




Article

Cultivation Method Effect on *Schizochytrium* sp. Biomass Growth and Docosahexaenoic Acid (DHA) Production with the Use of Waste Glycerol as a Source of Organic Carbon

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Abstract: Inexpensive carbon sources offering an alternative to glucose are searched for to reduce costs of docosahexaenoic acid production by microalgae. The use of waste glycerol seems substantiated and prospective in this case. The objective of this study was to determine the production yield of heterotrophic microalgae *Schizochytrium* sp. biomass and the efficiency of docosahexaenoic acid production in various types of cultures with waste glycerol. Cultivation conditions were optimized using the Plackett–Burman method and Response Surface Methodology. The highest technological performance was obtained in the fed-batch culture, where the concentration of *Schizochytrium* sp. biomass reached 103.44 ± 1.50 g/dm³, the lipid concentration in *Schizochytrium* sp. biomass was at 48.85 ± 0.81 g/dm³, and the docosahexaenoic acid concentration at 21.98 ± 0.36 g/dm³. The highest docosahexaenoic acid content, accounting for $61.76 \pm 3.77\%$ of total fatty acids, was determined in lipid bodies of the *Schizochytrium* sp. biomass produced in the batch culture, whereas the lowest one, accounting for $44.99 \pm 2.12\%$ of total fatty acids, in those of the biomass grown in the fed-batch culture.

Keywords: microalgae; heterotrophic culture; docosahexaenoic acid; glycerol; cultivation methods



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1. Introduction

The biomass of microalgae represents a source of valuable substances that may be used in medicine and pharmacy but also in the fertilizer, feedstuff, and food industries as well as in bioenergetics [1,2]. Microalgae are known for producing valuable compounds, like e.g., docosahexaenoic acid (DHA). It is an unsaturated fatty acid belonging to the Omega-3 family, which is an important structural component of cell membranes in certain tissues of a human body, e.g., in phospholipids that constitute neurons of the brain cortex or the retina [3]. The acyl group formed by DHA in phospholipids affects the thickness of the cytoplasmic membrane of cells, its looser structure, and also its permeability to ions and small molecules. In addition, it creates the environment for receptor, channel, and peripheral proteins condensed in neuronal membranes. DHA plays a meaningful neuroprotective role, attenuates inflammatory reactions, and prevents damage and apoptosis of neurons [4]. It has also been proven to prevent arterial hypertension development, minimize the incidence of cardiac diseases, and play a significant role in the proper brain development of newborns [5,6]. According to some studies, DHA increases calcium absorption, thereby positively affecting the osseous tissue; allows maintaining appropriate levels of the “bad” and the “good” cholesterol; aids functions of the circulatory and immune systems; and even affects the condition of our hair and nails [7,8]. Because DHA cannot be synthesized de novo by the human body, it needs to be provided with food [9,10].

Today, its main dietary sources include fish flesh lipids and plant oils [11]. The demand for fatty acids from the Omega-3 family is observed to increase due to the growing awareness of consumers and their care for a healthy lifestyle [12]. This justifies the need to search for alternative production methods that would be environmentally friendly and justifiable considering technical and economic concerns. One of the recently developing DHA production trends is the use of the biomass of heterotrophic algae from the genus *Schizochytrium* sp. [13]. Studies conducted so far have demonstrated glucose to be the optimal carbon source for the *Schizochytrium* sp. strain cultured for DHA isolation [14].

Unfortunately, the use of glucose as a source of organic carbon is cost-inefficient [15], because the yield of sugar conversion by microalgae is very low, i.e., 5 tons of sugar are needed to produce 1 ton of oil [16]. Therefore, cheap carbon sources are searched for as an alternative to glucose to optimize the costly DHA production by microalgae. As *Schizochytrium* sp. biomass can grow on various carbon sources, including wastes, works on the use of waste glycerol seem a justified and prospective trend in scientific research [17]. The supply of this waste increases successively due to the increasing global production of biodiesel, which is associated with the need of ensuring appropriate fractions of biocomponents in conventional fuels [18]. The global production of biodiesel approximates 41.3 billion liters annually; with waste glycerol accounting for 12% of total esters produced [19].

Glycerol has been thermochemically converted to hydroxyacetone [20] and dipropylene glycol [21]. Glycerol is also used to maintain the strains, for example, the strain thraustochytrid *Aurantiochytrium* sp. ICTFD5 was stored in glycerol (20%, *v/v*) at -80 °C [22]. Other processes have also been explored, such as the reformation of glycerin to produce synthesis gas and hydrogen [23], production of epichlorohydrin [24], hydrogenation [25], and etherification [26]. Glycerin can serve as a source of carbon in biochemical processes that produce omega-3 fatty acids or 1,3-propanediol colorants [27]. Waste glycerin is also commonly used in energy production as a substrate for fermentative methane production [28]. By harnessing specific strains of bacteria and synthesizing new enzymes, new conversion pathways become available, including ethanol production or β -carotene production [29].

The goal of this study was to verify the yield of biomass production by heterotrophic microalgae *Schizochytrium* sp. and the efficiency of docosahexaenoic acid (DHA) production in batch, semi-continuous, and continuous cultures fed with waste glycerol as a source of organic carbon.

2. Materials and Methods

2.1. Study Design

Research works were divided into three stages, depending on culture type. In stage 1, *Schizochytrium* sp. algae were cultivated in the batch culture, in stage 2—bioreactors were exploited in the fed-batch mode, whereas in stage 3—the microalgae were cultivated in the continuous culture. Analyses were carried out to determine the growth kinetics of the *Schizochytrium* sp. genus microalgae (r_{DCW}), the rate of lipid and DHA production (r_{DHA}), and the rate of glycerol consumption.

2.2. Cultivation Conditions

The cultivation conditions were established in previous works aimed at optimizing technological parameters that affect the *Schizochytrium* sp. biomass cultivation yield and DHA production yield, using glycerol as an external carbon source [30]. The values were identified through a series of experiments structured using Plackett–Burman design. After identifying the most significant parameters affecting the final results, the values of parameters ensuring the highest DCW and DHA concentrations were determined using the Response Surface Methodology. The modeled parameter values and predicted performance were then experimentally verified using a batch *Schizochytrium* sp. culture [30]. At all experimental stages, cultivation conditions were as follows: temperature 27 °C, initial glycerol concentration 150 g/dm³, oxygen concentration 50%, initial peptone concentra-

tion 10 g/dm³, initial culture pH 6.5, volumetric airflow rate 0.3 L_{air}/min·L_{react.}, salinity 17.5 PSU, initial yeast extract concentration 0.4 g/dm³, and turbine stirring speed 175 rpm. The dry cell weight (DCW) of the inoculum reached 4–6 g/dm³.

2.3. Materials

The study was conducted with *Schizochytrium* sp., a strain of single-cell heterotrophic microalgae from the family *Thraustochytriaceae*. The inoculum was obtained from the ATCC (American Type Culture Collection). *Schizochytrium* sp. cells were maintained in sterile agar slants of the ATCC790By+ medium containing 2% agar (*w/w*). The cells were spread into new agar slants every month. To obtain enough inoculum for the experiment, the cells were transferred from the agar slants to 50 cm³ conical flasks, containing 15 cm³ of the agarless ATCC790By+ liquid medium with a pH of 6.5, with the composition given in Tables 1 and 2.

Table 1. Composition of the ATCC790 By+ culture medium.

Component	Unit	Concentration
Glucose	(g/dm ³)	5.0
Yeast extract	(g/dm ³)	1.0
Peptone	(g/dm ³)	1.0
Artificial seawater	(dm ³)	1.0

Table 2. Characteristics of artificial seawater.

Component	Unit	Concentration
(NH ₄) ₂ SO ₄	(g/dm ³)	1.0
KH ₂ PO ₄	(g/dm ³)	3.0
Na ₂ SO ₄	(g/dm ³)	12.0
MgSO ₄	(g/dm ³)	5.0
K ₂ SO ₄	(g/dm ³)	7.0
KCl	(g/dm ³)	2.0
	Trace elements	
CaCl ₂	(mg/dm ³)	50
MnCl ₂	(mg/dm ³)	5.2
ZnSO ₄	(mg/dm ³)	5.2
CuSO ₄	(mg/dm ³)	0.8
Na ₂ MoO ₄	(mg/dm ³)	0.016
NiSO ₄	(mg/dm ³)	0.8
FeSO ₄	(mg/dm ³)	0.01
CoCl ₂	(mg/dm ³)	0.066
thiamine	(mg/dm ³)	0.76
vitamin B ₁₂	(mg/dm ³)	1.2
vitamin B ₅ (calcium salt of pantothenic acid)	(mg/dm ³)	25.6

The medium was autoclave-sterilized before use (121 °C, 15 min, Systec V-95 autoclave). The flasks were shaken on a temperature-controlled orbital shaker (Excella E24R, New Brunswick/Eppendorf) at 200 rpm and a temperature of 25 °C. After 120 h, 10 cm³ of the culture was transferred as the *Schizochytrium* sp. inoculum into a 250 cm³ Erlenmeyer flask containing 90 cm³ of the fresh ATCC790By+ medium. In another stage, the *Schizochytrium* sp. inoculum was passaged to a 500 cm³ conical flask. This way, 200 cm³ of the culture was obtained, which was further used as the inoculum fed into a bioreactor with a working capacity of 2 dm³ (Biostat B Twin, Sartorius Stedim), 1.8 dm³ of which was the culture medium.

Crude glycerin, obtained from the PKN Orlen Południe S.A. Plant (Trzebinia, Poland), was used as the sole source of carbon in the cultures. The most important information from

the characteristics' card (<http://www.rafineria-trzebinia.pl>, accessed on 14 May 2021) is presented in Table 3.

Table 3. Characteristics of crude glycerin.

Properties	Unit	Concentration
Color	(-)	light-brown
Odor	(-)	characteristic
pH	(-)	5
Glycerol	(% w/w)	80
Water	(% w/w)	15
Sulfated ash	(% w/w)	5
Methanol	(% w/w)	0.3
MONG (Matter Organic Non-Glycerol)	(% w/w)	6
Chlorides	(ppm)	10
Halogen derivatives	(ppm)	35
Acidity	(cm ³) of NaOH consumed	0.25
Esters	(cm ³) of HCl consumed	8–10
Heavy metals	(ppm)	5
Aldehydes	(ppm)	10
Temperature of melting/freezing	(°C)	18
Temperature of initial boiling	(°C)	290
Temperature of ignition	(°C)	177
Temperature of self-ignition	(°C)	429
Temperature of decomposition	(°C)	>290
Vapor pressure	(mbar)	0.01
Relative density/density converted to 20 °C	(mg/dm ³)	1.26
Viscosity at 20 °C	(mm ² /s)	1.5
Density at 15 °C	(mg/dm ³)	1.2
Explosive properties	(-)	-
Explosive properties	(-)	-

2.4. Experimental Station

The *Schizochytrium* sp. biomass was grown in a Biostat B Twin (Sartorius Stedim) bioreactor with a working capacity of 2 dm³. The bioreactor was fitted with acid, base, antifoaming agents, and organic substrate pumps. The substrate pump enabled controlling the volume of the additional substrate added to the culture, counting its volume (totalizer), and controlling its feeding rate. The system had a gas module for monitoring dissolved oxygen (DO), a system for stabilizing pH based on injecting acid or base via peristaltic pumps, and a system for temperature measurement and stabilization at 27 °C. The contents of the bioreactor were stirred by a six-bladed Rushton turbine (53 mm in diameter).

2.5. Experimental Procedures

At stage 1, the batch culture was continued for 120 h. A sample of microalgal biomass was collected every 20 h and determined for DCW concentration (g/dm³), lipid concentration (g/dm³), DHA concentration (g/dm³), and the concentration of glycerol left in the culture medium (g/dm³). Each determination was performed in triplicate.

At stage 2, biomass samples were collected every 5 h and determined for the concentration of waste glycerol left in the culture medium. If it was equal to or lower than 60 g/dm³, additional glycerol was fed into the bioreactor in the amount ensuring its concentration in the culture medium at 150 g/dm³. The threshold glycerol concentration in the medium was established at 60 g/dm³, because at this concentration *Schizochytrium* sp. entered into the stationary phase of growth. Feeding the additional glycerol prevented *Schizochytrium* sp. from entering into the decline phase and allowed increasing lipid concentration in the biomass. The other determinations, including the dry cell weight (DCW) concentration (DCW) (g/dm³), lipid concentration (g/dm³), and DHA concentration (g/dm³) were conducted every 20 h.

At stage 3, the continuous culture aimed to maintain the cells in the intensive growth phase, which was achieved by the successive supply of nutrients depleting from the medium and discharge of metabolites and excess cells from the culture. Maintaining process continuity required adjusting the amount of discharged biomass to the biomass growth rate so as the cell concentration in the bioreactor remained constant. The feeding rate of the substrate (dilution rate or dilution factor) was determined using the following formula: $D = F/V$, where: D —dilution rate (dilution factor) (1/day), F —substrate flow rate (m^3/day), and V —bioreactor tank volume (m^3). Once the cells had reached the log phase of growth, a fresh medium was fed into the bioreactor (containing $150 \text{ g}/\text{dm}^3$ of glycerol and all essential micro- and macro-elements). Four different cultures were carried out, differing in the dilution rate D that reached 0.2, 0.4, 0.6, and 0.8 1/day. This action was aimed at optimizing the feeding rate of the substrate in the form of glycerol and collecting the spent part of the culture medium along with microalgae cells so that the culture was maintained in the phase of the most intensive growth (the highest r_{DHA} and r_{DCW} values). When a given volume of the spent culture medium had been discharged from the bioreactor, a respective volume of fresh medium was fed into the bioreactor. Culture samples were collected every 8 h for dry cell weight (DCW) determination. The culture status was considered to be steady-state when 2 conditions were met: at least 3 volumes of the culture medium were exchanged in the bioreactor, and the DCW changed only by $\pm 5\%$. At that moment, final determinations were conducted for DCW, lipid concentration, and DHA concentration in *Schizochytrium* sp. cells.

2.6. Analytical Methods

The crude glycerol concentration in the culture medium was determined by pre-centrifugation ($8000 \times g$, 4 min, 10°C ; UNIVERSAL 320 R centrifuge, Hettich). The supernatant was then filtered (pore size of the filter = 0.2 mm), and the filtrate was assayed for glycerol levels using a Glycerol GK Assay Kit (Megazyme). The test involved phosphorylating the glycerol with adenosine 5'-triphosphate (ATP), with the reaction product (adenosine 5'-diphosphate, ADP) used to further phosphorylate D-glucose, which oxidizes producing nicotinamide adenine dinucleotide (NADH). The concentration of NADH was measured spectrophotometrically (Multiskan GO Microplate, Thermo Scientific) at a wavelength of 340 nm.

The dry cell weight (DCW) of the microalgae was determined according to the method described by Chang et al. (2013) [31]. The assay was done by transferring a 50-cm^3 sample of the culture to a pre-weighed centrifuge tube, which was then centrifuged ($8000 \times g$ for 15 min, UNIVERSAL 320 R centrifuge, Hettich). The supernatant was discarded, and the concentrated biomass was washed twice with distilled water, then dried at 60°C for 12 h in a moisture balance (MAR, Radwag) to stabilize the biomass.

The lipid content of the biomass was determined by adding 7 cm^3 of a 20% hydrochloric acid solution to 1.0 g of freeze-dried biomass (ALPHA 1-4 LD plus freeze dryer, Christ), which was then placed in a water bath (GFL 1003) at 75°C for 40 min. The sample was treated with 20 cm^3 of n-hexane to extract the lipids and placed in a vacuum evaporator (Hei-VAP Advantage G3, Heidolph) to evaporate the solvent. The lipid content of the sample was measured gravimetrically. The determination of fatty acids in the microalgal biomass was done using a modified direct transmethylation method described by Grayburn (1992) [32]. To this end, 20–100 mg of the freeze-dried microalgal biomass (ALPHA 1-4 LD plus, Christ) were transferred to a reaction vial and enriched with 2 mg of triheptadecanoylglycerol as an internal standard. Next, it was spiked with 2 cm^3 of a 1% H_2SO_4 methanolic solution, and then the vial was thoroughly mixed and heated to a temperature of 80°C for 2 h (MKR 13 block thermostat–Ditabis). After cooling, 2 cm^3 of chloroform and 1 cm^3 of distilled water were added. The vial was mixed at 1250 rpm (Vortex Reax top, Heidolph), and centrifuged at $1500 \times g$ (UNIVERSAL 320 R, Hettich). The organic phase containing fatty acid methyl esters (FAME) was harvested and analyzed by chromatography. A Clarus 680 GC (Perkin Elmer) gas chromatograph was used for FAME

analysis, with helium used as the carrier gas. The column temperature was raised from 150 °C to 250 °C at 10 °C/min, then kept at 250 °C for 10 min. The injector temperature was kept at 275 °C with an injection volume of 1 µL. Detection was made using a flame ionization detector (FID) at 280 °C. The peak areas were identified by comparing their retention times with those of standard mixtures.

2.7. Statistical Analysis

Each experimental variant was conducted in four replications. The statistical analysis of experimental results was conducted using a STATISTICA 13.1 PL package. The hypothesis concerning the normality of distribution of each analyzed variable was verified using the W Shapiro-Wilk test. One-way analysis of variance (ANOVA) was conducted to determine differences between the variables. Homogeneity of variance in groups was determined using a Levene test. The Tukey (HSD) test was applied to determine the significance of differences between the analyzed variables. In the tests, results were considered significant at $\alpha = 0.05$.

3. Results

The DCW concentration obtained at stage 1 of the study reached $66.43 \pm 1.29 \text{ g/dm}^3$. The greatest increase in biomass concentration, i.e., from $21.14 \pm 1.44 \text{ g/dm}^3$ to $61.49 \pm 1.89 \text{ g/dm}^3$, was observed in the log phase of growth between the 40th and the 80th h of the culture (Figure 1). In this phase, the microalgae growth rate constant was $\mu = 0.027 \text{ 1/h}$. From the 80th till the 120th h of the culture, DCW stabilized at 61.49 to 66.43 g/dm^3 (Figure 1), and the biomass growth rate reached $r_{\text{DCW}} = 0.55 \text{ g/dm}^3 \cdot \text{h}$. The logarithmic increase in lipid concentration in microalgal cells, ranging from $11.55 \pm 0.54 \text{ g/dm}^3$ to $37.16 \pm 0.68 \text{ g/dm}^3$, was observed between the 20th and the 80th h of the culture (Figure 1). After 80 h, the lipid concentration accounted for 60.43% of DCW. Afterward, it began to decrease, reaching $23.25 \pm 0.45 \text{ g/dm}^3$ after 120 h (Figure 1). Changes in the DHA concentration in the biomass were analogous to those observed for lipids. Between the 20th and the 80th h, it increased 6.5-fold, i.e., from $3.47 \pm 0.192 \text{ g/dm}^3$ to $22.29 \pm 0.41 \text{ g/dm}^3$ (Figure 1), and afterward decreased successively to $14.36 \pm 0.47 \text{ g/dm}^3$ in the 120th h of the culture, accounting for 21% of DCW. The determined rate of DHA increase reached $0.17 \text{ g/dm}^3 \cdot \text{h}$. Over the entire process, DHA concentration ranged from 30 to 70% of the total fatty acids (% TFA), with the 70% TFA value recorded in the 100th h of the culture. Glycerol consumption over the process was directly dependent on the varying DCW concentration. It increased along with an increasing DCW concentration in the culture medium. The initial concentration of this external carbon source in the culture medium was 150 g/dm^3 . During the log phase of growth, i.e., between the 20th and the 80th h of the culture, it decreased from $136.78 \pm 4.58 \text{ g/dm}^3$ to $8.73 \pm 1.21 \text{ g/dm}^3$ (Figure 1), at the consumption rate of $1.17 \text{ g/dm}^3 \cdot \text{h}$.

At stage 2 of the study, the highest DCW concentration reached $104.44 \pm 1.50 \text{ g/dm}^3$, that of lipids— $48.85 \pm 0.81 \text{ g/dm}^3$, and that of DHA— $21.98 \pm 0.36 \text{ g/dm}^3$ (Figure 2). The concentration of the added glycerol was at 416.23 g/dm^3 over the entire process. The greatest increase in the microalgal biomass growth was observed between the 40th and the 80th h of the culture and ranged from $20.23 \pm 1.55 \text{ g/dm}^3$ to $82.3 \pm 2.7 \text{ g/dm}^3$. In this phase, the biomass growth rate constant reached $\mu = 0.035 \text{ 1/h}$. In the stationary growth phase, i.e., between the 20th and the 80th h of bioreactor exploitation, biomass concentration was observed to increase from $82.3 \pm 2.7 \text{ g/dm}^3$ to $103.43 \pm 1.50 \text{ g/dm}^3$ (Figure 2), at the rate of $r_{\text{DCW}} = 0.86 \text{ g/dm}^3 \cdot \text{h}$. The accumulation of lipids at the logarithmic rate was recorded between the 40th and the 80th h of the culture. In that period, the lipid concentration increased from $11.12 \pm 0.85 \text{ g/dm}^3$ to $46.09 \pm 0.85 \text{ g/dm}^3$, which accounted for 44% of DCW (Figure 2). In the subsequent hours of the culture, the increase in lipid concentration in the microalgal biomass was insignificant, and its final value reached $48.85 \pm 0.81 \text{ g/dm}^3$, accounting for 47% of DCW. Between the 40th and the 80th h of the culture, the DHA concentration increased nearly three-fold, i.e., from the initial value of $5.01 \pm 0.38 \text{ g/dm}^3$

to $20.74 \pm 0.38 \text{ g/dm}^3$ (Figure 2), and then stabilized in the subsequent hours of cultivation. The rate of DHA concentration increase reached $0.18 \text{ g/dm}^3 \cdot \text{h}$, whereas DHA concentration accounted for 45% of TFA. The initial concentration of glycerol in the culture medium was at 150 g/dm^3 . Because in the 60th h of the culture, its concentration decreased below 60 g/dm^3 (i.e., was at $57.94 \pm 0.18 \text{ g/dm}^3$), its pulse feeding was begun. This technological treatment enabled maintaining a high ($46.09\text{--}48.85 \text{ g/dm}^3$) and more stable lipid concentration in the biomass, compared to the batch culture. Over the entire 120-h cultivation cycle, additional glycerol doses were fed to the bioreactor three times more.

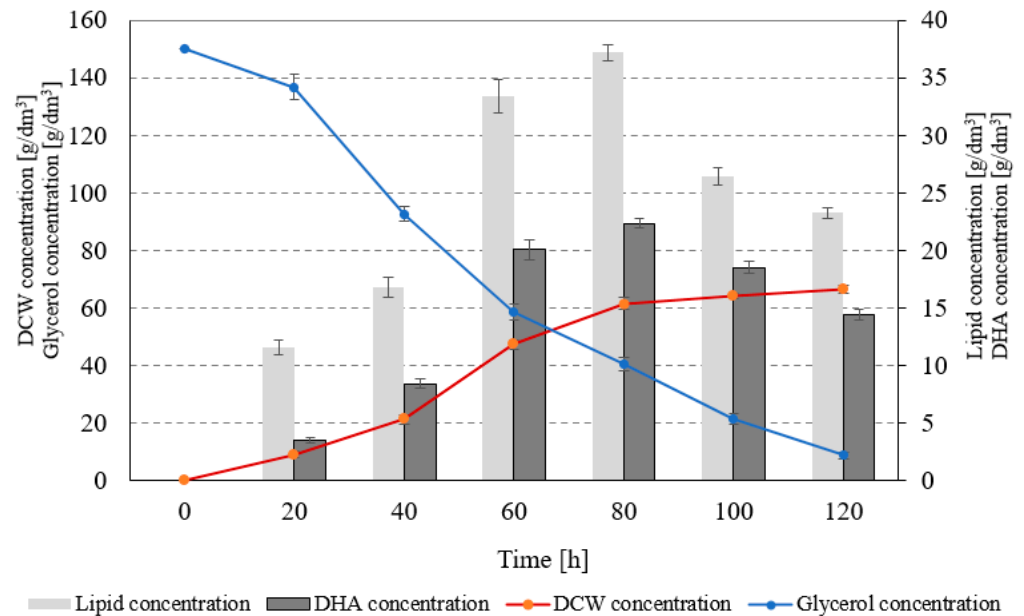


Figure 1. Changes in DCW concentration, lipid concentration, and DHA concentration in *Schizochytrium* sp. biomass and changes in glycerol concentration in the culture medium during the batch culture.

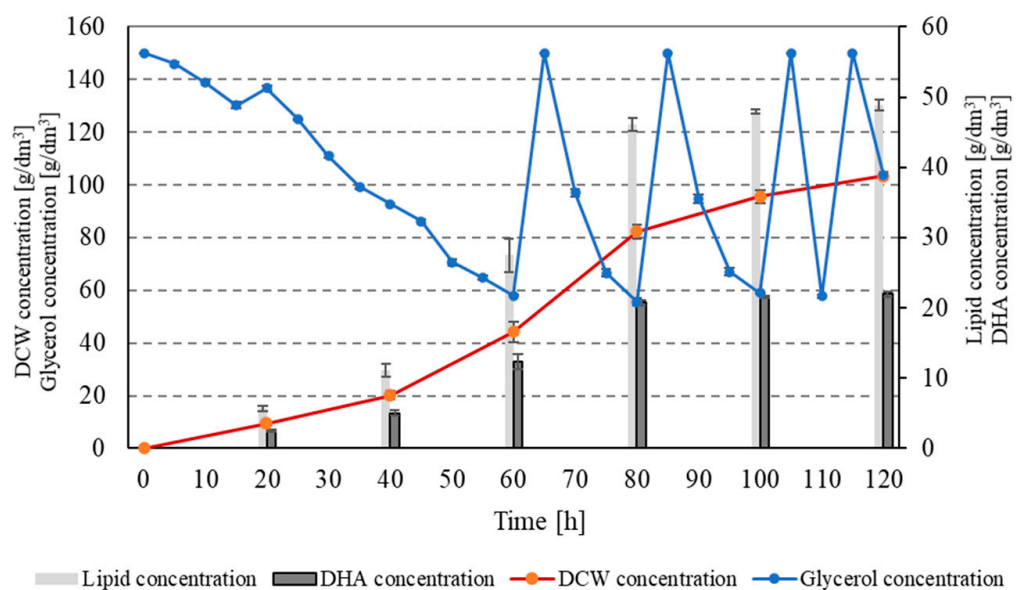


Figure 2. Changes in DCW concentration, lipid concentration, and DHA concentration in *Schizochytrium* sp. biomass and changes in glycerol concentration in the culture medium during the fed-batch culture.

Stage 3 of the study aimed to determine the effect of the selected dilution factor on the efficiency of *Schizochytrium* sp. biomass growth, and production of lipids and DHA. The concentration of *Schizochytrium* sp. DCW in the steady-state was observed to decrease along with an increasing dilution rate (Figure 3, Table 4). The highest DCW concentration, reaching $51.9 \pm 0.91 \text{ g/dm}^3$, was determined at $D = 0.2 \text{ 1/day}$, whereas its lowest value, reaching $16.02 \pm 0.85 \text{ g/dm}^3$, was recorded at $D = 0.8 \text{ 1/day}$ (Figure 3, Table 4). The lowest r_{DCW} value, reaching $r_{\text{DCW}} = 10.38 \pm 0.18 \text{ g/dm}^3 \cdot \text{day}$, was noted at $D = 0.2 \text{ 1/day}$, while the highest one, reaching $r_{\text{DCW}} = 17.63 \pm 0.42 \text{ g/dm}^3 \cdot \text{day}$, was recorded at $D = 0.4 \text{ 1/day}$ (Figure 3). The values indicating the production efficiency of those compounds were also the highest at $D = 0.4 \text{ 1/day}$ and reached $r_{\text{lipids}} = 16.32 \pm 0.25 \text{ g/dm}^3 \cdot \text{day}$ and $r_{\text{DHA}} = 8.37 \pm 0.22 \text{ g/dm}^3 \cdot \text{day}$. The lipid concentration in the *Schizochytrium* sp. cells decreased from $40.79 \pm 0.63 \text{ g/dm}^3$ to $5.23 \pm 0.39 \text{ g/dm}^3$ at D ranging from 0.4 to 0.8 1/day (Figure 3, Table 4). Its highest value ($40.79 \pm 0.63 \text{ g/dm}^3$), was determined at $D = 0.4 \text{ 1/day}$. The rate of the lipid concentration increase (r_{lipids}) decreased from $16.32 \pm 0.25 \text{ g/dm}^3 \cdot \text{day}$ to $4.27 \pm 0.31 \text{ g/dm}^3 \cdot \text{day}$ at D ranging from 0.4 to 0.8 1/day (Figure 3, Table 4). The concentration of DHA in *Schizochytrium* sp. cells in the continuous culture was also dependent on the dilution rate D , decreasing from $20.94 \pm 0.55 \text{ g/dm}^3$ to $2.07 \pm 0.23 \text{ g/dm}^3$ at $D = 0.4\text{--}0.8 \text{ 1/day}$. The rate of the DHA concentration increase also decreased over time, i.e., from $8.37 \pm 0.22 \text{ g/dm}^3 \cdot \text{day}$ at $D = 0.4 \text{ 1/day}$ to $1.66 \pm 0.18 \text{ g/dm}^3 \cdot \text{day}$ at $D = 0.8 \text{ 1/day}$ (Figure 3, Table 4).

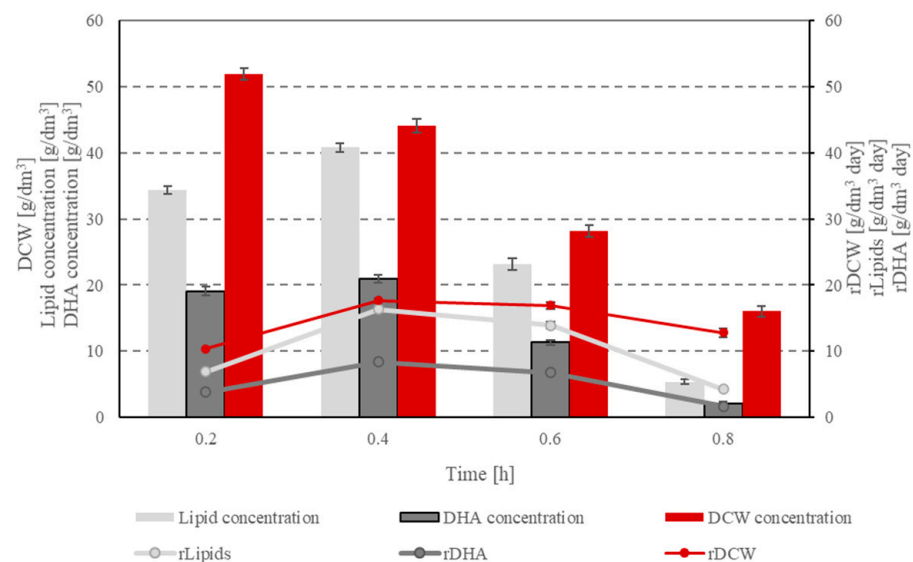


Figure 3. Changes in DCW concentration, lipid concentration, and DHA concentration in *Schizochytrium* sp. biomass and changes in glycerol concentration in the culture medium during the continuous culture.

Table 4. Parameters indicating the efficiency of the continuous culture depending on the dilution rate D (1/day).

Parameter	Unit	Value			
Dilution rate D	(1/day)	0.2	0.4	0.6	0.8
DCW	(g/dm^3)	51.90 ± 0.91	44.07 ± 1.06	28.19 ± 0.85	16.02 ± 0.85
r_{DCW}	($\text{g/dm}^3 \cdot \text{day}$)	10.38 ± 0.18	17.63 ± 0.42	16.92 ± 0.51	12.81 ± 0.68
DHA	(g/dm^3)	19.10 ± 0.68	20.94 ± 0.55	11.29 ± 0.34	2.07 ± 0.23
r_{DHA}	($\text{g/dm}^3 \cdot \text{day}$)	3.82 ± 0.14	8.37 ± 0.22	6.78 ± 0.21	1.66 ± 0.18
TFA	(g/dm^3)	34.37 ± 0.58	40.79 ± 0.63	23.16 ± 0.84	5.34 ± 0.39
r_{lipids}	($\text{g/dm}^3 \cdot \text{day}$)	6.87 ± 0.12	16.32 ± 0.25	13.89 ± 0.51	4.27 ± 0.31

The highest DCW concentration ($103.44 \pm 1.50 \text{ g/dm}^3$) and the highest biomass growth rate in the log phase ($\mu = 0.035 \text{ 1/h}$) were determined in the fed-batch culture

(Table 5). In the batch culture, the DCW concentration was at $66.43 \pm 1.29 \text{ g/dm}^3$ and the average specific growth rate reached $\mu = 0.027 \text{ 1/h}$. In turn, the continuous culture yielded the lowest DCW concentration, reaching $44.07 \pm 1.06 \text{ g/dm}^3$, which was 57% lower compared to the value determined in the fed-batch culture (Table 5). A similar dependency was observed after comparing biomass growth rates. In this case, the highest value was also noted in the fed-batch culture. The r_{DCW} values determined for the batch culture, fed-batch culture, and continuous culture reached $0.55 \pm 0.07 \text{ g/dm}^3 \cdot \text{h}$, $0.86 \pm 0.12 \text{ g/dm}^3 \cdot \text{h}$, and $0.37 \pm 0.03 \text{ g/dm}^3 \cdot \text{h}$, respectively. The fed-batch culture also turned out to be the most efficient considering the lipid concentration ($48.85 \pm 0.81 \text{ g/dm}^3$) and DHA concentration ($21.98 \pm 0.36 \text{ g/dm}^3$) produced in *Schizochytrium* sp. biomass. In this technological variant, the DHA production rate reached $r_{\text{DHA}} = 0.18 \text{ g/dm}^3 \cdot \text{h}$. In the continuous culture, the DHA increase rate was similar, reaching DHA $r_{\text{DHA}} = 0.17 \text{ g/dm}^3 \cdot \text{h}$, but the determined $r_{\text{DCW}} = 0.37 \text{ g/dm}^3 \cdot \text{h}$ was 57% lower than in the fed-batch culture (Table 5). The culture yield, determined as the ratio of dry cell weight (g_{DCW}) to the weight of consumed glycerol (g_{glycerol}), was the lowest in the fed-batch culture and the continuous culture, mainly due to the effect of culture dilution with a fresh culture medium added to the fermenter in a continuous mode.

Table 5. Comparison of parameters characterizing the efficiency of *Schizochytrium* sp. culture and DHA production obtained in the batch, fed-batch, and continuous cultures.

Culture Type	Unit	Batch	Fed-Batch	Continuous ($D = 0.4$)
Culture duration	(h)	120	120	Continuous culture
DCW	(g/dm^3)	66.43 ± 1.29	103.44 ± 1.50	44.07 ± 1.06
r_{DCW}	($\text{g/dm}^3 \cdot \text{h}$)	0.55 ± 0.07	0.86 ± 0.12	0.37 ± 0.03
r_{DHA}	($\text{g/dm}^3 \cdot \text{h}$)	0.12 ± 0.02	0.18 ± 0.01	0.17 ± 0.01
Culture yield	($g_{\text{DCW}}/g_{\text{glycerol}}$)	0.47 ± 0.11	0.25 ± 0.09	0.18 ± 0.03
Glycerol consumption rate	($\text{g/dm}^3 \cdot \text{h}$)	1.17 ± 0.21	3.47 ± 0.30	2.02 ± 0.17
Total fatty acids (TFA)	(g/dm^3)	23.25 ± 0.45	48.85 ± 0.81	40.79 ± 0.63
DHA	(g/dm^3)	14.36 ± 0.47	21.98 ± 0.36	20.95 ± 0.55
Growth rate constant in the log phase	(1/h)	0.027 ± 0.004	0.035 ± 0.001	-

Regardless of culture type, the saturated palmitic acid (C 16:0) and the unsaturated docosahexaenoic acid (C 22:6) belonging to the Omega-3 family were the major fatty acids of the lipid fraction produced by the microalgae. The highest DHA concentration ($61.76 \pm 3.77\%$ of TFA) was determined in the lipid bodies of *Schizochytrium* sp. biomass derived from the batch culture, whereas the lowest one ($44.99 \pm 2.12\%$ of TFA) in those of the biomass produced in the fed-batch culture (Table 6). The percentage composition of fatty acids was the same in all cultures. In all culture variants, the lowest concentration in microalgal cells was observed for stearic acid (C 18:0) and ranged from $1.47 \pm 0.13\%$ of TFA in the batch culture to $2.72 \pm 0.43\%$ of TFA in the continuous culture (Table 6).

Table 6. Composition of fatty acids produced by *Schizochytrium* sp. microalgae in different culture types.

Fatty Acid	Unit	Batch Culture	Fed-Batch Culture	Continuous Culture ($D = 0.4$)
C 14:0		2.38 ± 0.21	3.51 ± 0.64	2.79 ± 0.28
C 16:0		27.88 ± 1.37	41.01 ± 2.84	38.57 ± 1.75
C 18:0	(% TFA)	1.47 ± 0.13	2.16 ± 0.47	2.72 ± 0.43
C 22:5 (DPA)		5.52 ± 0.98	8.13 ± 0.93	7.24 ± 0.94
C 22:6 (DHA)		61.76 ± 3.77	44.99 ± 2.12	51.33 ± 3.78

4. Discussion

The conducted experiments inscribe into pioneer research on the use of waste organic substrates to grow heterotrophic microalgae from the genus *Schizochytrium* sp. for industrial purposes [33,34]. The use of this technological solution can reduce industrial greenhouse gas emissions and significantly decrease the costs of producing high-value compounds, like Omega-3 fatty acids [33]. The greatest amounts of waste glycerol are generated from biodiesel production. Because the purification of crude glycerol generated in this process is expensive, its alternative applications are looked after. One of these can be its use as an external carbon source in microalgae cultivation [35,36].

One of the little-explored approaches of utilizing the waste glycerol fraction and converting it into value-added biocomponents is to use the biomass of *Schizochytrium* sp. heterotrophic microalgae. These algae accumulate large quantities of DHA in their cells, making them a prime resource for use in food, pharmaceutical, and animal feedstuff industries [37]. The *Schizochytrium* sp. biomass has been shown to grow on various carbon sources [38], substantiating efforts to develop efficient methods for growing it on waste glycerol as a carbon source. Studies to date have examined the growth of the *Thraustochytriaceae* family microalgae on such waste materials as spent brewer's yeast [39], breadcrumbs [40], coconut water [41], empty palm fruit bunches [42], sweet sorghum juice [43], okara powder [44], and beer and potato processing residues [45].

Each contaminant present in waste glycerol affects the cultivation of microalgae, which utilize this compound as the main carbon source. For instance, microalgae utilize free fatty acids, representing these contaminations, to synthesize longer polyunsaturated fatty acids (PUFAs), like e.g., DHA [46,47]. The production of PUFAs by microalgae with glycerol as a substrate is also aided by certain strains, like *Nitzschia closterium* or *Cryptocodinium cohnii*, which are capable of accumulating lipids, DHA in particular [48–50]. Other organisms that can produce PUFAs and grow on waste glycerol include the following microalgae/fungi: *Skelotema costatum*, *Pythium ultimum*, *Pythium irregulare*, *Mortiriella alpine*, *Chlorella stigmatophora*, and *Nannochloropsis salina*, and also various *Codium* sp. species [51,52].

The literature works have described multiple strategies implemented to intensify DHA synthesis by microalgae. A study by Wu et al. (2005) [53] has demonstrated glucose to be the optimal carbon source for the *Schizochytrium* sp. S31 strain is cultured for DHA isolation. In turn, Bailey et al. (2003) [54] have focused not only on the carbon source that would intensify DHA production but also on the mode of controlling oxygen concentration in the culture medium, which allowed them to obtain the DCW and DHA concentrations at 171.5 g/dm³ and 35.33 g/dm³, respectively. Ganuza et al. (2008) [55] also described *Schizochytrium* sp. G13/2S cultivation was performed based on the pH-auxostat, in which the final DCW concentration reached 63.3 g/dm³, and DHA concentration was at 5.47 g/dm³. Furthermore, Qu et al. (2010) [56] used in their study a two-stage cultivation method based on selected values of the oxygen diffusion coefficient ($k_L a$), which allowed them to obtain *Schizochytrium* sp. HX-308 biomass concentration at 92.72 g/dm³ and DHA concentration at 17.7 g/dm³.

Previous research works have demonstrated that the highest DHA concentration obtained during microalgae cultivation on the medium with glycerol reached 20.3 g/dm³ [57], whereas the highest DHA production yield reached 0.138 g/dm³·h [58]. In addition, the extent of glycerol conversion into microbiological oil was reported at 0.10 ± 0.02 g/g, which is lower than the glucose conversion rate. This difference was probably due to the relatively poor regulation of enzymes involved in the metabolic processes associated with glycerol assimilation [59]. However, Pyle et al. (2008) [60] have demonstrated the conversion rate into microbiological oil approximating 0.15 g/g in the case of a heterotrophic *Schizochytrium limacinum* strain. The above findings indicate that it is necessary to search for novel cultivation methods that would contribute to an increase in the conversion rate of a cheap carbon source (like glycerol) into a high-value microbiological oil (having a

higher concentration of DHA) and, consequently, to increased cost-effectiveness of the whole cultivation process.

The present study demonstrated that a crude glycerol concentration of 150 g/dm³ led to the highest DCW levels at 67.55 g/dm³, and DHA concentration increase in *Schizochytrium* sp. cells to 17.25 g/dm³. Other researchers have also noted how the type and concentration of the external carbon source affected microalgal cultures [61–63]. Those authors reported that the carbon source affected biomass growth and could influence PUFAs synthesis and that the glycerol-based cultures produced higher yields than the processes based on glucose, coconut oil, brewery wastes, or wastewater from soymilk production. It needs to be emphasized that the cultivation processes performed with glucose as a carbon source have not been optimized so far. The *Thraustochytriaceae* microalgae biomass (Y) grown on crude (waste) glycerol was found to produce higher DHA yields (166–550 mg/g) than a pure glycerol-based culture (110–223 mg/g) [62,63]. It is worth noting, however, that the final cell DHA levels are mainly determined by the cell growth phase at the time of DHA extraction.

So far, many studies have been described in the literature on the search for cultivation methods that will ensure an increase in the production of microbial lipids. It has been proven that fed-batch cultures are highly effective, as they allow for the significant growth of microorganism biomass and contribute to a high lipid content in cells [64,65]. This culture type was used to produce DHA by *Cryptothecodinium cohnii* and *Aurantochytrium* sp. KRS101 microalgae, with acetic acid and glucose as external carbon sources [66].

The present study aimed to verify which of the culture variants tested (batch culture, fed-batch culture, and continuous culture) promoted the growth of *Schizochytrium* sp. biomass and ensured the high accumulation of lipids, including docosahexaenoic acid. The most effective technological solution turned out to be the fed-batch culture. Over the 120-h cultivation period, it ensured DCW concentration at 103.44 ± 1.50 g/dm³, with the biomass growth rate reaching $r_{DCW} = 0.86 \pm 0.12$ g/dm³·h. Ultimately, the microalgal cells accumulated 48.85 ± 0.81 g/dm³ of lipids, with DHA concentration accounting for 45% *w/w* ($C_{DHA} = 21.98 \pm 0.36$ g/dm³), and its concentration increase rate reaching $r_{DHA} = 0.18$ g/dm³·h.

It has been reported in the literature that the production efficiency of bioactive compounds by microorganisms during a fed-batch culture can be significantly improved by keeping the concentration of the test substrate constant [67]. Maintaining a constant concentration of sucrose was considering the best method ensuring a high yield of 2,3-butanediol in the *Serratia marcescens* culture [68]. A study conducted by Bailey et al. (2003) [54] proved that under strictly controlled pH value, oxygen level, and glucose concentration in the fed-batch culture, the *Schizochytrium* sp. microalgae were able to produce 200 g/dm³ of biomass (during 90–100 h of the culture), simultaneously accumulating 40–45 g/dm³ of DHA. The presented works confirm that the fed-batch culture is the most effective cultivation method allowing to obtain high concentrations of both the microalgal biomass and the desired secondary metabolite–DHA. The advantage of this cultivation method in the production of a wide range of bioproducts, such as amino acids, antibiotics, unicellular proteins, vitamins, enzymes, etc., lies in the elimination of the inhibiting effect of the excess concentration of substrates on the growth of microorganisms [68].

During the continuous culture of *Schizochytrium* SR21, Ethier et al. (2010) [61] recorded the rate of dry cell weight increase at $r_{DCW} = 0.145$ g/dm³·h, and DHA increase rate at $r_{DHA} = 21.7$ mg/dm³·h. In the present study, the continuous culture yielded over 2.5-fold higher biomass growth and almost 8-fold higher DHA concentration increase, proving that the final culture performance parameters are also affected by the microalgae strain used. Similar concentrations of *Cryptothecodinium cohnii* biomass (109 g/dm³) and DHA (19 g/dm³) were obtained by Ratledge (2001) [69]. However, this culture was conducted on a laboratory scale (2 dm³) using the pH-auxostat method. In turn, the *Schizochytrium* sp. HX-308 biomass concentration reaching 92.72 g/dm³ and DHA concentration reaching 17.70 g/dm³ were obtained in a pilot-scale study (a bioreactor with a working capacity of

50 dm³) by Qu (2010) [56], who optimized oxygen diffusion into cells by the appropriate choice of the $k_L a$ coefficient value.

The profile of fatty acids produced by *Schizochytrium* sp. microalgae did not differ, regardless of the process scale. An identical observation was made by Hu et al. (2008) [70], who demonstrated that the qualitative profile of fatty acids with a chain length of 10–24 atoms was similar for the species of the same class or division and did not depend on the culture scale. In turn, the fatty acid composition was reported to change depending on the growth phase; with an increase observed in their concentration in the logarithmic growth phase, and stabilization or a slight decrease—in the stationary growth phase [71]. It is worth emphasizing that the unsaturation degree of fatty acids produced by microorganisms can be modified by a few parameters, including carbon source, concentrations of carbon and nitrogen compounds, dilution rate (in the case of the continuous culture), saturation with oxygen, or temperature [55,72]. According to literature data available regarding the laboratory-scale cultures, the *Schizochytrium* sp. biomass growth rate (r_{DCW}) ranged from 0.15 to 1.3 g/dm³·h [60,73], whereas DHA concentration increase rate (r_{DHA}) from 0.010 to 0.55 g/dm³·h [16].

Many technological treatments are recommended to ensure a high density of *Schizochytrium* biomass and a high rate of DHA concentration increase when scaling up from laboratory studies. According to the Martek Biosciences company, the modifications concerning the nutrient composition of the culture medium, processes conducted in the chemostat, and two-stage processes with limited concentrations of nitrogen compounds, can increase the biomass concentration of *Schizochytrium* genus microalgae (a genetically modified strain) from 21 g/dm³ (laboratory scale) to even 170–210 g/dm³ (commercial scale), and DHA concentration from 2 g/dm³ (laboratory scale) to 40–50 g/dm³ (pilot scale) [16].

5. Conclusions

The analysis of the results obtained in the present study proved the feasibility of utilizing waste glycerol, generated during biodiesel production, to produce DHA in the culture of microalgae from the genus *Schizochytrium* sp.

The highest technological effects were obtained in the fed-batch culture. The concentration of *Schizochytrium* sp. biomass reached 103.44 ± 1.50 g/dm³, the lipid concentration in *Schizochytrium* sp. biomass was at 48.85 ± 0.81 g/dm³, whereas the DHA concentration at 21.98 ± 0.36 g/dm³.

The highest DHA concentration, accounting for $61.76 \pm 3.77\%$ of TFA, was determined in lipid bodies of the *Schizochytrium* sp. biomass produced in the batch culture, whereas the lowest one, accounting for $44.99 \pm 2.12\%$ of TFA, in those of the biomass grown in the fed-batch culture. The percentage composition of fatty acid was the same in all cultures.

The culture yield, determined as the ratio of dry cell weight to the weight of consumed glycerol, was the lowest in the fed-batch culture and the continuous culture, mainly due to the effect of culture dilution with a fresh culture medium added to the fermenter in a continuous mode.

The high efficiency of the technological solution tested should be further confirmed in fractional-technical and then on the commercial scale. It is also necessary to perform a thorough analysis of the economic effectiveness of the proposed technology based on data obtained from the installation exploited on the large scale.

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Abbreviations

DHA	docosahexaenoic acid
TFA	total fatty acids
DCW	dry cell weight
ATCC	American Type Culture Collection
D	dilution rate (dilution factor)
F	substrate flow rate
V	bioreactor tank volume
NADH	nicotinamide adenine dinucleotide
FID	flame ionization detector
r_{DHA}	rate of DHA production by microalgae
r_{DCW}	growth rate of the microalgal biomass
r_{lipids}	rate of lipids production

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