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Concept of Batch and Fed-Batch Cultures of *Yarrowia lipolytica* as a Valuable Source of Sterols with Simultaneous Valorization of Molasses and Post-Frying Rapeseed Oil

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Abstract: Food byproduct streams can potentially be transformed into value-added products such as microbial lipids in bioprocesses based on the non-conventional *Yarrowia* yeast. The effect of culture conditions of *Y. lipolytica* KKP 379 wild strain in waste media on the efficiency of lipid accumulation, fatty acid composition, presence of selected sterols, yield and elemental composition of biomass has been studied. Batch and fed-batch bioreactor cultures were carried out in media with molasses hydrolysate (MH) and post-frying rapeseed oil. It was determined that biomass grown in MH contained more minerals than in medium with rapeseed post-frying oil. Considering the PDSC study, the T_{max} of oxidation induction ranged from 10.04–26.36 min for the analyzed samples. The biomass from fed-batch cultures with MH had the highest total sterol content (68.40 mg/g_{oil}), dominated by ergosterol at 60.16 mg/g. Feeding with post-frying rapeseed oil with new doses of mineral medium promoted maintaining the cellular lipid content at a high level (30.75–31.73%) for 50 h, with maximum yield at 37.50%. The results of the experiment showed that the cellular lipid accumulation efficiency of *Y. lipolytica* yeast and the content of sterols in the cell membrane can be manipulated by selecting waste substrates and culture mode.

Keywords: *Yarrowia lipolytica*; microbial lipids; sterols; thermal analysis; waste valorization; molasses; post-frying rapeseed oil

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1. Introduction

The circular economy concept includes a model to support responsible management of available resources, and, thus, promotes the 10th Sustainable Development Goal (SDGs) introduced by the United Nations. Many companies around the world, as participants in the food system, use a linear management model to minimize the generation of waste as leakage in the production cycle [1]. Food waste and by-products are generated at every stage of the food procurement process. The food industry generates about 1.3 billion tons of broad-based waste annually and about 38% of food waste comes from the food processing stage. Statistics show an important environmental and ethical problem, but also an economic loss due to undeveloped resources with untapped potential [2,3].

Biotechnological valorization of agri-food industry waste is closely related to sustainable resource reuse technologies. Food side-streams could be potentially transformed into added value products by biotechnological processes based on non-conventional microorganisms, such as *Yarrowia lipolytica* yeast [4–7]. The physiological characteristics of

Y. lipolytica make the species able to utilize both simple and complex wastes as sources of carbon, nitrogen, phosphorus and other essential components [8,9].

The main byproduct of sugar mills is the brown leachate, obtained when sucrose crystals are separated in the final stage of sugar production, called molasses. Generally speaking, the production of 1 ton of raw sugar involves the production of 0.38 tons of molasses. The thick and viscous syrup consists of up to 20% (*w*/*w*) water and 80% (*w*/*w*) dry matter. Molasses contains large amounts of sucrose and reducing sugars (20–60% *w*/*w*), in smaller amounts of inorganic salts, nitrogenous compounds, vitamins and colour-forming substances. Because of the composition, molasses is used as a low-cost substrate necessary for yeast cell growth [10,11].

Regarding the ability of oleaginous *Y. lipolytica* cells to grow and accumulate significant amounts of microbial lipids in media with hydrophobic carbon sources, there was established, that waste cooking oils (WCOs) may be considered as an efficient source of carbon stimulating lipase activity and promoting the Single Cell Oils (SCOs) accumulation process [9,12,13]. In the food industry, microbial oils have gained popularity as additives in infant formula and animal feed. SCOs are primarily a source of triacylglycerols, sterols and sterol esters similar to those from plant sources. Monounsaturated fatty acids stand out in the fatty acid profile of microbial oils with saturated and polyunsaturated acids in smaller amounts, depending on culture conditions and medium composition. The best-known sterol of microbial origin is ergosterol, which can be present in the form of esters and free sterol. The highest content of this compound has been reported for yeasts of the genera *Saccharomyces*, *Metschnikowia*, *Kluyveromyces*, *Pichia* and *Torulaspora* [14,15].

The aim of the present study was a multifaceted evaluation of the concept of batch culture in comparison with fed-batch culture modes in terms of the growth and the efficiency of microbial oil accumulation by *Yarrowia lipolytica* yeast. The ability of SCOs accumulation in yeast cells over time has been analyzed, depending on culture conditions i.e., medium supplementation, to highlight the potential of yeast biomass as a source of bioactive components, the fatty acid profile and sterols content in extracted oil samples were also determined. Moreover, the oxidative stability of the extracted oils was studied.

2. Materials and Methods

2.1. Microorganisms

The wild type yeast strain *Yarrowia lipolytica* KKP 379 from the Collection of Industrial Microorganisms Cultures belonging to Institute of Agricultural and Food Biotechnology (IAFB, Warsaw, Poland) was used in the study. The yeast strain was stored in 20% (*v*/*v*) glycerol solution in YPG medium (1% yeast extract, 2% peptone, 2% glucose) at −20 °C.

2.2. Culture Conditions

Inoculum culture was provided in YPG medium (yeast extract 1%, peptone 1%, glucose 1%, pH 5.0). The flasks were incubated at 28 °C for 24 h with a rotation amplitude of 140 rpm. The batch and fed-batch cultures were conducted in a BIOFLO 3000 laboratory bioreactor from New Brunswick Scientific (44 Talmadge Road, Edison, NJ, USA) (28 °C, 0.025% (*v*/*v*) inoculum) with a working volume of 4 L. The bioreactor was equipped with temperature, pH and oxygenation control. The dissolved oxygen content was measured using an oxygen electrode and regulated using compressed air to maintain the relative degree of oxygenation at a level not lower than 30% of the initial oxygen concentration (variable agitation speed 300–600 rpm). Changes in pH values were monitored using a selective electrode. Parameters for yeast cultures were calculated according to Fabiszewska et al. [16]. Culture conditions were chosen on the basis of our previous researches [4,9,16].

Media M1-b and M2-fb contained molasses 50 g/L, Na_2HPO_4 1.5 g/L and yeast extract 0.5 g/L, which were added at 0 h. Additionally, the M2-fb culture was fed with molasses hydrolysate (MH) after 16, 24, 40 and 48 h. The composition of the O1-fb medium at 0 h was as follows: KH_2PO_4, 3.0 g/L; $(NH_4)_2SO_4$, 8.0 g/L; Na_2HPO_4, 2.5 g/L; $MgSO_4$, 1.5 g/L; $CaCl_2$, 0.15 g/L; $FeSO_4xH_2O$, 0.16 g/L; $MnCl_2x4H_2O$, 0.08 g/L; $ZnSO_4$, 0.02 g/L and of

post-frying rapeseed oil, 50.0 g/L. In the next stages of O1-fb cultivation was supplemented with post-frying rapeseed oil and concentrated YNB (Yeast Nitrogen Base) (26.8 g dissolved in 250 mL) with the following composition: $(NH_4)_2SO_4$, 5.0 g/L; biotin, 2.0µg/L; calcium pantothenate, 400 µg/L; folic acid, 2.0 µg/L; inositol, 2.0 mg/L; nicotinic acid, 400 µg/L; *p*-aminobenzoic acid, 200 µg/L; pyridoxine HCl, 400 µg/L; riboflavin, 200 µg/L; thiamine HCl, 400 µg/L; H_3BO_3, 500 µg/L; $CuSO_4$, 40 µg/L; KI, 100 µg/L; $FeCl_3$, 200 µg/L; $MnSO_4$, 400 µg/L; Na_2MoO_4, 200 µg/L; $ZnSO_4$, 400 µg/L; K_3PO_4, 1.0 g/L; $MgSO_4$, 0.5 g/L; NaCl, 0.1 g/L; $CaCl_2$, 0.1 g/L.

2.3. Wastes

The waste from post-frying rapeseed oil originated from a fish processing company in Podlaskie Voivodship, Poland. Cod fillets were fried at 170 °C in full immersion in the oil. The oily waste was filtered and the solid particles were separated. Given that wild *Yarrowia* does not produce the enzyme invertase, the molasses obtained from the sugar refinery was hydrolyzed with 98% sulfuric acid after double dilution. As a result, the by-product consisted of 58% reducing sugars. The dried molasses had the following composition: C—348.87 g/kg, N—22.3 g/kg, S—2.16 g/kg, P—0.14 g/kg, K—45.59 g/kg, Na—7.25 g/kg, Ca—1.61 g/kg, Mg—92.50 mg/kg, Fe—80.63 mg/kg, Al—19.09 mg/kg, Mn—33.11 mg/kg, Cu—2.53 mg/kg, Zn—30.39 mg/kg, Ni—4.46 mg/kg, Pb—2.39 mg/kg, Cr—1.77 mg/kg, V—0.30 mg/kg, Sr—7.10 mg/kg, Ba—6.24 mg/kg, Ti—2.17 mg/kg, Zr—0.30 mg/kg.

2.4. Analytical Methods

Yeast biomass yield was determined by dry cell weight measured by thermogravimetric method. Cells were harvested by centrifugation (8000 rpm, 4 °C for 10 min) washed with redistilled water and dried at 105 °C to a constant weight.

The residual waste oil was extracted from 30 mL of medium by double extraction with portions of *n*-hexane. Cellular lipids were extracted from dry biomass in Soxhlet apparatus; *n*-hexane was used as a solvent. The solvent was removed by distillation under reduced pressure of 360 mbar (Buchi Rotavapor R-200 evaporator, Flawil, Switzerland).

Dried samples of yeast biomass were crushed and homogenized in a mortar. Total C, N and S contents were determined by dry combustion (Vario MacroCube, Elementar, Langenselbold, Germany). Total content of P, K, Na, Ca, Mg, Fe, Al, Mn, Cu, Zn, Ni, Pb, Cr, V, Sr, Ba, Ti and Zr was measured by inductively-coupled plasma atomic emission spectrometry—ICP-OES (Avio 200, Perkin Elmer, Waltham, MA, USA) after samples digestion in a mixture of HNO_3 and HCl (3:1 v/v) using the microwave digestion system (Milestone Ethos Up, Sorisole, Italy).

Reducing sugars were analyzed using a modified chemical method of Jain et al. [17] The reagent DNS (3,5-dinitrosalicylic acid) reacts with reducing sugars at elevated temperatures to form a reddish-brown product, 3-amino-5-nitrosalicylic acid. 1 mL of DNS and 1 mL of medium was incubated in a boiling water bath for 5 min, cooled rapidly and 5 mL of redistilled water was added. The concentration of the color compound was determined by measuring the absorbance at 540 nm.

2.5. Fatty Acids Profile and Sterols Determination

The fatty acid composition was determined by gas chromatography with a flame ionization detector (GC-FID) as described by Wierzchowska et al. [9].

Oil samples or standards (stigmasterol, cholesterol, ergosterol) were weighed and dissolved in 2 mL of hexane. 100 µL of 5α-cholestane (10.5 mg/25 mL chloroform) was added as an internal standard. Measured compounds were derivatized with 0.5 mL of 2M KOH in methanol for 1 h. 1mL of upper layer was collected carefully and transferred to a glass vial. The sample was evaporated in a stream of nitrogen and 100 µL of silylating reagent (BSTFA + TMCS, 99:1) and 100 µL pyridine was added to the samples. The prepared sample was shaken and left for 24 h at room temperature in order to fulfill derivatization process.

0.2 mL of hexane was added and the trimethylsilylether sterols content was analyzed. The separation of sterol derivatives was performed with the use of GC coupled with mass spectrometer Shimadzu—QP-2010S and capillary column ZB-5 ms (30 m × 0.25 mm × 0.25 µm) stationary phase (5%—phenyl-arylene—95%—dimethylpolysiloxane). Column temperature procedure: initial 60 °C for 3 min, temperature rate 15 °C/min to 250 °C, second temperature rate 3 °C/min to 310 °C and hold for 10 min. Injector and ion source temperature were 250 °C and 240 °C, respectively. Carrier gas was helium with the flow 0.7 mL/min. Interface temperature of GC-MS was 250 °C. The ionization energy was 70 eV. The Total Ion Current (TIC) was used to detect sterols (m/z ranged 100–600). The qualitative analysis of trimethylsilylether was made on the basis of a comparison of their retention time with retention time of available standards and mass spectra as well as literature data. The internal standard 5α-cholestane was used to quantify sterols. Results were presented in mg/g of oil. Each sample was analyzed in duplicate [18].

2.6. PDSC Analysis of Extracted Oils

Pressure differential scanning calorimetry (PDSC) study was conducted using a DSC Q20 TA Instrument (TA Instruments, New Castle, DE, USA). Samples of oil (3 mg) in aluminum pans were placed in the cell under an oxygen atmosphere (50 mL/min flow rate) with an initial pressure of 1400 kPa. The empty pan was used as a reference. The measurements were carried out in isothermal conditions of 120 °C. The induction time of the samples was determined based on onset time of oxidation reaction (T_{on}) and the maximum rate of oxidation (T_{max}). Parameters are used to characterize different stages of oil oxidation process. Diagrams were registered and analyzed using TA Universal Analysis 2000 software [19,20].

2.7. Statistical Analyses

Statistical analyses of the results was performed using Statistica 13.0 set plus software (Statsoft, Cracow, Poland). Determination of sterols content was performed of repeated measurements with one-way ANOVA followed by Tukey's multiple comparison test and $p \leq 0.05$ were considered to be statistically significant.

3. Results

3.1. Batch and Fed-Batch Yarrowia lipolytica Cultures

Three experiments were carried out in media containing by-products of the agro-food industry in a laboratory bioreactor under the conditions outlined in the methodology section (Section 2.2). There was evaluated the effect of the culture mode on the growth of yeast strain *Y. lipolytica* KKP 379 and its ability to accumulate lipids. Microbial utilization of hydrophilic and hydrophobic low-cost substrates from the culture media (molasses and rapeseed oil after fish frying) was also investigated.

In M1-b and M2-fb media, molasses hydrolysate was the only source of carbon and the main source of minerals. In the case of M1-b batch culture (Figure 1a), the lag phase lasted about 10 h. High oxygen demand of yeast cells was observed throughout the culture period. When the cells entered the logarithmic growth phase, the sugar content in the medium had already been at a low level of 8.29 g/L. After 16 h of culture in molasses medium, the biomass yield was only 2.9 g_{DCW}/L, and after 24 h its value slightly increased to 3.0 g_{DCW}/L. The low content of the carbon source in the cultivation medium made biomass growth constricted. With a very low sugar content of 3.81 g/L, the biomass yield after 40 h of culture was only 3.5 g_{DCW}/L. In the final stage of culture, the yeast consumed almost the entire pool of available carbon source in the medium, reducing its content from 29 g/L to 2.7 g/L. The pH of the medium remained relatively constant at 5.5 until about 40 h, after which time it began to decrease slightly to 2.89 after 62 h.

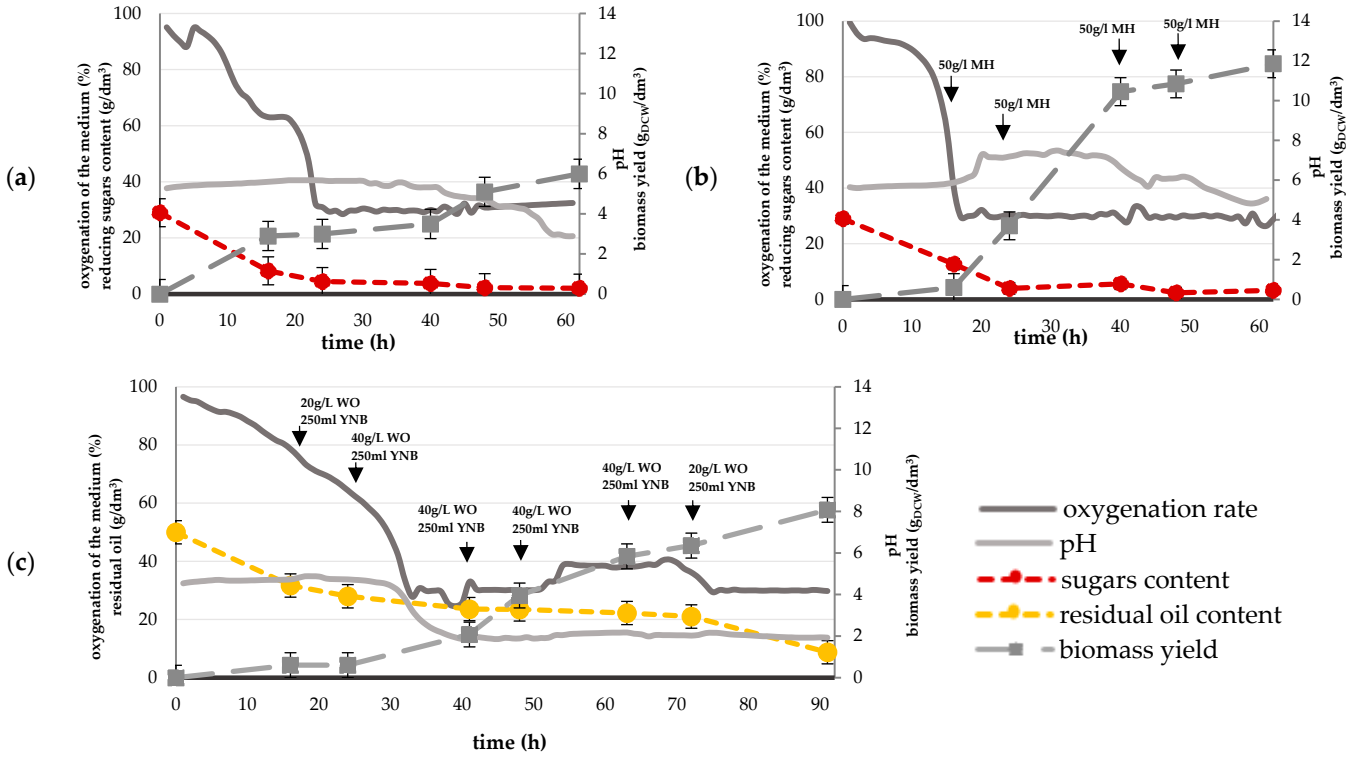

Figure 1. Changes in the oxygenation of the medium, pH, biomass yield of *Y. lipolytica* KKP 379 and content of reducing sugars in medium (**a**) M1-b and (**b**) M2-fb with hydrolyzed molasses hydrolysate (MH) as a hydrophilic carbon source and content of residual oil in (**c**) O1-fb medium with post-frying rapeseed oil (WO) as hydrophobic carbon source and supplemented with YNB medium.

In M2-fb yeast culture (Figure 1b), the medium was supplemented with molasses hydrolysate after 16, 24, 40 and 48 h. It is worth noting that feeding the culture at the beginning of the logarithmic phase of growth promoted biomass growth. Admittedly, the adaptation phase was longer than in M1-b medium and the biomass yield after 16 h of culture was several times lower than at the same point in M1-b culture. After 48 h the biomass yield was 10.84 g_{DCW}/L, compared to 5.10 g_{DCW}/L in the no-feeding mode. Finally, supplying hydrolysate to the culture medium as a source of essential components for the cells resulted in a biomass yield of 11.85 g_{DCW}/L. Taking into account that wild yeast *Y. lipolytica* is unable to break down sucrose, which is the main sugar of molasses, the cheap substrate was treated with acid to hydrolyze the disaccharide. Thus, the hydrolysate of molasses required neutralization before being added to the culture. Therefore, supplementing the medium with ingredients necessary for growth caused periodic fluctuations in the pH. As a result of the 62-h yeast culture, the content of simple sugars in the M2-fb medium decreased to 3.28 g/L. The initial content of hydrolyzed molasses was 50 g/L, which corresponded to 29 g/L of simple sugars. Taking into account the sum of molasses portions with which the M2-fb medium was supplemented during the culture, the yeast utilized a total of 141.72 g/L of simple sugars from the waste medium.

The subsequent O1-fb culture was conducted in mineral medium with 5% post-frying rapeseed oil as a hydrophobic source of carbon for *Yarrowia* cells (Figure 1c). In contrast to cultures conducted in molasses media, the culture was carried out for 90 h. Twice a day, 250 mL of medium was withdrawn from the bioreactor, and then the culture was supplemented with the defined amount of oily waste, as well as 250 mL of sterile concentrated YNB medium. The adaptation phase lasted nearly 24 h. After 16 h of culture, the level of waste oil in the culture medium decreased to 31.7 g/L, while the biomass yield was 0.6 g_{DCW}/L. In an effort to provide the cells with the needed energy source, an addition of 20 g/L of post-frying rapeseed oil was used. Nonetheless, over the next 8 h, the yeast

utilized a significant portion of the lipid carbon source, leaving an unused waste of 28 g/L, with an unchanged biomass yield. After 24 h, the medium was enriched with an increased dose (40 g/L) of waste oil. At 40 h of culture, the residual oil content in the substrate was 23.6 g/L, with a biomass yield of 2.08 g_{DCW}/L, which increased to 3.96 g_{DCW}/L by the end of the second day. After 62 h, the biomass yield was 5.84 g_{DCW}/L. It is worth mentioning that this value was similar to the level of biomass growth in M1-b batch culture with molasses, in which no additional substrate supplementation was used, after 62 h the yield was 6.0 g_{DCW}/L. On the third day of culture, the M1-fb medium was enriched with a dose of post-frying oil at 40 g/L, and part of the medium was replaced. The applied culture conditions and substrate composition caused the biomass yield of *Y. lipolytica* yeast by fed-batch culture with post-frying rapeseed oil to reach 8.08 g_{DCW}/L after 90 h. It is emphasized that, despite periodic dosing of the carbon source into the medium, the yeast effectively utilized a significant amount of waste oil. At the end of the culture, 8.76 g/L of industrial waste remained in the medium, with an initial amount of 50 g/L. During the process, a total of 250 g/L of waste post-frying oil was added to the O1-fb culture.

3.2. Effect of the Culture Mode on Lipid Accumulation

The experiment also compared the efficiency of microbial lipid biosynthesis in cells of *Y. lipolytica* strain KKP 379 in batch and fed-batch media containing molasses hydrolysate and fed batch culture with post-frying rapeseed oil. Figure 2. shows changes in the lipid content of yeast cells over time. For each culture variant, the efficiency of the accumulation process reached a value of more than 30% per gram of dry biomass. Interestingly, the dynamics of microbial lipid biosynthesis for each culture varied. In the case of media in which molasses hydrolysate M1-b and M2-fb were used, the yield was highest on the first and second days of culture. For the culture in medium O1-fb with lipid carbon source on the third day of culture the maximum accumulation yield was observed. After 16 h of biomass culture in M1-b medium, the content of cellular lipids was 21% of dry cell weight, and after 24 h it was already 35%. At the same time (16 h) in M2-fb medium, the efficiency of lipid accumulation was only 8% and did not increase over the next eight hours. Probably, the medium turned out to be deficient in carbon atoms and other necessary components to cover the demand of cells in a culture environment. This type of limitation forced cells cultured in M1-b medium to accumulate lipids as an energy reservoir. In M2-fb medium, the addition of molasses hydrolysate promoted biomass growth (Figure 1b). After 48 h of cultivation, the SCO content of the cells reached a maximum (37%). A gradual decrease in the cellular lipid content of biomass was observed from 24 h of culture in M1-b medium. After 40 h, the SCO accumulation efficiency was at 26%, and after 48 h, only 10%. The observed decrease can be explained by the mobilization of cellular energy reserves in the form of lipids, due to the lack of availability of the necessary components in the substrate. In the end, after 62 h, only 2% fat per dry weight remained inside the cells. In the case of yeast culture in M2-fb medium, a decrease in cellular lipid content to 5% after reaching the maximum efficiency of the SCO accumulation process has been noted. The composition of the medium did not meet the increased biomass energy demand, which also resulted in the exploitation of cellular resources. A different mechanism was observed in O1-fb medium. At the 40st hour for this culture variant, the SCO content of the biomass was the highest at 31.73% and remained relatively high until 90 h, reaching the efficiency maximum at 63 h (37.50%).

The synthesis of microbial oil proceeded intensely during the first days in cultures with molasses as opposed to yeast culture in medium with post-frying rapeseed oil, where the accumulation took place in the stationary growth phase. One of the most important parameters describing the kinetics of SCOs accumulation is concentration of lipid coefficient (L) (Table 1). The highest value of this parameter was observed in 48 h of fed-batch culture with molasses (M2-fb), when L amounted to 4.01 g/L. Lipids concentration in M1-b medium without supplementation was lower, the L_{max} 1.05 g/L was reached in 24 h. The value accounted for approximately 25% of the parameter calculated for the M2-fb medium. In

the case of the O1-fb culture, the maximum amount of microbial oil that could be extracted from the medium was noted at the end of culture, L parameter reached 2.48 g/L.

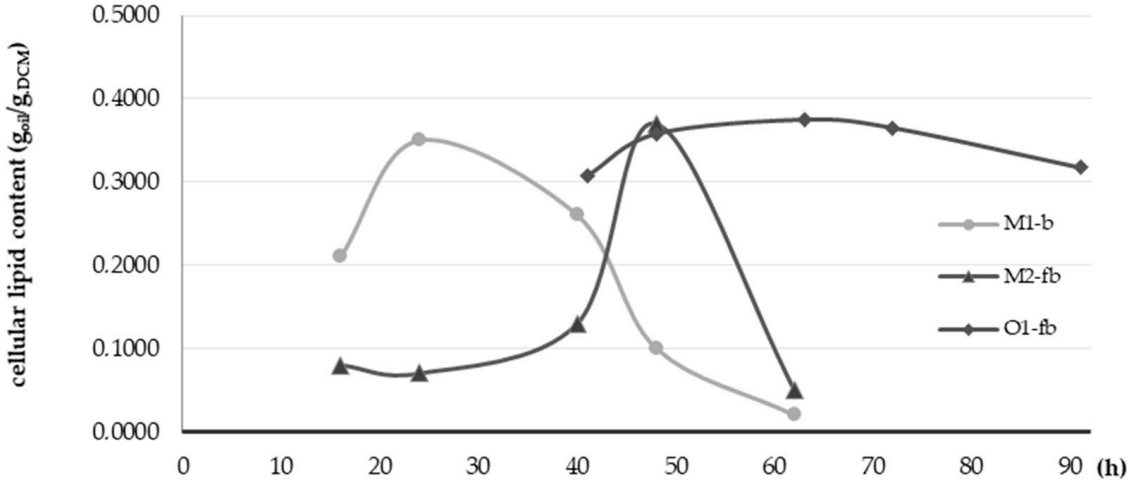

Figure 2. Cellular lipid content of *Y. lipolytica* KKP 379 during batch culture in medium M1-b and fed-batch cultures in medium O1-fb and M2-fb.

Table 1. Parameters of *Y. lipolytica* KKP 379 yeasts strain culture in media M1-b, M2-fb and O1-fb.

Parameter	Unit		M1-b	M2-fb	O1-fb
Initial concentration of carbon source	g/L		50	50	50
Time (t)	h		62	62	91
Biomass yield (X)	g_{DCW}/L		6.00	11.82	8.08
Conversion biomass yield per carbon substrate ($Y_{X/S}$)	g_{DCW}/g		0.1200	0.2364	0.1616
Concentration of lipids produced (L)	g/L	16 h	0.6090	0.0480	-
		24 h	1.0500	0.2590	-
		40 h	0.9100	1.3585	0.6600
		48 h	0.5100	4.0108	1.4454
		62 h	0.1200	0.5925	2.1900
		72 h	-	-	2.2762
		90 h	-	-	2.4846

3.3. Fatty Acids Composition of Cellular Lipids from Y. lipolytica Cultures

To compare the fatty acid composition of cellular lipids obtained by de novo accumulation in molasses media and by the ex novo pathway in culture with post-frying waste, oil samples from M1-b, M2-fb and O1-fb (Table 2) cultures were analyzed. For both microbial oils accumulated in biomass cultured in molasses media and in cultures with post-frying oil, the fatty acid with the dominant proportion was oleic acid (C18:1). After 62 h, in oil samples from M1-b culture, the C18:1 content was 52.66%, in M2-fb—44.43%, and in O1-fb—61.82%. Linoleic acid (C18:2) was another fatty acid with a significant proportion. The highest percentage of C18:2 was observed in SCO from the M2-fb culture (30.75%). In the oil sample from the M1-b culture as well as the M2-fb medium, linolenic acid (18:3) from the omega-3 fatty acids was present, 7.68% and 6.48%, respectively. Saturated fatty acids such as palmitic acid (C16:0) and stearic acid (C18:0) were also determined in each of the oil samples.

Table 2. The fatty acids profile (%) of microbial lipids extracted from biomass cultured in M1-b, M2-fb and O1-fb media.

Culture Variant	Time	C16:0	C18:0	C18:1	C18:2	C18:3	C20:0	C22:0
M1-b	62 h	11.67	5.51	52.66	22.49	7.68	n.d.	n.d.
M2-fb	62 h	13.69	4.65	44.43	30.75	6.48	n.d.	n.d.
O1-fb	40 h	3.01	9.25	60.36	18.23	4.20	4.09	1.06
	48 h	4.09	11.08	58.43	18.88	2.94	3.75	0.92
	62 h	4.21	10.86	61.81	16.41	1.76	4.15	1.05
	72 h	5.20	10.13	63.17	15.26	2.23	3.61	0.68
	90 h	4.09	8.49	66.94	14.41	0.72	4.93	0.51

n.d.—not detected.

For microbial oil extracted from biomass cultured in O1-fb medium changes in fatty acid composition were observed after 40, 48, 62, 72 and 90 h. The cellular oil extracted from the biomass after 90 h of culture containing the most C18:1 acid (66.94%) was at the same time characterized by the lowest proportion of C18:2 acid from the omega-6 group (14.41%). Considering the change in the content of C18:2 in the total fatty acids profile of microbial oils from successive O1-fb culture stages, its percentage decreased with the time of cultivation. The highest amount of C18:2 was noted on the second day of the culture stage (18.88%).

Oil samples from O1-fb cultures were characterized by a small proportion of C18:3 acid. Its content after 40 h was 4.20%. In the following hours its content decreased, and at the end of the culture it was only 0.72% of all fatty acids. Microbial oils from O1-fb culture were more differentiated in fatty acid composition. In contrast to cellular lipid samples from strain cultures in molasses media, arachidonic acid (C20:0) and behenic acid (C22:0) was detected in SCOs from O1-fb cultures.

3.4. Effect of the Culture Mode on the Sterol Content and the Elemental Composition of the Biomass

The content of sterols in microbial oils extracted from biomass cultured in molasses media and in medium with post-frying rapeseed oil has been also described. The content of cholesterol, dehydroergosterol, ergosterol, campesterol, stigmasterol and β-sitosterol determined in analyzed oils samples has been shown in Table 3. The lowest total sterol content 3.25 mg/g_{oil} was noted for microbial lipids from O1-fb culture, in which yeast biomass was fed with rapeseed oil after frying. In SCO derived from medium with post-frying oil, β-sitosterol proved to be the dominant sterol at 44.29%, followed by campesterol (25.22%), ergosterol (13.86%) and dehydroergosterol (12.67%). Stigmasterol accounted for 2.96% of the total sterols content and cholesterol 1.02%.

In SCO sample from M1-b culture of *Yarrowia* yeast in molasses medium, the total sterol content was 8.34 mg/g_{oil}, while in M2-fb culture sterol content amounted to 68.40 mg/g_{oil}. After 62 h in both variants of molasses culture M1-b and M2-fb, the sterol with the highest proportion was ergosterol, with a content of 57.95% and 87.94% respectively. In the culture M2-fb with molasses hydrolysate, the ergosterol content was 60.16 mg per gram of lipids extracted from biomass. In the M1-b medium without feeding mode, the ergosterol content at the end of the culture was 5.00 mg/g of oil. The another sterol with a significant percentage was β-sitosterol (18.25%) and campesterol (10.24%). Dehydroergosterol, cholesterol and stigmasterol were determined in smaller amounts (Table 3).

Table 3. Content of the major sterols in microbial oil samples extracted from cultures of *Y. lipolytica* KKP 379 biomass in medium M1-b, M2-fb and O1-fb.

Oil Samples	M1-b		M2-fb		O1-fb	
	%	mg/g_{oil}	%	mg/g_{oil}	%	mg/g_{oil}
Cholesterol	5.34 ± 0.50	0.45 ± 0.13 ^{b}	0.32 ± 0.05	0.22 ± 0.04 ^{a}	1.02 ± 0.26	0.04 ± 0.02 ^{a}
Dehydroergosterol	6.51 ± 0.31	0.54 ± 0.08 ^{a}	1.85 ± 0.24	1.26 ± 0.11 ^{b}	12.67 ± 1.28	0.42 ± 0.23 ^{a}
Ergosterol	57.95 ± 2.58	5.00 ± 1.33 ^{a}	87.94 ± 0.91	60.16 ± 3.33 ^{b}	13.86 ± 1.74	0.40 ± 0.00 ^{c}
Campesterol	10.24 ± 0.91	0.90 ± 0.10 ^{a}	2.15 ± 0.17	1.47 ± 0.05 ^{b}	25.22 ± 3.22	0.84 ± 0.49 ^{a}
Stigmasterol	2.67 ± 0.53	0.22 ± 0.01 ^{a}	2.90 ± 0.38	1.98 ± 0.17 ^{b}	2.96 ± 0.57	0.12 ± 0.09 ^{a}
β-sitosterol	18.25 ± 3.81	1.50 ± 0.02 ^{a}	4.86 ± 0.16	3.32 ± 0.04 ^{b}	44.29 ± 0.38	1.44 ± 0.69 ^{a}
∑	100	8.34 ± 1.66	100	68.40 ± 3.75	100	3.25 ± 1.55

Lowercase letters indicate significant differences between treatments ($p < 0.05$).

The elemental composition of *Y. lipolytica* yeast biomass cultured in waste media was dominated by carbon, nitrogen, potassium, sodium, phosphorus and sulfur (Table 4). Yeast biomass fed with a lipid carbon source in the form of post-frying rapeseed oil was characterized by a higher content of carbon (601.12 g/kg), iron (0.59 g/kg) and aluminum (55.47 mg/kg) compared to cultures in molasses media. In contrast, biomass from M1-b and M2-fb cultures was richer in minerals such as nitrogen, sodium, sulfur, potassium, manganese, copper, zinc, cobalt and strontium. All biomass samples contained similar amounts of phosphorus (9.84–11.96 g/kg), magnesium (0.65–0.84 g/kg), lead (0.90–1.13 mg/kg) and barium (0.54–0.77 mg/kg). Cells fed with molasses hydrolysate obtained from M2-fb medium were richer than biomass from M1-b batch culture in potassium (47.54 g/kg), phosphorus (11.96 g/kg), calcium (2.13 g/kg), manganese (197.35 mg/kg), zinc (209.80 mg/kg) and strontium (6.45 mg/kg). On the other hand, biomass samples from the M1-b culture compared to the M2-fb culture contained more carbon (447.87 g/kg), nitrogen (65.56 g/kg), sulfur (13.61 g/kg), iron (0.46 g/kg), aluminum (19.25 mg/kg), chromium (10.71 mg/kg), nickel (15.08 mg/kg) and zirconium (10.71 mg/kg).

Table 4. Elemental composition of dry biomass of *Y. lipolytica* KKP 379 from M1-b, M2-fb and O1-fb media.

Biomass Samples	C g/kg	N g/kg	K g/kg	Na g/kg	P g/kg	S g/kg	Ca g/kg	Mg g/kg	Fe g/kg	Al mg/kg	Ti mg/kg
M1-b	447.87	65.56	25.63	18.47	9.84	13.61	0.87	0.69	0.46	19.25	3.41
M2-fb	376.26	43.21	47.54	16.89	11.96	8.76	2.13	0.65	0.20	7.98	1.09
O1-fb	601.12	20.78	16.48	0.46	10.05	2.04	0.08	0.84	0.59	55.47	2.03
	Mn mg/kg	**Cu mg/kg**	**Zn mg/kg**	**Cr mg/kg**	**Ni mg/kg**	**Pb mg/kg**	**V mg/kg**	**Zr mg/kg**	**Co mg/kg**	**Sr mg/kg**	**Ba mg/kg**	**Li mg/kg**
M1-b	136.05	6.90	168.64	10.71	15.08	1.13	0.62	3.94	2.43	2.77	0.54	0.18
M2-fb	197.35	5.77	209.80	3.12	2.54	0.90	0.53	1.27	2.23	6.45	0.77	0.29
O1-fb	50.79	0.79	93.34	2.29	1.08	1.20	0.14	1.30	0.01	0.40	0.66	0.01

3.5. PDSC of Cellular Oil Samples

In this experiment, PDSC measurements were conducted at a temperature of 120 °C at isothermal conditions. The PDSC curves for cellular lipids from M1-b, M2-fb and O1-fb are presented in Figure 3. It can be noted that the oxidation reaction time values for the extracted oil samples are different. At 120 °C, cellular oil from fed-batch culture with molasses (M2-fb) had the shortest T_{on} and T_{max} lasting 2.58 min and 10.04 min, followed by oil from batch culture with molasses (M1-b) with results of about 8.41 min and 16.32 min, respectively. The highest value of T_{on} and T_{max} parameters are observed in the oil sample from the culture fed with post-frying rapeseed oil (O1-fb), which are 14.03 and 26.23 min.

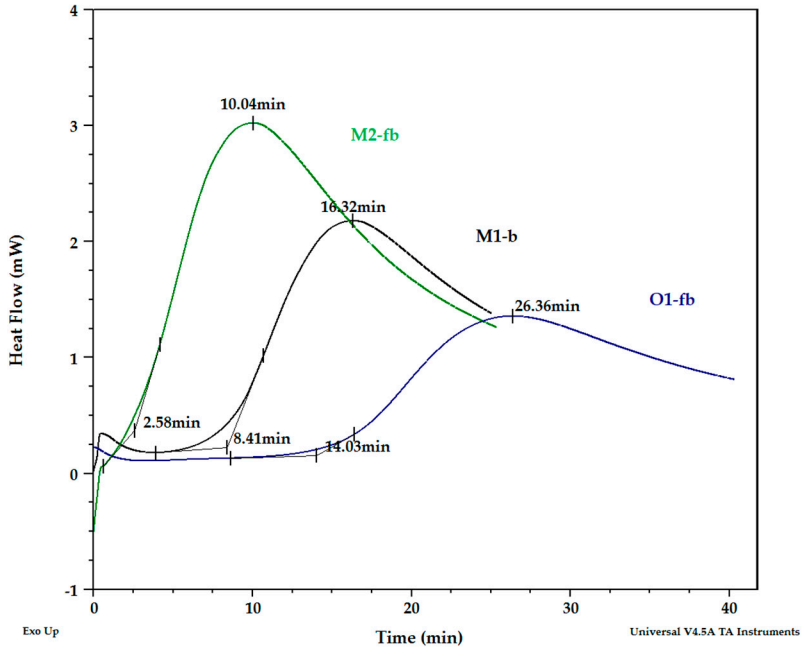

Figure 3. PDSC curves showing oxidation induction times T_{on} and T_{max} of cellular lipid samples from M1-b, M2-fb and O1-fb cultures.

4. Discussion

In the current experiment, among the factors differentiating the culture variants was type of waste substrate, as well as the culture mode. It is worth to note the difference in the supplementation method of the two fed-batch cultures. In the M2-fb culture variant, molasses was the only supplemented medium component. In the culture with post-frying rapeseed oil, in addition to supplementing the hydrophobic carbon source, the mineral medium was enriched with YNB medium, which was primarily a source of nitrogen in the form of ammonium sulfate and amino acids.

4.1. Effect of Culture Mode on Lipid Accumulation in Fed-Batch Culture with Post-Frying Rapeseed Oil

In a study by Wierzchowska et al. [9], *Y. lipolytica* KKP 379 yeast cells were cultured in a mineral medium containing 3.0 g/L KH_2PO_4, 1.1 g/L Na_2HPO_4 and 8g/L $(NH_4)_2SO_4$. The assumed composition of the medium corresponded to the base of the O1-fb culture medium in the current experiment. Comparing the results of the two studies, a higher biomass yield was obtained in the culture where the medium was not supplemented with a carbon source and enriched with minerals and amino acids—11.10 g_{DCW}/L after 88 h. Meanwhile, the M2-fb culture lasting 90 h resulted in a biomass yield of 8.08 g_{DCW}/L. However, a higher final microbial lipid accumulation efficiency of 30.75% was observed for the M2-fb culture, while the 88-h batch culture resulted in a biomass lipid content of 20.9%. Providing cells with access to a carbon source in the form of post-frying rapeseed oil in the medium promoted lipid accumulation during the stationary phase of biomass growth. These observations support the theory already described in the literature that post-frying wastes are a good feedstock for promoting SCO accumulation [4,5,12]. Cyclically taking away a certain volume of medium and replacing it with a portion of YNB concentrate also had a beneficial effect on maintaining a reservoir of spare lipids in the biomass. Taking into account the L parameter, for both cultures the authors obtained the same result of 2.48 g of oil per liter of medium [9]. This shows that for practical and economic reasons of obtaining microbial oil, batch cultures without feeding can be more cost-effective solution to reduce interference in the culture process making it more laborsaving. Nevertheless, fed-batch cultures using lipid waste as carbon sources allow the use of significantly larger amounts of substrates difficult to store and dispose of [9].

4.2. Comparison of Batch and Fed-Batch Cultures in Molasses Media

The addition of five times the dose of the molasses hydrolysate in portions in the M2-fb culture compared to the M1-b culture resulted in twice the biomass yield—11.82 g_{DCW}/L after 62 h. In a study by Imatoukene et al. [21], batch culture of *Y. lipolytica* yeast JMY5578 in YNB medium without amino acids and ammonium sulfate with molasses and ammonium chloride lasting 72 h, resulted in a biomass yield of 14.2 g_{DCW}/L. To be mentioned, the C/N molar ratio of the molasses hydrolysate used in the current study was 18.3, while in the cited research the C/N value for the culture medium was 35. There is an assumption that, hydrolysate may have contained insufficient amounts of sugars available to the cells. The biomass yield from the M1-b batch culture was more than half the result of the batch culture in the study by Imatoukene et al. [21] In comparison, the value of biomass yield after 62 h of the M2-fb culture was similar to the batch culture of the JMY5578 strain. What's important, the auxotrophic JMY5578 strain was unable to degrade fatty acids and remobilize triacylglycerols from cellular reserves. Despite this, the content of lipids accumulated in the cells was finally at 6.7% (L = 0.95 g/L). After 62 h of batch and fed-batch culture with molasses hydrolysate, cellular lipid content was negligible. Despite the unsatisfactory final result, wild yeast strain KKP 379 generally accumulated higher amounts of intracellular lipids reaching maximum yields for M1-b and M2-fb after 24 and 48 h in both cases above more than 35%. After this time, due to the depletion of the necessary carbon source in the M1-b culture, there was a significant decrease in cellular lipid content. Nevertheless, the decrease in this case may have been due to the osmotic pressure of the molasses solution and the inadequate C/N ratio of the hydrolysate. Moreover, the M2-fb culture feeding strategy contributed to a gradual decrease in the C/N ratio as a result of medium supplementation with molasses hydrolysate as a source of nitrogen and a shift in the stage of cellular lipid accumulation as a consequence, compared to M1-b. According to the literature, in order to effectively stimulate lipid accumulation in the cells of oleaginous microorganisms, the C/N molar ratio should be above 20. Higher C/N molar ratios, as a consequence of nitrogen limitation, favor the accumulation of SCOs [9,22]. It should be noted that, cell physiology in waste substrates can be disrupted due to reactions with their various components. An example is furfural, which is formed by acidic and thermal treatment of sugars, considered as one of the inhibitors of growth and the process of cellular lipid biosynthesis [23].

4.3. Effect of Culture Conditions on Microbial Sterols Content

The role of preparing hydrolysate of molasses to make it more digestible for yeast cells through the process of acid hydrolysis and following neutralization should be emphasized. The pretreatment process contributed to the high extracellular salt concentrations. Hence, the high osmotic pressure of the culture medium was a stress factor for the yeast. This may explain why, in the current experiment, the highest sterol content, especially high ergosterol content, was observed in yeast cells from molasses-fed culture. The yeast biomass from the fed-batch culture in molasses medium (M2-fb) contained significantly more dehydroergosterol, ergosterol, campesterol, stigmasterol and β-sitosterol compared to the other cultures conducted in the experiment. Ergosterol is an essential yeast metabolite responsible for adapting cell membranes by altering their fluidity and permeability. This fungal component not only promotes membrane lipids resistance to peroxidation, but most importantly increases the resistance of cell membranes to osmotic stress when cells are environmentally exposed [24,25]. The regulation of membrane sterols is significant in many cellular processes. As demonstrated by Walker et al. [26], the tolerance of *Y. lipolytica* yeast to high concentrations of ionic liquids used as green solvents was associated with the maintenance of cell membrane homeostasis and impaired migration of cations which was possible through the regulation of cellular sterol biosynthesis including ergosterol. The survival rate of *Saccharomyces cerevisiae* mutants incapable of ergosterol synthesis was significantly reduced under osmotic shock conditions [27].

4.4. Analysis of Fatty Acid Composition and Oxidative Stability

It is well known that microbial oils extracted from the oleaginous yeast *Y. lipolytica* are rich in oleic and linoleic acid, which has also been confirmed by conducted studies. The composition of all extracted lipids was dominated by these two acids, as mentioned. Differences in fatty acid composition between biomass oil samples obtained from cultures in molasses hydrolysate media and post-frying rapeseed oil media were noticeable as well. In lipid samples from cultures with molasses hydrolysate, no C20:0 and C22:0 acid content was recorded in opposition to C16:0 and C18:1, whose percentages were increased relative to the other samples. In general, microbial oils from molasses-based media were less diverse in composition than samples from cultures with waste rapeseed oil. When Gajdoš et al. [28] cultured three mutant strains of *Y. lipolytica* overexpressing *DGA2* in medium with 8% (*w*/*w*) molasses, palmitic acid accounted for 14.0–19.6% of all fatty acids after 3 days of culture. The dominant was oleic acid 53.9–55.7%, but linoleic acid content was significantly lower than in the current experiment with wild strain, ranging from 3.9–9.4%. Very similar results were obtained by the cited authors for cultures in sucrose medium.

Oils with a high content of unsaturated fatty acids, which include the cellular oils of oleaginous yeast, are particularly easily oxidized. Oxidative stability is one of the most important parameters regarding the asses of oil quality. Oxidation of fatty acids under the influence of heat treatment, extraction conditions or during storage causes quality deterioration [29]. The extracted oil samples were less stable compared to conventional rapeseed oil or olive oil for which T_{max} measured at 120 °C ranged from 66.61–74.78 min and 134.15–180.07 min, respectively [30,31]. While in our study, T_{max} parameter ranged from 10.04–26.36 min for the analyzed samples. On the other hand, similar oxidative stability was observed for measurements on linseed oil and sunflower oil. The maximum time of oxidative reaction for sunflower oil at 120 °C was 33.4 min [20]. For linseed oil, especially rich in linolenic acid, oleic acid and linoleic acid, the maximum time of induction was 21.20–24.72 min [32], making it the closest to the values obtained in the current experiment.

4.5. Elemental Composition of Y. lipolytica Dry Biomass from Waste Media

Through elemental composition analysis, it was possible to found that biomass from media containing molasses hydrolysate was more abundant in minerals such as nitrogen, sodium, sulfur, potassium, manganese, copper, zinc, cobalt and strontium. In contrast, yeast biomass from cultures with post-frying rapeseed oil contained more carbon, iron and aluminum. Generally speaking, all biomass samples were abound in phosphorus (9840–11,960 mg/kg), magnesium (650–840 mg/kg), calcium (80–2130 mg/kg), zinc (93.34–168.64 mg/kg) iron (500.40–594.18 mg/kg), copper (0.01–2.43 mg/kg), manganese (50.79–197.35 mg/kg) than commercial baker's yeast *Saccharomyces cerevisiae*, where the content of the mentioned elements was as follows: P—7270 mg/kg, Mg—795 mg/kg, Ca—121 mg/kg, Zn—55.0 mg/kg, Fe—35.4 mg/kg, Cu—14.3 mg/kg, Mn—4.9 mg/kg [33].

5. Conclusions

The results of the present study showed that the cellular lipid accumulation efficiency of *Y. lipolytica* yeast and the content of sterols in the cell membrane can be manipulated by selecting waste substrates and culture mode. Due to the unique ability of the species to degrade and utilize a wide range of complex substrates, choosing a mode of yeast culture with feeding may allow for the disposal of larger amounts of waste. However, it is probably impossible to completely exclude the possibility that biomass growth may be disrupted by certain components acting as inhibitors. On the other hand, the complex nature of the waste may affect the modification of the composition of cell membranes, and, thus, the accumulation of components of interest to researchers, such as ergosterol. The authors showed that the fed-batch mode of culture with molasses hydrolysate allowed to obtain higher amounts of microbial oil from the medium by favoring biomass growth. On the other hand, in culture with a post-frying oil, feeding the biomass with both the carbon source and fresh medium promoted maintaining the lipid content of the cells at a high

level for a long time, further leveling the negative impact of harmful metabolites in the medium. Thus, based on the observation of the process of microbial lipid biosynthesis over time, it can be concluded that the choice of culture mode determines the time to achieve maximum accumulation efficiency and the success of the culture process directed at obtaining oil-rich biomass.

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