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Effect of the Concentration of Extracellular Polymeric Substances (EPS) and Aeration Intensity on Waste Glycerol Valorization by Docosahexaenoic Acid (DHA) Produced in Heterotrophic Culture of *Schizochytrium* sp

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Abstract: The study aimed to determine the effectiveness of docosahexaenoic acid (DHA) production by *Schizochytrium* sp. biomass fed with waste glycerol depending on the concentration of extracellular polymeric substances (EPS) in the culture medium and medium aeration effectiveness. The microalgae from the genus *Schizochytrium* sp. were proved to be capable of producing EPS composed of glucose, galactose, mannose, fucose, and xylose. The highest EPS concentration, reaching 8.73 ± 0.09 g/dm³, was determined at the stationary growth phase. A high EPS concentration caused culture medium viscosity to increase, contributing to diminished oxygen availability for cells, lower culture effectiveness, and reduced waste glycerol conversion to DHA. The *Schizochytrium* sp. culture variant found optimal in terms of the obtained technological effects and operating costs was performed at the volumetric oxygen mass transfer coefficient of $k_L a = 600$ 1/h, which enabled obtaining dry cell weight (DCW) of 147.89 ± 4.77 g/dm³, lipid concentration of 69.44 ± 0.76 g/dm³, and DHA concentration in the biomass reaching 29.44 ± 0.36 g/dm³. The effectiveness of waste glycerol consumption in this variant reached 3.76 ± 0.31 g/dm³·h and 3.16 ± 0.22 g/gDCW.

Keywords: waste glycerol; microalgae; *Schizochytrium* sp.; docosahexaenoic acid (DHA); extracellular polymeric substances (EPS); lipids

1. Introduction

Microalgae from the genus *Schizochytrium* sp. are known for producing valuable compounds, e.g., docosahexaenoic acid (DHA)—belonging to the Omega-3 family of fatty acids. So far, its main dietary sources have included fish flesh lipids and plant oils [1]. As the demand for Omega-3 family fatty acids increases due to the growing awareness of consumers and their care for a healthy lifestyle [2], a need emerges to search for competitive methods of their production that would be environmentally friendly as well as technologically and economically viable. The biomass of heterotrophic microalgae from the genus *Schizochytrium* sp. seems to offer a fine alternative in this respect [3].

To optimize, boost yield, and improve cost-effectiveness of DHA production by *Schizochytrium* sp., studies have analyzed multiple technological parameters having a significant impact on the final production results. These included: culture medium pH [4], temperature [5], turbine speed [6,7], nutrient concentration [8], oxygen concentration [9], initial inoculum concentration [10], and salinity [11]. Unfortunately, values of these basic parameters characterizing *Schizochytrium* sp. culture conditions determined under labora-

tory conditions are not always consistent with respective values obtained from large-scale installations [12,13].

Inexpensive carbon sources offering an alternative to glucose are also searched for. Given the fact that *Schizochytrium* sp. biomass can grow on various carbon sources, including wastes, studies on the use of waste glycerol seem a justified prospective trend in scientific research [14]. So far, glycerol has been used as a carbon source in biochemical processes [15] and thermochemically converted to dipropylene glycol [16] and hydroxyacetone [17]. Other explored processes included its reformation to produce hydrogen and synthesis gas [18], esterification [19], hydrogenolysis [20], and production of epichlorohydrin [21]. Other ways of waste glycerol management for energetic purposes that have recently spurred great interest include production of ethanol (*Saccharomyces cerevisiae*) or β -carotene (*Blakeslea trispora*) [22–24].

Technological advance necessitates scale enlargement and verification of data obtained, that can be achieved via exploitation of pilot bioreactor installations followed by tests in the full technical scale [25–28]. During culture scale enlargement, it is impossible to keep all technological parameters at the levels established and employed in the laboratory scale [29,30]. Additionally, new difficulties and limitations often emerge, which determine the course and effectiveness of the whole process [31]. It is then indispensable to know the critical values of technological parameters having the greatest impact on the course of the entire incubation process [32]. One of the exploitation treatments posing severe difficulties in the large-scale facilities is the technologically and economically effective oxygen supply to bioreactors [33]. These difficulties stem from the cubature of facilities and growing viscosity of the culture medium during intensive production of *Schizochytrium* sp. Biomass [34,35].

Fluid viscosity affects the mixing process, mass transfer, and gas diffusion in the culture medium, and consequently, the Reynold number value, and correlates with the oxygen mass transfer coefficient [36,37]. In high-density microbiological cultures with a high biomass concentration, the culture liquid turns viscous, which significantly influences hydrodynamics, heat and mass transfer, cell growth kinetics, and formation and accumulation of secondary metabolites in the culture medium [38–40]. It is believed that the increase in culture medium viscosity observed in the microorganism-mediated processes, including *Schizochytrium* sp., is largely due to the production of extracellular polymeric substances (EPS) [41,42]. Apart from technological difficulties and complications in establishing a commercial scale of high-density cultures, the viscosity increase caused by polysaccharide production may suppress lipid accumulation in cells and DHA production [43]. Therefore, it seems advisable to conduct a study to monitor culture viscosity and adjust aeration parameters to ensure the highest effectiveness of oxygen transfer to cells as the method of oxygen supply determines its solubility in the culture medium in aerobic biotechnological processes [44,45].

The study aimed to determine the effectiveness of a heterotrophic culture of *Schizochytrium* sp. fed with waste glycerol as an organic carbon source depending on the concentration and characteristics of extracellular polymeric substances (EPS) produced by the microalgal biomass and on medium aeration effectiveness. Experimental works aimed at verifying the influence of these parameters on the viscosity of the culture, the growth of biomass and the production of docosahexaenoic acid (DHA) were carried out on a laboratory scale.

2. Methods

2.1. Materials

The study used *Schizochytrium* sp. strain obtained from the ATCC (American Type Culture Collection). The method harnessed for preliminary biomass proliferation using the ATCC790By+ culture medium was presented in our previous works [46,47]. The exact experiment was conducted in a bioreactor with an active volume of 20 dm³ (Biostat C, Sartorius Stedim, France).

2.2. Cultivation Conditions

Crude glycerin, obtained from the PKN Orlen Południe S.A. Plant in Trzebinia, Poland, was used as the sole source of carbon in the cultures. The most important information from its characteristics' card (<http://www.rafineria-trzebinia.pl>, accessed on 1 September 2021) is presented in Table 1.

Table 1. The properties and characteristics of the glycerol used in the experiments.

Properties	Unit	Concentration
Color	[-]	light-brown
Odor	[-]	characteristic
pH	[-]	5
Glycerol	[% w/w]	80
Water	[% w/w]	15
Sulphated 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 NaOH 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	[kg/dm ³]	1.26
Viscosity at 20 °C	[mm ² /s]	1.5
Density at 15 °C	[kg/dm ³]	1.2
Explosive properties	[-]	-

The culture was incubated for 120 h, and the initial concentration of waste glycerol was 150 g/dm³. Throughout the process, biomass samples were collected every 5 h and determined for the concentration of waste glycerol left in the culture medium. If the concentration was equal to or lower than 60 g/dm³, fresh waste glycerol (density of 1.26 g/cm³) was fed 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 [46,47]. Feeding the additional glycerol prevented *Schizochytrium* sp. from entering into the decline phase and allowed the continuous increase of lipid concentration in the biomass.

The other, initial culture conditions were established in our earlier optimization works [46,47]. The Plackett–Burman method and Response Surface Methodology were used for the two-stage identification. The modeled values of parameters and the expected final effects were verified in a laboratory scale [46]. The initial culture conditions were as follows: glycerol concentration 150 g/dm³, temperature 27 °C, peptone concentration 10 g/dm³, oxygen mass transfer rate k_La 150 1/h, salinity 17.5 psu, culture pH 6.5, yeast extract concentration 0.4 g/dm³, turbine speed 185 rpm, and inoculum DCW 5.0 g/dm³.

As culture medium viscosity significantly affects oxygen transport to cells, and, consequently, the value of oxygen mass transfer rate (k_La), it turned out necessary to verify

those initial culture conditions, which have a direct impact on its values, i.e., turbine speed and volumetric air flow rate (the other culture parameters remained unchanged). Given the above, five cultures were performed differing in $k_L a$ values due to the modification of mixing and aeration conditions. Afterward, the final biomass concentration, lipid, and DHA concentration were determined for these cultures. The results obtained allowed determining the rate of DHA production by microalgae (r_{DHA}) and the growth rate of the microalgal biomass (r_{DCW}). The values of mixing and aeration parameters used in the successive experimental variants are presented in Table 2.

Table 2. The $k_L a$ values and conditions of *Schizochytrium* sp. culture mixing and aeration used in the study.

Culture Variant	$k_L a$ [1/h]	Turbine Speed [rpm]	Volumetric Air Flow Rate [dm ³ /h]	Bioreactor Tank Volume [dm ³]
1	150	185	0.80	20.0
2	300	370	1.60	20.0
3	450	555	2.40	20.0
4	600	740	3.20	20.0
5	750	925	4.00	20.0

2.3. Analytical Methods

Determinations of: glycerol concentration in the culture medium, dry cell weight (DCW) of microalgae, and lipid concentration in the biomass, as well as the qualitative analysis of microalgal biomass fatty acids were conducted following the methodology presented in our previous works [46,47]. Crude glycerol concentration in the culture medium was determined by centrifugation ($8000 \times g$, 4 min, 10 °C; UNIVERSAL 320 R centrifuge, Hettich, Tuttlingen, Germany) and analysis of the supernatant using the Glycerol GK Assay Kit (Megazyme, Bray, Ireland). The concentration of nicotinamide adenine dinucleotide (NADH) was measured spectrophotometrically (Multiskan GO Microplate, Thermo Scientific, Vantaa, Finland) at a wavelength of 340 nm. The dry cell weight (DCW) of the microalgae was determined from the concentrated biomass that was washed twice with distilled water, then dried at 60 °C for 12 h in a moisture balance (MAR, Radwag, Poland) to stabilize the biomass. The lipid content of the biomass was determined by adding a hydrochloric acid solution to 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 n-hexane for lipid extraction and placed in a vacuum evaporator (Hei-VAP Advantage G3, Heidolph, Schwabach, Germany) to evaporate the solvent. The lipid content of the sample was measured gravimetrically. The determination of fatty acids was done using a modified direct transmethylation method. The organic phase containing fatty acid methyl esters (FAME) was harvested and analyzed by chromatography (Clarus 680 GC (Perkin Elmer, Waltham, MA, USA) gas chromatograph) [46,47].

Culture viscosity was determined using a Cannon-Fenske capillary viscosimeter (SCHOTT, Mainz, Germany). The $k_L a$ value was determined using a method described by Swaaf et al. (2001) [40]. To this end, two solutions were prepared, including a supernatant of the culture medium and a solution of marine salt having the same concentration as in the culture medium (reference solution). To decrease partial pressure to 2% in both solutions, they were flushed with nitrogen. Then, each solution was aerated at the rate of 5 dm³/min and mixed at the speed of 300 rpm. The $k_L a$ value was determined as the inclination of the curve of partial oxygen concentration in the solution dependence on time.

Samples (5 cm³) of the *Schizochytrium* sp. culture were collected, transferred to a centrifuge tube, and centrifuged at a room temperature for 12 min at $15,000 \times g$ (UNIVERSAL 320 R, Hettich). After completed centrifugation, the supernatant was determined for the concentration of saccharides representing the polymeric part of EPS, i.e., the so-called non-attached EPS [48]. The saccharides were isolated from the supernatant by 12-h precipitation

in cold ethanol ($-20\text{ }^{\circ}\text{C}$), the final concentration of which after sample addition reached 80%, and then by nitrogen drying. Afterward, their concentration was determined using the phenol-sulfate method [49]. To this end, a 1-cm^3 sample of precipitated polysaccharides dissolved in distilled water was collected, to which 1 cm^3 of a 5% phenol solution was added, and the whole sample was mixed at the speed of approximately 1250 rpm (Vortex Reax top, Heidolph). Next, 5 cm^3 of concentrated H_2SO_4 were added using an automatic pipette, and the sample was again mixed at the speed of approximately 1000 rpm (Vortex Reax top, Heidolph) and left to stand. After 10 min, the sample was mixed again and placed in a water bath with a temperature of $27\text{ }^{\circ}\text{C}$ for 15 min (GFL 1003 digital water bath). After the fixed time, the sample was shaken, and then its absorbance was measured at the wavelength of 488 nm (Multiskan GO Microplate spectrophotometer, Thermo Scientific), using a glucose solution as a standard solution.

The composition of monosaccharides constituting the EPS polysaccharide fraction was analyzed with the gas chromatography method. To this end, the polysaccharide fraction of extracellular EPS was hydrolyzed, whereas saccharide monomers were methylated with acid methanol containing 2 M HCl. The hydrolysis was continued for 16 h at a temperature of $85\text{ }^{\circ}\text{C}$. The resulting samples, containing methylglycosides, were converted into trimethylsilyl derivatives and separated in a silica column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$) in a chromatograph (Perkin Elmer, Clarus 680 GC) equipped in a split-splitless injector and a flame ionization detector (FID), according to the method by Chaplin and Kennedy (1986) [50]. Injector's and detector's temperatures were set at 220 and $250\text{ }^{\circ}\text{C}$, respectively. Pure helium was used as a carrier gas, at the flow rate of $25\text{--}30\text{ cm}^3/\text{min}$, whereas mannitol was used as the internal standard.

2.4. Statistical Analysis

All experiments were conducted in four replications. Measurements of the analyzed parameters and indicators were carried out in duplicate or triplicate depending on the repeatability of the obtained results. The statistical analysis of experimental results was carried using a STATISTICA 13.1 PL package. One-way analysis of variance (ANOVA) was performed to determine the significance of differences between variables. HSD Tukey test was used for post hoc analysis. In the tests, results were considered significant at $\alpha = 0.05$.

3. Results and Discussion

3.1. EPS Concentration and Composition

Many factors determine EPS production by microorganisms, including nutrients present in the culture medium—such as carbon or nitrogen [51,52] and their concentration ratio, and also physical parameters, such as temperature, pH, or mixing speed [53,54]. EPS are essential for proper microalgae growth in the medium as they increase their mobility, serve as a carbon source for bacteria and microfauna, and finally elicit various effects on physical and chemical parameters of the culture medium, including porosity, mechanical stability, or density [55,56]. Besides, they preserve the structure of cell aggregates and protect them against drying out [57].

During the culture of *Schizochytrium* sp. biomass under optimized initial conditions, culture medium viscosity increased from $7.15 \pm 0.91\text{ mm}^2/\text{s}$ to $20.97 \pm 0.74\text{ mm}^2/\text{s}$. Its most dynamic increase, from $11.11 \pm 0.17\text{ mm}^2/\text{s}$ to $17.77 \pm 0.16\text{ mm}^2/\text{s}$, was observed from the 60 h of incubation till the culture reached the stationary growth phase (Figure 1). A linear correlation was found between DCW of *Schizochytrium* sp. biomass, viscosity of the culture medium, and EPS concentration (Figure 2). After 120 h of incubation, DCW concentration reached $103.44 \pm 1.5\text{ g/dm}^3$ (Figure 1). The initial EPS concentration in the culture medium was $2.93 \pm 0.11\text{ g/dm}^3$ and increased to $4.63 \pm 0.14\text{ g/dm}^3$ in the 60 h of incubation. Afterward, the *Schizochytrium* sp. population reached the stage of the stationary growth, which determined a ramped increase in EPS concentration to $7.25 \pm 0.11\text{ g/dm}^3$, followed by its stabilization, and its final concentration of $8.73 \pm 0.09\text{ g/dm}^3$ after 120 h of incubation (Figure 1). Different reviews concerning microalgae EPS production have been published [58,59]. Most of results

were obtained in laboratory, with perfectly controlled conditions. Both the EPS productivity and the EPS concentration in the culture medium vary significantly depending on the strain studied. Nevertheless, the maximum productivity found in literature for a cyanobacteria (*Anabanea* sp.) is $2.9 \text{ g/dm}^3/\text{d}$, for a microalga grown in autotrophy (*Porphyridium* sp.) is $0.19 \text{ g/dm}^3/\text{d}$ and for a microalga grown in heterotrophy (*Chlorella* sp.) is 0.36 g/L/d . The average concentrations are most of the time between 0.5 and 1 g/dm^3 [60]. Jain et al. (2005) [61] observed a lower yield of EPS production by microalgae from the *Thraustochytriaceae* family, reaching $0.3\text{--}1.1 \text{ g/dm}^3$, compared to that determined in the present study. Additionally, Chang et al. (2014) [62] reported on the possibility of EPS production by the biomass *Schizochytrium* sp. S31 and the effect of this process on the increase in the viscosity of the culture medium.

Figure 1. Changes in *Schizochytrium* sp. DCW concentration, medium viscosity, and EPS concentration in the culture medium during incubation.

Figure 2. Dependence of DCW and medium viscosity on EPS fraction concentrations culture.

Concentrations of saccharide monomers of the EPS fraction remained stable over the incubation period. The monosaccharides present in the EPS fraction included hexoses, except for xylose which is classified among pentoses. The major monosaccharide turned out to be glucose, with the concentration accounting for $60.64 \pm 1.93\%$ mol. In contrast, the lowest concentration, reaching $2.66 \pm 1.28\%$ mol, was found for xylose. The qualitative and quantitative composition of monomer saccharides is presented in Table 3. Similar conclusions were formulated by Jain et al. (2005) [61], who additionally demonstrated that glucose, galactose, mannose, and xylose of the EPS fraction were constituents of the cell walls of the *Thraustochytriaceae* family microalgae.

Table 3. Composition of saccharide monomers of the EPS polysaccharide fraction.

Saccharide Monomer	Molar Percentage [% mol]
Glucose	60.64 ± 1.93
Galactose	18.00 ± 1.13
Mannose	11.47 ± 0.71
Fucose	7.23 ± 0.62
Xylose	2.66 ± 1.28

3.2. Effect of Oxygen Mass Transfer Rate (k_La) on Culture Technological Effectiveness

The method of oxygen supply determines its solubility in the culture medium in aerobic biotechnological processes [44,63]. Investigations conducted thus far have shown that providing the appropriate oxygen supply to the culture significantly increases the growth of *Schizochytrium* sp. cells and DHA synthesis in the biomass [35,62,64]. Oxygen transfer to cells has been found to be strongly affected by culture medium viscosity, whose increase observed in microbiological cultures is due to EPS synthesis [65].

The increased viscosity of the culture medium diminishes the effectiveness of oxygen transfer to cells, which affects the effectiveness of waste glycerol consumption and its conversion to DHA. Hence, it is essential to search for a method of air supply to the system that would be justified by both final technological effects and operating costs.

Regardless of the applied k_La coefficient value, each culture entered into three growth phases: adaptation phase (1) spanning from 0 to 40th hour, logarithmic growth phase (2) observed between the 40th hour and 80th–100th hour, and stationary growth phase (3) observed since the 80th–100th hour to 120th hour of incubation. In the variant with $k_La = 150$ 1/h, biomass DCW increased from 5.0 ± 1.0 g/dm³ to 22.42 ± 1.09 g/dm³ in phase 1, from 22.42 ± 1.09 g/dm³ to 87.60 ± 2.79 g/dm³ in phase 2, and reached the final value of 112.77 ± 2.65 g/dm³ in phase 3 (Figures 3a and 4, Table 4). The concentrations of lipids and DHA peaked in the logarithmic growth stage to reach the final values of 48.90 ± 1.56 g/dm³ and 21.02 ± 0.73 g/dm³, respectively (Figure 4). Similar values of biomass production, lipid accumulation, and DHA synthesis were obtained in variant 2 with $k_La = 300$ 1/h (Figures 3b and 4, Table 4). Glycerol consumption yield in both these variants approximated 3.6 g/dm³·h and 4.0 g/gDCW (Table 4). Ren et al. (2010) [35] confirmed in their study that $k_La = 150$ 1/h promoted cell growth within the first 40 h of incubation and that decreasing the value of this coefficient to 88.5 1/h increased final DHA concentration in cells. The above observations were presumably due to the microalgae capability for lipid accumulation under conditions of substrate stress [66]. In the culture of *Schizochytrium* sp. described by Chang et al. (2013) [67], the use of $k_La = 143 \pm 19$ 1/h ensured biomass DCW concentration at 25.56 ± 1.60 g/dm³ and DHA concentration at barely 2.28 g/dm³. The low values of the above parameters can be explained by poor availability of oxygen molecules to microalgal cells and, consequently, by the impaired metabolic activity in the cycle of tricarboxylic acid or/and lipogenesis.

Figure 3. Changes in concentrations of *Schizochytrium* sp. DCW, lipids, and DHA in the biomass at $k_L a$ values of: (a) 150 [1/h], (b) 300 [1/h], (c) 450 [1/h], (d) 600 [1/h], and (e) 750 [1/h].

Figure 4. Concentrations of DCW, DHA, and lipids depending on k_La value.

Table 4. Parameters characterizing the effectiveness of *Schizochytrium* sp. culture depending on k_La value.

Parameter	Unit	$k_La = 150$ [1/h]	$k_La = 300$ [1/h]	$k_La = 450$ [1/h]	$k_La = 600$ [1/h]	$k_La = 750$ [1/h]
Time [h]	[h]	120	120	120	120	120
DCW	[g/dm ³]	112.77 ± 2.65	113.45 ± 2.05	139.90 ± 3.86	147.89 ± 4.77	149.03 ± 3.31
Lipid concentration	[g/dm ³]	48.90 ± 1.56	49.34 ± 1.25	60.03 ± 1.24	69.44 ± 0.76	69.88 ± 0.81
DHA	[g/dm ³]	21.02 ± 0.73	22.15 ± 0.65	25.32 ± 0.56	29.44 ± 0.36	30.05 ± 0.71
r_{DCW}	[g/dm ³ ·h]	0.94 ± 0.15	0.95 ± 0.09	1.17 ± 0.21	1.23 ± 0.25	1.24 ± 0.13
r_{DHA}	[g/dm ³ ·h]	0.18 ± 0.08	0.18 ± 0.05	0.21 ± 0.02	0.25 ± 0.15	0.25 ± 0.09
Glycerol consumption	[g/dm ³ ·h]	3.57 ± 0.32	3.60 ± 0.26	3.68 ± 0.28	3.76 ± 0.31	3.82 ± 0.19
EPS	[g/gDCW]	3.97 ± 0.76	3.99 ± 0.64	3.28 ± 0.59	3.16 ± 0.22	3.18 ± 0.15
Viscosity	[mm ² /s]	8.73 ± 0.09	8.62 ± 0.12	8.94 ± 0.06	9.17 ± 0.23	9.08 ± 0.17
		20.97 ± 0.74	21.03 ± 1.02	21.33 ± 0.68	21.07 ± 0.91	21.43 ± 1.13

In the culture variant with $k_La = 450$ 1/h, DCW of microalgae increased from 5.0 ± 1.0 g/dm³ to 35.63 ± 3.83 g/dm³ in the adaptation phase, while at the end of phase 2 (after 100 h of incubation) it reached 130.46 ± 4.53 g/dm³. The stationary growth phase yielded the DCW concentration of 139.90 ± 3.86 g/dm³ at the end of the incubation cycle (Figures 3c and 4, Table 4). In turn, the logarithmic growth stage brought about the most intense processes of lipid accumulation in cells and DHA synthesis (Figure 3c). Their final concentrations in the biomass reached 60.03 ± 1.24 g/dm³ and 25.32 ± 0.56 g/dm³, respectively (Figure 4, Table 4). In this variant, on average 442.0 g/dm³ of glycerol were fed to bioreactors during the entire incubation cycle, resulting in its consumption yield at 3.68 g/dm³·h, 3.28 g/gDCW (Table 4).

The best, statistically comparable technological effects associated with DCW increase, lipid synthesis, and DHA accumulation in cells were observed in the culture variants 4 and 5 with k_La values ranging from 600 1/h to 750 1/h. After 40 h of the adaptation phase, the DCW of *Schizochytrium* sp. reached 37.37 ± 3.14 g/dm³ in variant 4 and 38.31 ± 2.92 g/dm³ in variant 5. After 120 h, the respective values reached 147.89 ± 4.77 g/dm³ (Figures 3d and 4, Table 4) and 149.03 ± 3.31 g/dm³ (Figures 3e and 4, Table 4). The final lipid concentration was determined at approximately 70.0 g/dm³, whereas the final DHA concentration in biomass approximated 30.0 g/dm³ (Figures 3d,e and 4, Table 4). Increasing the volumetric oxygen mass transfer rate and turbine speed in these variants contributed to the highest recorded values of r_{DHA} at 0.25 g/dm³·h and r_{DCW} at ca. 1.2 g/dm³·h (Table 4). Glycerol consumption yield in both these variants approximated 3.8 g/dm³·h and 3.2 g/gDCW (Table 4). In the study by Chang et al. (2013) [67], increasing the value of k_La coefficient from 143 ± 19 1/h to 568 ± 24 1/h and 1805 ± 105 1/h resulted in DCW concentration exceeding 100 g/dm³.

Those authors also showed that the value of $k_L a$ coefficient affected assimilation of nutrients, microalgal biomass growth, and DHA synthesis.

Literature data and the present study results enable the conclusion that higher $k_L a$ values stimulate cell proliferation and lipid accumulation in microalgal biomass. The present study revealed very strong correlations between DCW, DHA, and lipid concentrations and $k_L a$ values (Figure 5). A further increase in the $k_L a$ value from 600 1/h to 750 1/h caused no statistically significant differences in the values of the analyzed parameters (Figures 4 and 5). However, the variant with $k_L a = 750$ 1/h proved less technologically and economically viable.

Figure 5. Correlations between DCW, DHA, and lipid concentrations and $k_L a$ values.

4. Conclusions

The *Schizochytrium* sp. biomass incubated in the culture with waste glycerol produced EPS, the highest concentration of which was determined in the stationary growth phase. Concentrations of saccharide monomers of the EPS fraction remained stable over the incubation period. The monosaccharides present in the EPS fraction included hexoses, except for xylose which is classified among pentoses. The major EPS component turned out to be glucose, with the concentration reaching $60.64 \pm 1.93\%$ mol.

EPS synthesized by *Schizochytrium* sp. biomass cause a significant increase in culture viscosity, which in turn contributes to diminished oxygen availability for the cells and to reduced effectiveness of waste glycerol utilization, lipid production, and DHA accumulation in cells.

Increased effectiveness of culture medium aeration boosts technological effects. The highest culture efficiency was observed with volumetric oxygen mass transfer of $k_L a = 600$ 1/h. The increase of its value to 750 1/h did not cause any significant changes in the values of the analyzed parameters. This variant was not justified by either the technological effects achieved nor by the operating costs. Glycerol consumption was increased linearly depending on the applied oxygen mass transfer coefficient, ranging from $k_L a$ 150 1/h to $k_L a$ 600 1/h.

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