08	308.61	7.26	3.49	29.63	135.57

*Conc., Concentration

Table 2: Lipid analysis of different cultivation modes at different temperatures

		Biomass (g/L)	Total Lipid (%)	Content (mg/g)					
				C14	C16	C18	C20:5	C22:5	C22:6
Pure Glycerol	H 20 °C	6.88	58.28	21.97	325.91	5.95	6.63	29.80	169.03
	H 25 °C	6.38	57.80	20.49	310.01	7.60	6.80	35.72	175.25
	H 30 °C	5.59	57.33	17.39	289.00	9.77	7.58	46.41	173.11
	M/H 20 °C	7.59	67.51	26.69	369.64	7.48	6.76	34.10	211.54
	M/H 25 °C	7.51	68.12	25.36	373.58	9.54	6.92	45.92	194.11
	M/H 30 °C	5.87	65.27	19.23	335.95	12.00	6.40	54.55	189.55
	M 20 °C	7.99	70.71	25.96	373.29	7.83	7.14	43.85	228.25
	M 25 °C	8.13	68.20	23.53	377.70	10.34	5.67	51.05	191.79
	M 30 °C	5.51	68.00	20.23	354.54	13.49	5.11	59.26	188.14
Crude Glycerol	H 20 °C	6.63	64.51	28.19	369.76	7.57	7.84	28.22	185.12
	H 25 °C	6.34	65.20	24.80	356.48	9.61	5.79	40.79	193.06
	H 30 °C	5.78	63.86	20.23	335.70	10.82	5.17	49.51	201.28
	M/H 20 °C	7.84	62.60	20.00	295.63	5.69	8.63	39.24	233.73
	M/H 25 °C	7.52	65.63	28.37	364.42	9.43	5.46	43.13	183.22
	M/H 30 °C	7.15	71.86	30.40	457.95	14.96	7.06	63.90	222.87
	M 20 °C	7.94	67.66	30.41	355.70	6.84	6.35	41.08	217.02
	M 25 °C	7.67	65.37	29.08	360.08	9.61	6.13	46.66	184.63
	M 30 °C	6.49	59.04	22.48	320.66	10.23	7.66	44.43	162.55

*H, Heterotrophic; M, Mixotrophic

4. Conclusions

S. limacinum SR21 can metabolize crude glycerol as carbon source for growth and DHA accumulation. The addition of 3 % crude glycerol in Medium 2 enabled a maximum biomass growth and specific growth rate of 7.8 g/L and 0.71 d^{-1}. DHA accumulation in *S.* limacinum SR21 was affected by temperature and the mode of cultivation. Mixotrophic cultivation showed a greater DHA accumulation compared to

heterotrophic. The highest DHA productivity of 233.73 mg/g was obtained using crude glycerol with mixo/heterotrophic cultivation at 20 °C. This study proved that a green alternative route for dealing with excess crude glycerol was DHA production using microalgae. The next step for this research would be to scale up using outdoor photobioreactors. Scaling up would allow for proper financial analysis, which determine the commercial viability of the large scale culture system.

Acknowledgements

This research was supported by Ministry of Science and Technology (Taiwan) with funding number 103-2221-E-155-055. This work is also supported financially by Fundamental Research Grant Scheme (Malaysia, FRGS/1/2013/SG05/UNIM/02/1) and Ministry of Science, Technology, Innovation (Malaysia, MOSTI-02-02-12-SF0256) from the University of Nottingham in Malaysia.

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