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Application of Aqueous Two-Phase Systems with Thermoseparating Polymers (EOPO) as a Method for Extractive Fermentation with *Neochloris oleoabundans*

Ana Patricia Garza-Chapa [1,2], Carlos Iván Ávila-Velasco [1,2], José González-Valdez [3] and Alma Gómez-Loredo [1,2,*]

[1] Facultad de Ciencias Químicas, Universidad Autónoma de Nuevo León, Pedro de Alba, S/N, San Nicolas de los Garza 66455, NL, Mexico
[2] Centro de Investigación en Biotecnología y Nanotecnología, Facultad de Ciencias Químicas, Universidad Autonoma de Nuevo Leon, Parque de Investigacion e Innovación Tecnologica, Km. 10 Autopista al Aeropuerto Internacional Mariano Escobedo, Apodaca 66629, NL, Mexico
[3] Tecnologico de Monterrey, School of Engineering and Science, Ave. Eugenio Garza Sada 2501 Sur, Monterrey 64849, NL, Mexico; jose_gonzales@tec.mx
* Correspondence: alma.gomezlr@uanl.edu.mx

Abstract: Extractive fermentation is an in situ method for the production and recovery of biomolecules of interest. Aqueous two-phase systems (ATPS) allow the product to be recovered in one phase of the system, reducing unit operations in the bioprocess. Thermosensitive polymers such as EOPOs are an interesting alternative to be applied in ATPS. In this work, different EOPOs were tested in an extractive fermentation strategy with the green microalgae *Neochloris oleoabundans* to provide a basis for future implementations of these systems in microalgae bioprocesses. Extractive fermentations were carried out with two EOPOs of different molecular weights (3900 and 12,000 g/mol) at concentrations of 10% and 15% (*w*/*v*). The microalga was incubated axenically under two different sets of conditions for 21 and 45 days, respectively. Cell counts were performed, and cell growth curves were obtained. Additionally, a semi-continuous and batch extractive fermentation assay was performed. The extractive fermentation with EOPO showed lower cell growth and a longer adaptation time of the microalgae in the fermentation, and EPS production yields of up to 8–23 g/L were obtained. Extractive fermentation is an interesting method to be implemented in microalgae cultures; however, further conditions need to be explored to achieve an appropriate bioprocess.

Keywords: extractive fermentation; aqueous two-phase system; thermoseparating polymers; *Neochloris oleoabundans*

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1. Introduction

In an industrial bioprocess, microorganisms are often used in a controlled environment to produce molecules of interest. The choice of the most suitable extraction and purification methods for said bioproducts and the most appropriate sequence in which they appear to maximize yield and purity are key points in bioprocess design [1]. At the same time, some of the important factors to consider in the selection of these procedures are economic feasibility, sustainability, and their environmental impact [2].

One methodology that has been widely studied for recovery or purification stages in a bioprocess are aqueous two-phase systems (ATPS), which involve the mixing of two immiscible aqueous liquid solutions to form a biphasic system, in which the desired bioproduct will be distributed to either phase according to its nature and the way it physicochemically interacts with the different system design parameters and characteristics [3]. Typically, a combination of polymer–polymer, polymer–salt, or other compound solutions such as alcohols, organic acids, surfactants, and ionic liquids, among others, are used in ATPS phase formation [4,5].

Several recent scientific reports have described the use of "intelligent" polymers in ATPS to achieve better performances in the operation. In this context, thermosensitive polymers (TP), such as EOPOs, can provide interesting bioprocessing advantages when used in ATPS [6]. EOPOs consist of random diblock or triblock structures of ethylene oxide (EO) and propylene oxide (PO) that vary in their molecular weights. ATPS with this TP are formed by combining liquid solutions of EOPO and inorganic salts and increasing the temperature in the system. However, the temperature required to form the biphasic system varies depending on the polymeric molecular structure [7]. The higher the EO content in it, the less hydrophobicity it presents, which results in a lower temperature needed to generate the phases. An efficient ATPS design will then consider the most suitable EOPO for the procedure regarding its potential yield and capacity to maintain the integrity of the molecule of interest since its use could provide several additional advantages, such as rapid compound exclusion, prevention of product biodegradation, and the potential for its reuse, contributing to the sustainability and economy of the bioprocess [8].

For its part, extractive fermentation (EF) is a combined in situ production and primary recovery procedure for biomolecule procurement where microorganisms are allowed to grow in one liquid phase while their extracellular bioproducts are simultaneously separated in the opposite phase [9]. The use of TP-containing ATPS in EF has been previously employed for the extraction of biomolecules produced by different microorganisms [10–12]. This provides a large design space for novel bioprocesses where the purification of the intended bioproduct could benefit from the use of TP in a combined EF strategy.

In this context, microalgae are photosynthetic unicellular organisms that can produce high-added-value biomass and biomolecules. Microalgae can grow under different cultivation conditions, at any time of the year, and using natural or artificial light [13]. The biomass of microalgae can be utilized as feed for aquaculture and/or livestock [14]. Furthermore, proteins, polysaccharides, lipids, and pigments extracted from these microorganisms can be of interest in various industrial sectors such as energy, pharmaceuticals, cosmetics, and food [15]. It has been reported that exopolysaccharides (EPS) produced by green microalgae have potential applications as bioactive molecules, biosurfactants, bioemulsifiers, and bioabsorbents, among others [16–20].

The methods for extracting EPS produced by microorganisms have been developed and adapted according to the needs and characteristics of the product. Depending on the microorganism, some EPS are freely suspended in the culture broth, while others may be attached to the cell membrane. Therefore, extraction procedures could require the use of physical, chemical, or combined operations. Within the physical methods for recovering EPS suspended in the culture broth, centrifugation or ultra-centrifugation are primarily used to separate the biopolymer from the cells. Additionally, a thermal pretreatment may be applied if the EPS is heat-resistant. This thermal treatment can also be beneficial for denaturing proteins and preventing their precipitation when using organic solvents. On the other hand, if the EPS is attached to the cells, chemical methods are employed to extract it. For instance, alkaline substances like sodium hydroxide can be used to separate EPS from the cells before centrifugation. Finally, organic solvents such as ethanol or acetone are used to precipitate EPS suspended in the culture broth. In these cases, contaminants like salts or proteins may be present, so desalting and deproteinization are recommended to obtain pure EPS.

Considering the advantages that ATPS extraction could provide in the design of a potentially industrialized bioprocess to recover EPS and other high added-value molecules found in microalgae and how this could be potentialized in an EF operation, this study aimed to test the use of thermosensitive polymer-based ATPS in the EF of the green microalga *Neochloris oleoabundans*. The goal was to facilitate the extraction and primary recovery of EPS and other high-added-value biomolecules that remain in the spent biomass from the system with the final vision of designing a biorefinery with this purpose in the near future.

2. Materials and Methods

2.1. Microalgal Cultures

2.1.1. Pre-Inoculum Cultures and Control Conditions

Neochloris oleoabundans (UTEX 1185; University of Texas at Austin, Austin, TX, USA) was used for this project. Liquid freshwater-based culture medium for pre-inoculum and control conditions was prepared according to the method described by Salim et al. [21]. Briefly, the medium was composed as follows: 3 g/L KNO_3 (DEQ, García, NL, Mexico), 0.26 g/L $NaH_2PO_4 \cdot 2H_2O$ (PQM Fermont, Monterrey, NL, Mexico), 0.74 g/L KH_2PO_4 (JT BAKER, Phillipsburg, NJ, USA), 2.38 g/L HEPES (Merck Sigma-Aldrich, St. Louis, MO, USA), 61.8 µg/L H_3BO_3 (DEQ, García, NL, Mexico), 0.11 g/L EDTA-Fe(III)-Na (Merck Sigma-Aldrich, St. Louis, MO, USA), 37 mg/L EDTA-Na_2 (Merck Sigma-Aldrich, St. Louis, MO, USA), 3.2 mg/L $ZnSO_4 \cdot 7H_2O$ (JT BAKER, Phillipsburg, NJ, USA), and 13 mg/L $MnCl_2 \cdot 4H_2O$ (Merck Sigma-Aldrich, St. Louis, MO, USA). Said components were added to the deionized water and mixed homogeneously; pH was adjusted to 6.8 with NaOH (DEQ, García, NL, Mexico), and the mixture was then autoclaved at 121 °C and 15 psi for 15 min. Subsequently, the following components were added to the culture medium under sterile conditions: 1.83 mg/L $CuSO_4 \cdot 5H_2O$ (JT BAKER, Phillipsburg, NJ, USA), 0.4 g/L $MgSO_4 \cdot 7H_2O$ (DEQ, García, NL, Mexico), 13 mg/L $CaCl_2 \cdot 2H_2O$ (DEQ, García, NL, Mexico), 1 µg/L Vitamin B_{12} (Merck Sigma-Aldrich, St. Louis, MO, USA), 1 µg/L D-Biotin (Merck Sigma-Aldrich, St. Louis, MO, USA), and 200 µg/L Thiamine-HCl (Merck Sigma-Aldrich, St. Louis, MO, USA). Pre-inoculum cultures were incubated until the exponential phase was reached (around 7 days, 1×10^6 cell/mL approximated) under the following conditions: 25 °C ± 2, 150 rpm, LED light (3000 K) with a photon flux of 40–60 µmol photons m^{-2} s^{-1}.

2.1.2. Extractive Fermentation with Thermosensitive Polymers

For cultures in the presence of thermosensitive polymers, EOPOs (Merck Sigma-Aldrich, St. Louis, MO, USA) with different molecular weights were used in different concentrations: 3900 g/mol at 10% and 15% (*w*/*v*), and 12,000 g/mol at 10% (*w*/*v*) (Figure 1). The EOPOs were placed in Erlenmeyer flasks with 4 mL of deionized water and sterilized by autoclaving at 121 °C and 15 psi for 15 min. Afterwards, 100 mL of sterile base culture medium was added and gently mixed until fully homogenized. Then, 10% (*v*/*v*) of pre-inoculum culture of *Neochloris oleoabundans* was added, and the flasks were incubated under the same conditions described in Section 2.1.1. for 21 days. Additionally, the cultivation with thermosensitive polymers was scaled to 1.5 L at room temperature (29 °C ± 2, approximately in the summer season) with an airflow rate of 1.5 L/min, LED light (4000 K), and photon flux of 60–90 µmol photons m^{-2} s^{-1} (Figure 1) for 45 days. The 100 mL cultures will be referred to as conditions A, and the 1.5 L cultures as conditions B, respectively. The cultures were conducted in duplicate with their respective control culture medium.

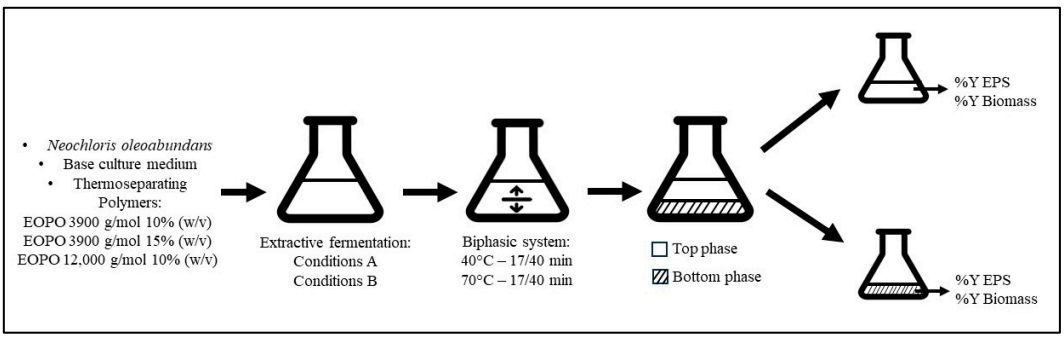

Figure 1. Simplified scheme of ATPS as a method in an extractive fermentation with *Neochloris oleoabundans* and thermosensitive polymers. It illustrates the formation of the biphasic system and the recovery of valuable products from each of the phases.

2.2. Cell Viability in Extractive Fermentation

Samples were taken from the extractive fermentation cultures with EOPO every 2 days, and cell counts of *Neochloris oleoabundans* were performed using a Neubauer chamber (Hausser Scientific, Horsham, PA, USA) under an optical microscope (ZEISS, Oberkochen, Germany) with a 40× objective. The calculations for cell viability were made using Equation (1) [22].

$$[C] = \overline{x} \times DF \times Vf \quad (1)$$

where

CCell concentration (cells/mL);
DF: Dilution factor;
$\overline{x}$: Average number of cells counted per quadrant;
Vf: Volume factor (10,000 mm^3).

Parameter estimation and cell growth curves was performed using the corrected Gompertz mathematical model (Equation (2)) [23] with the assistance of statistical software (Statistica 12; TIBCO®, Palo Alto, CA, USA). The constants b and c are generated by the statistical software.

$$Y = a \times \exp(-\exp(b - c \times T)) \quad (2)$$

where

Y: Log(N(T)/N_0);
$N(T)$: Number of cells at a certain time;
N_0: Initial number of cells;
a: Value of maximum growth;
b: Constant (generated by software);
c: Constant (generated by software);
T: Time (h).

2.3. Generation of the In Situ Biphasic System for EPS Extraction

At the end of the fermentation procedures, the culture flasks were placed in a water bath to form the ATPS and begin EPS recovery with the conditions we previously identified in the research group: ATPS phase formation and extraction were carried out at 40 °C for cultures with EOPO 3900 g/mol at 10% and 15% (w/v), and at 70 °C for cultures with EOPO 12,000 g/mol at 10% (w/v) (Figure 1). This process was conducted for 17 min in 100 mL cultures and for 45 min in 1.5 L cultures, respectively. Once the phases were formed, the EF flasks were agitated for 1 min to promote EPS extraction. When the system was reequilibrated, the volume ratio (i.e., volume of the top phase/volume of the bottom phase) for each fermentation was calculated and 50 mL samples of each phase were placed into conical tubes using sterile serological pipettes. If an interface was observed, it was considered as part of the bottom phase. The generation of the in situ biphasic system is depicted in Figure 1.

2.4. Exopolysaccharide and Biomass Quantification

The resulting *Neochloris oleoabundans* biomass recovered in each phase of the EF operation was obtained as a wet biomass pellet by centrifugation at 9500 rpm and 4 °C for 20 min using a Legend XF centrifuge (ThermoFisher Scientific Sorvall, Waltham, MA, USA). The pellet was frozen for future projects. From the initial supernatant, EPS were recovered adding 2 parts of absolute ethanol at 4 °C. The mixture was left to settle for approximately 24 h at 4 °C. Afterwards, centrifugation was carried out at 9500 rpm and 4 °C for 20 min using the same centrifugation equipment. The supernatant was discarded, and the EPS pellets were washed twice with 70% ethanol. After each wash, solutions were centrifuged again at 9500 rpm and 4 °C for 20 min [11]. The supernatant was discarded, and the pellet was retained. Finally, the resulting EPS pellet was dried using an Integrated Speed Vac (ThermoFisher, Waltham, MA, USA) at 45 °C and 50 Torr for 1.5 h. Once dried, the

EPS pellet was stored for future characterizations. The EPS yield was calculated using Equation (3). For the microalgal biomass yield, Equation (4) was used.

$$\%Y = \frac{DE}{WB} \times 100 \quad (3)$$

where

Y: yield;
DE: dry weight of EPS;
WB: wet weight of biomass.

$$\%Y = \frac{WB}{V} \times 100 \quad (4)$$

where

Y: yield;
WB: wet weight of biomass;
V: volume.

2.5. Extractive Fermentation with TP in a Semi-Continuous System

A semi-continuous extractive fermentation test was performed to compare it with a batch extractive fermentation system. This was carried out in four cultures of *Neochloris oleoabundans* with a 12-day incubation period under the incubation conditions described in Section 2.1.2. Two of the cultures were supplemented with 25%(w/v) EOPO 12,000 g/mol, while the other two were used as control batch cultures without EOPO addition. Subsequently, phase separation of the system was performed in the flasks containing EOPO at 70 °C for 20 min, and the upper phase was removed to extract EPS. The same volume of fresh culture medium was added to replace the extracted volume, and it was further incubated for 8 additional days. At the end of the incubation, the control batch flasks were heated at 80 °C for 10 min and then transferred to 50 mL conical tubes for centrifugation and EPS extraction. The extraction of EPS and biomass was conducted as described in Section 2.4, obtaining the EPS pellet from the upper phase of the system and the biomass from the lower phase. Both pellets were dried using an Integrated Speed Vac at 45 °C and 50 torr for 5 h.

2.6. Statistical Analysis

The obtained data were analyzed using analysis of variance (ANOVA) and Tukey's test when required, with a significance value of $p > 0.05$. The statistical analysis was performed using Excel® (version 2401, Microsoft Corporation, Redmond, WA, USA) and Minitab® (version 19.1, LLC, State College, PA, USA) software.

3. Results

3.1. Extractive Fermentation and Cellular Viability

Cellular viability was monitored in the control cultures and the extractive fermentation cultures in conditions A and B. The flasks corresponding to the extractive fermentation with EOPO 12,000 g/mol at 10% (w/v) in conditions A did not show cellular growth, while the flasks with EOPO 3900 g/mol at 10% and 15% (w/v) showed cellular growth by visual assessment. Cells were observed through microalgae cell count quantification under the microscope. Cells that were exposed to the TP visually displayed an increase in cell size and cell wall thickness. Additionally, cell clustering or aggregates were observed for cultures using EOPO 3900 g/mol at 15% (w/v) (Figure 2).

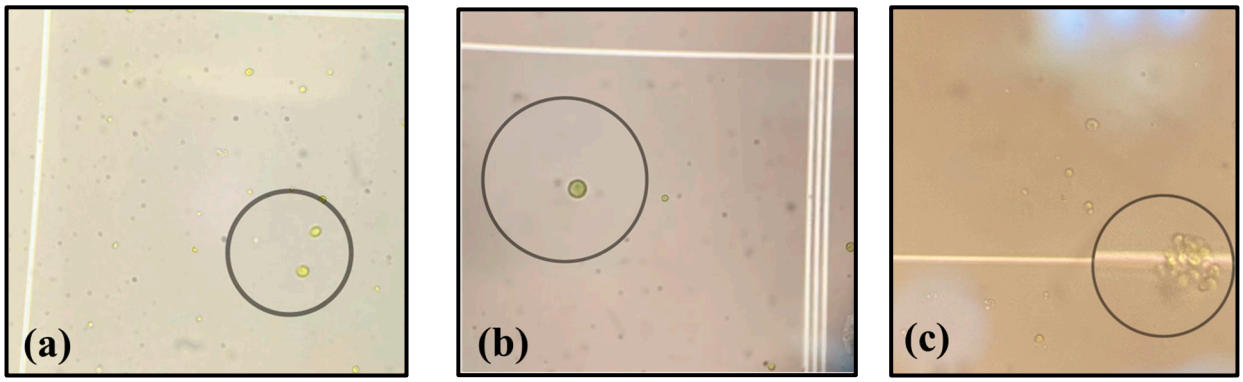

Figure 2. *Neochloris oleoabundans* cells in extractive fermentation with EOPO under a 40× objective on an optical microscope. The microalgae cells can be observed within the circles in the figures: (**a**) cells under control culture conditions, (**b**) cells in the presence of 10% (*w*/*v*) EOPO 3900 g/mol, showing thickening of the cell wall, and (**c**) cells in the presence of 15% (*w*/*v*) EOPO 3900 g/mol, forming cellular aggregates.

The control culture medium and 10% (*w*/*v*) EOPO 3900 g/mol in 100 mL (conditions A) exhibited the highest cell growth among the cultures tested under different conditions. The control culture medium presented the shortest lag phase and generation times, with the highest maximum growth and maximum growth rate. For its part, the culture with 10% (*w*/*v*) EOPO 3900 g/mol displayed a more prolonged cell growth curve, with a longer lag phase and an exponential growth phase extending beyond 300 h, as evident in the cell growth graphs (Figure 3).

The 1.5 L cultures showed cell growth in both the control medium and in the 10% (*w*/*v*) EOPO 12,000 g/mol system. However, cell death was observed in cultures with 10% and 15% (*w*/*v*) EOPO 3900 g/mol (Figure 3). Additionally, these last systems presented coagulation and viscosity changes during fermentation, along with atypical cell morphological features that were observed under the microscope.

Growth kinetic parameters were determined (Table 1), including maximum growth rate (a), lag phase (λ), generation time (G), and maximum velocity rate (μmax). *Neochloris oleoabundans* in the presence of the TP exhibited an extended lag phase compared to the control culture medium. Furthermore, it can be observed that the microalgae in the presence of EOPO showed reduced cellular growth and an elongated exponential phase (Figure 3). This behavior is evident in the parameters obtained from the Gompertz equation, where the generation phase and lag phase of microalgae with TP exhibited higher values compared to the control culture.

Table 1. Calculated growth parameters for the extractive fermentation cultures with *Neochloris oleoabundans* under conditions A (25 °C ± 2, 150 rpm, LED light, and photon flux of 40–60 μmol photons m^{-2} s^{-1}) and B (29 °C ± 2, airflow rate of 1.5L/min, LED light, and photon flux of 60–90 μmol photons m^{-2} s^{-1}).

	Condition A				**Condition B**			
Fermentation	**a**	**μmax**	**λ**	**G**	**a**	**μmax**	**λ**	**G**
Control	1.49	0.03	33.46	18.92	0.98	0.00	−2.55	99.84
EOPO 3900 g/mol 10% (*w*/*v*)	1.29	0.02	71.01	30.21	−2.45	−0.01	249.10	−35.64
EOPO 3900 g/mol 15% (*w*/*v*)	−0.06	−0.05	71.21	13.14	−2.39	−0.15	253.62	−4.50
EOPO 12,000 g/mol 10% (*w*/*v*)	0.00	-	00.79	-	0.40	0.00	232.85	106.35

a: maximum growth rate; μmax: maximum velocity rate; λ: lag phase; G: generation time.

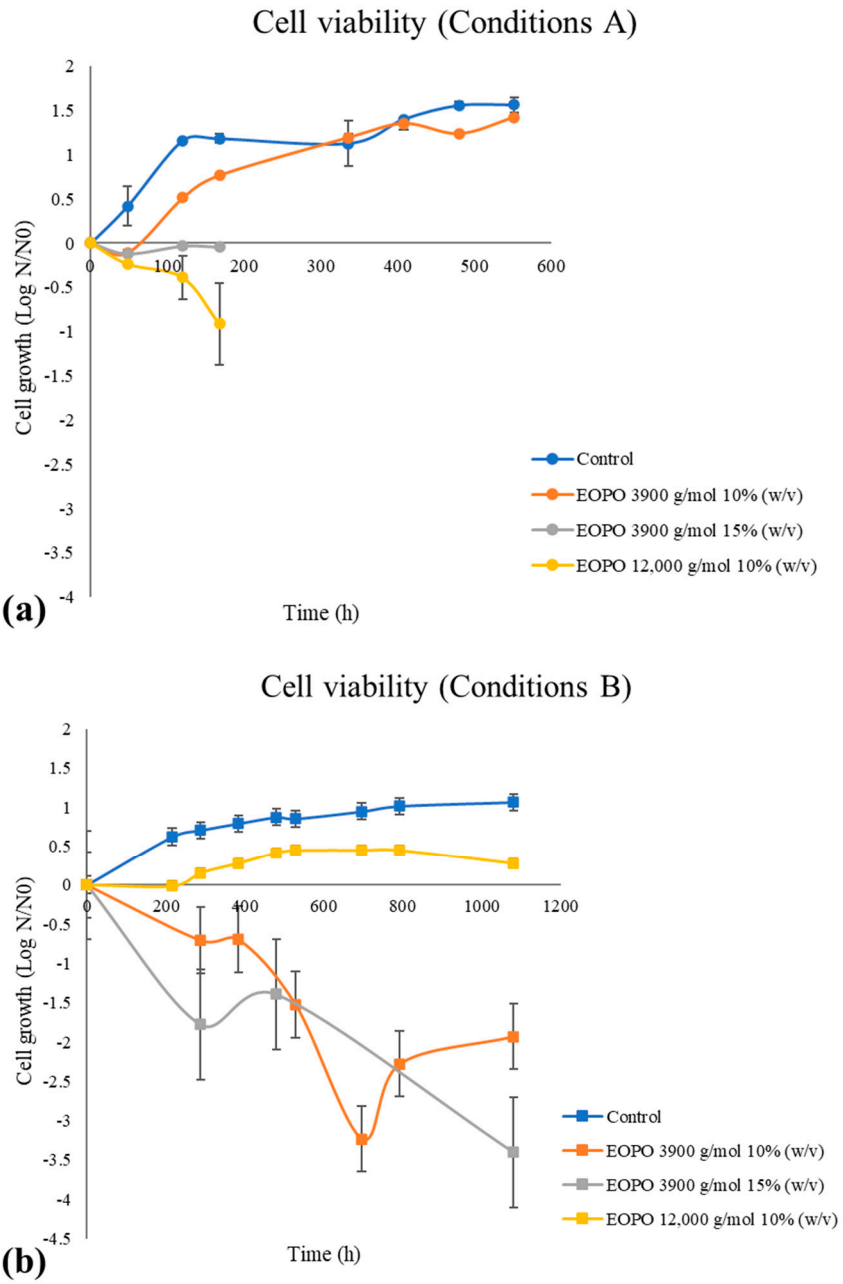

Figure 3. Cell growth curves of extractive fermentation of *Neochloris oleoabundans* in the presence of EOPOs. (**a**) Fermentation results for conditions A (25 °C ± 2, 150 rpm, LED light, and photon flux of 40–60 µmol photons m^{-2} s^{-1}). (**b**) Fermentations results in conditions B (29 °C ± 2, airflow rate of 1.5 L/min, LED light, and photon flux of 60–90 µmol photons m^{-2} s^{-1}). Error bars show standard error of 2 replicates.

3.2. Generation of the Biphasic System

As mentioned, the extractive fermentation flasks containing TPs were placed in a water bath, and the system temperature was monitored. The transition temperature (TT) of the culture with 10% and 15% (*w*/*v*) EOPO 3900 g/mol occurred at 40 °C, while TT of the 10% (*w*/*v*) EOPO 12,000 g/mol was observed at 70 °C. During the thermal treatment, separation of phases was observed and an apparent interphase was formed. After the time of heat treatment and reequilibration, no further changes in the formed phases were observed (Figure 4). Visually, the biomass shows an affinity at the formed interface; however, this interface was considered as part of the lower phase.

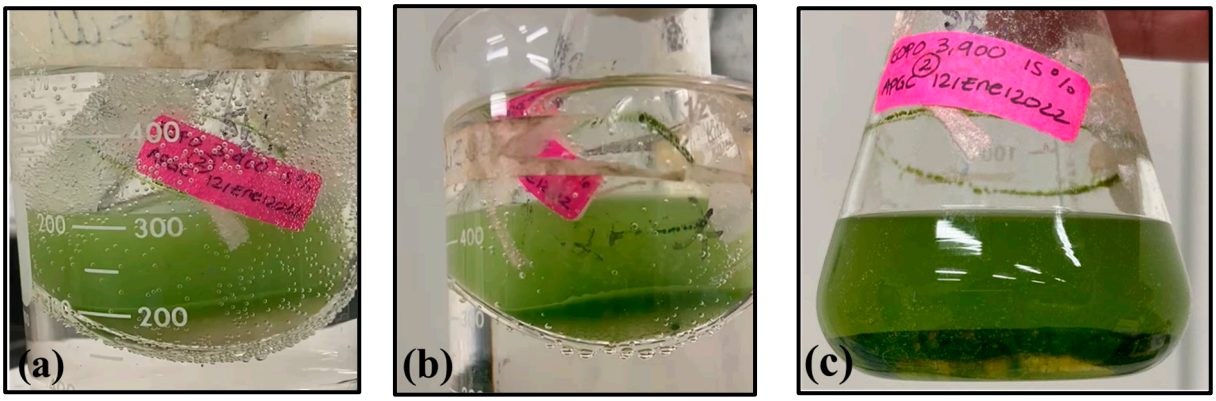

Figure 4. Extractive fermentation products of *Neochloris oleoabundans* after ATPS formation and system reequilibration with 15% (*w*/*v*) EOPO 3900 g/mol in conditions A (25 °C ± 2, 150 rpm, LED light, and photon flux of 40–60 µmol photons m^{-2} s^{-1}). (**a**) Separation at t = 6 min; (**b**) separation at t = 17 min; (**c**) separation at t = 2 h.

3.3. Extraction of EPS and Biomass

After the thermal treatment for phase formation and mixing, the flasks were allowed to rest for approximately 2 h at room temperature to allow system reequilibration. When extracting EPS and biomass from the obtained phases, the biomass was primarily distributed in the lower phase. For its part, the EPS was present in both phases, but was found in larger amounts in the top phase. Particularly, extractive fermentations under conditions A exhibited an EPS production ranging from 8 to 14 g/L with yields of up to 113.56% in relation to the obtained biomass amounts. In contrast, cultures under conditions B showed higher EPS productions of between 22 and 23 g/L, with yields ranging only from 31 to 45% (Table 2). The yield of wet biomass obtained from the EF under both conditions ranged between 1 and 3.20% (Table 3).

Table 2. Recovered EPS after extractive fermentation of *Neochloris oleoabundans* with EOPO 3900 and 12,000 g/mol systems in conditions A (25 °C ± 2, 150 rpm, LED light, and photon flux of 40–60 µmol photons m^{-2} s^{-1}) and B (29 °C ± 2, airflow rate of 1.5L/min, LED light, and photon flux of 60–90 µmol photons m^{-2} s^{-1}).

		Conditions A				Conditions B			
Fermentation	**V_R**	**[TP]**	**Y_{TP}**	**[BP]**	**Y_{BP}**	**[TP]**	**Y_{TP}**	**[BP]**	**Y_{BP}**
Control	N/A	13.63 ± 0.000	63.85	N/A	N/A	22.97 ± 0.002	21.54	N/A	N/A
EOPO 3900 g/mol 10% (*w*/*v*)	13.67	11.33 ± 0.120	108.97	-	-	22.92 ± 0.000	44.62	22.31 ± 0.003	43.42
EOPO 3900 g/mol 15% (*w*/*v*)	7	8.93 ± 0.094	113.56	-	-	22.89 ± 0.002	43.85	22.34 ± 0.005	42.81
EOPO 12,000 g/mol 10% (*w*/*v*)	4	-	-	-	-	22.30 ± 0.000	30.24	21.95 ± 0.003	29.76

V_R: volume ratio; [TP]: top phase concentration (g/L); Y_{TP}: top phase yield (%); [BP]: bottom phase concentration (g/L); Y_{BP}: bottom phase yield (%); N/A: not applicable.

Table 3. The yield of *Neochloris oleoabundans* biomass extracted from the lower phase of the system after extractive fermentation under conditions A and B.

		Condition A	Condition B
Fermentation	**V_R**	**Y_{BP}**	**Y_{BP}**
Control	N/A	3.20	3.20
EOPO 3900 g/mol 10% (*w*/*v*)	13.67	1.56	1.54
EOPO 3900 g/mol 15% (*w*/*v*)	7.00	1.18	1.57
EOPO 12,000 g/mol 10% (*w*/*v*)	4.00	-	2.21

V_R: volume ratio; Y_{BP}: bottom phase yield (%); N/A: not applicable.

3.4. Extractive Fermentation in a Semi-Continuous System

After incubation for 8 days, following EPS extraction and replacement of the culture medium, cell growth decreased significantly, which eventually led to cell death after the temperature treatment for phase formation was performed, and a yellowish color was observed. Therefore, a single extraction of the upper phase was conducted for both the semi-continuous and batch systems. Figure 5 shows the EPS obtained in each system, after drying. A total of 56% of the EPS was recovered after the batch system, while only 24.6% was recovered in the semi-continuous system.

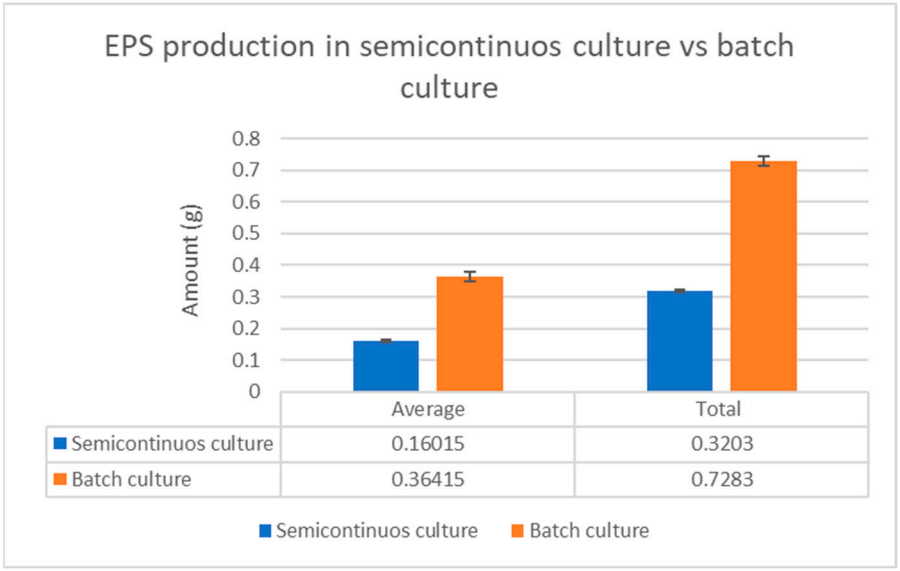

Figure 5. Production of EPS in semi-continuous and batch cultivation. The extractive fermentation was carried out with 20% (*w*/*v*) EOPO 12,000 g/mol for 12 days under sterile conditions, at 25 °C ± 2, 150 rpm, LED light (3000 K), with a photon flux of 40–60 µmol photons m^{-2} s^{-1}. The average of the two cultures from each system is shown, as well as the sum of the two cultures from each system.

4. Discussion

Understanding the growth kinetics of microorganisms during fermentation is crucial to obtaining an effective bioprocess. The presence of TP in an extractive fermentation induces stress in the microalgae, which can be observed in the behavior of the cell growth curves and their kinetic parameters (Figure 3, Table 1) when compared to the control culture medium. However, stress situations in microorganisms can be beneficial from a bioengineering point of view. It has been reported that green microalgae, when subjected to stress conditions such as nutrient depletion, pH variations, salinity, etc., can enhance the production of various high added-value biomolecules, such as lipids, pigments, and exopolysaccharides, among others [24–27].

Similarly, as presented by our results in Figure 2, cell swelling and thickening of the cell wall have been reported in *Neochloris oleoabundans* under stress conditions, particularly in the presence of high salt concentrations [28]. This thickening is attributed to the presence of sulfated polysaccharides in the cell wall structure, which provide protection to the cell against such harsh environments. Also, cell swelling can be related to accumulation of intracellular lipids, which are produced as a response to stress [29,30] or to cellular osmotic pressure, which should be considered for future research.

Among the differences in the culture conditions used in this study (Section 2.1.2); light intensity (an increase of approximately 50% in photon flux in conditions B) and temperature (an increase of approximately 30% in summer temperature in conditions B) are of relevance in microalgal growth [31]. On some occasions, an increase in light intensity in a microalgae culture can inhibit its growth, a phenomenon known as photoinhibition. This light oversaturation can damage cells and their photosynthetic capacity by producing

reactive oxygen species [32,33]. According to various authors, photoinhibition in *Neochloris oleoabundans* cells typically occurs at photon fluxes above 180 µmol photons m^{-2} s^{-1} [34]. On the other hand, light intensity in the range of 50–100 µmol photons m^{-2} s^{-1} has been previously reported to be more suitable for *Neochloris oleoabundans* growth [35]. Since light conditions of cultures of *Neochloris oleoabundans* in the present study are 40–60 µmol photons m^{-2} s^{-1} in conditions A and 60–90 µmol photons m^{-2} s^{-1} in conditions B, less than the reported inhibitory light intensity, photoinhibition is not likely to be the main factor contributing to the growth differences observed in the cultures.

On the other hand, temperature is also a limiting factor in the growth of microalgae. An increase in temperature in microalgae cultures has been reported to affect cells physiologically and biochemically. A study demonstrated that at the photosynthetic level, a decrease in the net photosynthesis rate was observed at temperatures above 30 °C, reducing the affinity of CO_2 for the enzyme ribulose-1,5-bisphosphate (Rubisco), thereby limiting biomass production in microalgae [36]. Studies have reported that the microalgae *Neochloris oleoabundans* can experience growth inhibition at 32 °C, with optimal temperatures for its growth ranging from 20–30 °C [37]. Since the cultures under condition B were at temperatures around 29 to 32 °C during the summer season, it could be inferred that the microalgae might have experienced growth inhibition, leading to lower cell growth compared to the cultures under condition A. Furthermore, studies have reported that *Neochloris oleoabundans*, when incubated at 35 °C for more than 11 days, exhibited inhibition [38]. In the case of the cultures under conditions B, which lasted up to 45 days in extractive fermentation, temperature likely played a crucial role in the growth inhibition.

It is important to note that in an extractive fermentation with TP, the two phases of the system will present proper separation when the polymer concentration and the culture medium components are in a concentration above the binodal curve [33]. Moreover, since the polymer is activated by temperature, it is necessary to reach or surpass said temperature to form two immiscible phases. Several extractive fermentation studies using EOPOs have been explored in order to obtain biomass and extracellular compounds, where cultures have been subjected to temperatures above 30 °C for phase separation of the system. In a study of extractive fermentation with *Rhodotorula mucilaginosa* UANL-001L to obtain EPS, EOPO 970 g/mol at 40% (*w*/*v*) was used, with the optimal conditions for phase formation being at 30 to 45 °C for 4–6 min. This resulted in a recovery of 42% EPS and 7% biomass on the top phase and 58% of EPS and 93% of biomass in the bottom phase [11]. In the extractive bioconversion of poly(R)-3-hydroxybutyrate (PHB) using *Cupriavidus necator* with 5% (*w*/*v*) EOPO 3900 g/mol, the culture medium was subjected to 65 °C to form the biphasic system, with a yield of 97% of PHB on the top phase [12]. Similarly, in extractive fermentation with *Burkholderia cepacia* for lipase production, the fermentation broth was placed in a water bath at 50 °C for 15 min to separate the phases of the system, resulting in a production of up to 55 U/mL recovered from the top phase of the system [10]. It is important to note that in an extractive fermentation with TP, the two phases of the system will present proper separation when the polymer concentration and the culture medium components are in a concentration above the binodal curve [3]. Moreover, since the polymer is activated by temperature, it is necessary to reach or surpass said temperature to form two immiscible phases. Our extractive fermentation with *Neochloris oleoabundans* using 10% and 15% (*w*/*v*) EOPO 3900 g/mol as a polymer for ATPS required 40 °C and 17 min for system phase formation and to recover the biomass from the bottom phase and the EPS from the top phase of the system. In both conditions, EOPO 3900 g/mol at 15% (*w*/*v*) exhibited a higher %Y recovery in the top phase of the system, 113.56% and 43.85% in conditions A and B, respectively (Table 2), yielding higher returns compared to those reported by Medina-Ramirez et al. of 42% in top phase and 58% in bottom phase [11]. However, the biomass recovered in the bottom phase of the system was 2.21% in cultures with EOPO 12,000 g/mol 10% (*w*/*v*) under condition B (Table 3), which was lower than the reported value of 93% by Medina-Ramirez et al. [11] in the bottom phase of the system.

It is important to note that system conditions can lead to significant differences on the partition of the molecules towards the bottom or upper phases. In our study, the extraction of EPS in both phases of the system under conditions B was very similar, with a difference of approximately 1–2, while in condition A, the EPS is mainly distributed in the top phase of the system, with extraction yields of up to 113.56% in the culture with EOPO 3900 g/mol at 15% (*w*/*v*) (Table 2). The partitioning of biomolecules in ATPS is influenced by the characteristics of the biomolecule itself as well as the characteristics of the system, such as polymer, temperature, and pH, among other factors. In the case of biomolecules such as proteins, temperature plays an important role in bioseparation, as increasing temperatures for the biphasic system can lead to protein denaturation or induce aggregation, affecting their distribution in the system. For EPS produced by *Neochloris oleoabundans*, it has been reported that some of them have monosaccharides in their molecular structure, including glucose, mannose, galactose, xylose, ribose, arabinose, and rhamnose, along with 0.59% peptide [20]. Therefore, the distribution is likely influenced by the nature of the molecules and the characteristics of the thermosensitive polymers.

Regarding the characteristics of the polymers used for the system, EOPO 3900 g/mol contains $EO_{50\%}$ and $PO_{50\%}$ in its structure, unlike EOPO 12,000 g/mol, which consists of $EO_{20\%}$ and $PO_{80\%}$. The ratio between the EO and PO blocks in its molecular structure contributes to the hydrophobicity of EOPO, as the amount of PO blocks increases, the hydrophobicity is higher, and likewise, higher PO content increases the viscosity of the polymer. The cloud point in the phase separation of the system is identified by turbidity and the beginning of the formation of the biphasic system. This cloud point is usually lower in EOPOs with higher hydrophobicity, meaning that they contain more PO blocks in their structure. In this study, we were able to identify the cloud point of $EO_{20\%}PO_{80\%}$ 12,000 g/mol at 70 °C to form the aqueous two-phase system, while $EO_{50\%}PO_{50\%}$ 3900 g/mol reached its cloud point at a lower temperature at 40 °C.

However, the temperature required to form the two phases of the system may vary if salts are added to the system. The base culture medium used for the growth of *Neochloris oleoabundans* in our ATPS contains a mixture of different salts necessary for its growth (Section 2.1.1). The overall composition of the salts in the medium in the system or within the microalga cells could influence the cloud point of the ATPS.

System separation and product partition can also vary due to other factors that affect the binodal curve of the system, such as pH, salinity, and sample composition [7], which in this case would be the biomass and the extracellular components such as EPS produced by the microalgae. The molecular weight of the polymer also influences the distribution of biomolecules; studies have reported that higher molecular weight improves the partition coefficient in large biomolecules, such as bromelain [39]. However, further characterization studies of the ATPs in *Neochloris oleoabundans* cultures are needed to accurately determine the factors influencing EPS partitioning in the system phases.

Extractions of various valuable biomolecules such as EPS produced by microorganisms have been performed using other ATPS. The extractive fermentation with *A. pullulans* for pululan production using classic ATPS of PEG-salt and PEG-dextran has been studied. Different concentrations of the system components and pH were tested for system partitioning, finding that biomass had an affinity for the upper PEG-rich phase, while the pululan polysaccharide was mostly distributed in the lower phase. A maximum yield of 36.47 g/L of pululan was reported in the ATPS system with PEG-dextran [4]. Aqueous two-phase systems have been used as a feasible method for EPS extraction post-fermentation. EPS produced by *Lactobacillus plantarum* was extracted using a system of 22.5% PEG 600 (*w*/*w*), 17% NaH_2PO_4, and a pH of 6, with a partition coefficient of 5.21 ± 0.05 and a recovery yield of 71.57 ± 0.039%. The EPS obtained from this bacterium had a molecular weight of 95,276 Da and was primarily composed of mannose, glucose, and galactose, with potential applications in industries such as pharmaceuticals or functional foods [40]. Unlike pullulan and EPS produced by *Lactobacillus plantarum* in ATPS with PEG-salt, the EPS from *Neochloris oleoabundans* with EOPO is mainly distributed in the upper phase of the system

under conditions A and evenly distributed in both phases of the system under conditions B (Table 2). Therefore, strategies need to be explored to enhance the partitioning of EPS in ATPS with *Neochloris oleoabundans* under conditions B to increase EPS extraction in the upper phase of the system.

The production and extraction of EPS in the cultures yielded quantities ranging from 8 to 23 g/L (Table 2). Studies on EPS production in *Neochloris oleoabundans* using mixotrophic culture media with lactose or glucose as a carbon source have reported EPS concentrations between 1 to 5 g/L approximately [20,25]. In this study, we obtained a higher quantity of EPS, greater than 20 g/L under condition B, compared to what has been previously reported. As mentioned, exopolysaccharides are produced as a defense mechanism in microalgae. Therefore, the increased production of EPS with thermosensitive polymers, coupled with high temperatures, could increase stress on *Neochloris oleoabundans*, favoring EPS production.

In the extractive fermentation in the semi-continuous system, cell viability was compromised when phase separation of the system was conducted at temperatures of 70 °C, resulting in a yellowish color, which is associated with cellular death of the microalgae [41] and a decrease in EPS production to 24.6% (Figure 5). Upon the addition of fresh culture medium, the microalgae were not able to reactivate their cell growth, confirming the suspicion of the loss of cell viability. Although thermosensitive polymers with a higher number of PO blocks and higher molecular weight, such as $EO_{20\%}PO_{80\%}$ 12,000 g/mol, are the most favorable for polymer recycling in an ATPS due to their greater efficiency in forming the biphasic system because of their hydrophobicity, we can conclude that the conditions of 70 °C for the phase separation of the system with EOPO 12,000 g/mol do not favor either the biomass recovery process or the recycling of the thermosensitive polymer in this semi-continuous system. However, in studies conducted on the recovery of biomolecules such as lipase [10], lysozyme [42,43], and ciprofloxacin [44], thermosensitive polymers in ATPS have been successfully recycled up to three times by adding fresh aqueous phase again in each cycle. Extractive fermentation as a method for producing and recovering EPS with thermosensitive polymers with *Neochloris oleoabundans* requires an extended time due to the solubility changes generated by the system components. Also, the temperature needed for the phase separation of the system affects the migration of the biomolecules to be extracted and the components of the medium [45]. EOPO 12,000 g/mol requires high temperatures for the formation of the biphasic system, so maintaining the microalgae under these conditions for extended periods is not a favorable strategy for a semi-continuous system. As a perspective, other thermosensitive polymers could be explored to implement a semi-continuous system with *Neochloris oleoabundans* for the recovery of bioproducts and their recycling over multiple cycles.

5. Conclusions

For in situ extractive fermentation, it is important to work with temperatures at which the microorganism remains viable. In this study, an EPS production of up to 22.9 g/L was achieved in 1.5 L cultures of *Neochloris oleoabundans* under conditions of 29 °C ± 2, airflow rate of 1.5 L/min, LED light, and photon flux of 60–90 µmol photons m^{-2} s^{-1} (conditions B) with 10%/*w*/*v* EOPO 3900 g/mol, with a biomass recovery yield of 1.54% from the lower phase and 44.62% EPS recovery from the upper phase of the system. Additionally, it was identified that EOPO 3900 and 12,000 g/mol in the culture medium can form the biphasic system at temperatures above 40 °C in less than 20 min. However, this temperature is still above the optimal temperature for the growth of *Neochloris oleoabundans*, so further assays are needed to reduce these conditions.

In the semicontinuous extractive fermentation using EOPO 12,000 g/mol, the biomass could not be reused due to cell death caused by prolonged exposure to high temperatures. Given the behavior of this EOPO in the extractive fermentation with *Neochloris oleoabundans*, it could be used for the extraction and purification of biomass or biomolecules after fermentation. For an extractive fermentation, it is necessary to find a thermosensitive polymer that

does not require high temperatures and shorter times for the generation of the biphasic system, thus avoiding compromising the viability of the biomass.

Therefore, extractive fermentation using TP in ATPS is an interesting strategy to be implemented in various microalgae bioprocesses for in situ extraction and purification of biomolecules. However, further studies need to be conducted to optimize the temperature and phase separation time, as the viability of the microalgae should not be compromised for their application in continuous or semi-continuous fermentation systems.

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