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Purification of the Lipidic Fraction of a Microalgal Extract by Multi-stage Process with Aqueous Two-phase Systems

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In the last decades, microalgal biomasses as a source of bioactive compounds represented an object of interest for researchers. To this end, studies were carried out on the extraction processes for the recovery of high value-added molecules. The excessive use of hazardous organic solvents and the growing environmental concern, led to the exploration of new extraction routes. Among these, innovative green solvents, such as natural deep eutectic solvents (NaDES) were studied. However, the difficulty of the fractionation NaDES microalgal extracts in separate streams rich in lipids, proteins, carbohydrates still represents a major drawback for a possible industrial application. In the present contribution, the feasibility to use aqueous two-phase systems (ATPS) for the fractionation of the main bioactive compounds (lipids, proteins, carbohydrates) contained in a NaDES extract from the green microalga *Chlorella vulgaris* was explored. Furthermore, a process flow diagram, containing a three-stage separation unit of ATPS, formulated with two aqueous solutions of polyethylene glycol 600 and choline chloride, was designed. Overall, the process designed, allowed obtaining separation yields higher than 70 %.

1. Introduction

Microalgae are unicellular eukaryotic organisms able to colonize several habitats, while preferring aquatic ones. The biochemical composition is related to the species, to the phase and growing conditions (light, temperature, soil composition), in suitable growth conditions they can accumulate significant quantities of oils, carbohydrates, starch and vitamins. Given the enormous biodiversity, which is still in largely unexplored and the abundance and ease with which they can be grown, microalgae represent a very interesting resource for new application and for the production of biomass from which metabolites and fine chemicals with high added value can be obtained. Among microalgae, *Chlorella vulgaris* a green microalga with a spherical shape (diameter equal to 2-10 µm) and it grows in freshwater. In its genus it is the most cultivated and exploited species to extract metabolites and chemicals of interest. The total protein content in *Chlorella vulgaris* is around 42-58% of the biomass according to the growing conditions. The lipid content in *Chlorella vulgaris* can vary between 5% and 40% of the dry weight of the biomass; under unfavorable growing conditions, the lipid content (especially triglycerides) can reach the 58% of the dry weight (Mata et al., 2010). The fatty acid profile also varies according to the growth conditions, in agreement with Yeh and Chang (2012), cultivation in mixotrophic condition of *Chlorella vulgaris* leads to the accumulation of 60-68 % of saturated and monounsaturated fatty acids, such as: palmitic acid, stearic acid, palmitoleic acid and oleic acid (Zheng et al., 2010). Carbohydrates are present as reducing sugars and polysaccharides, of which starch is the most abundant (Lordan et al., 2011). In nitrogen limiting conditions, carbohydrates can reach 12-55 % of dry weight (Choix et al., 2012). As for pigments, the most abundant in *Chlorella vulgaris* are chlorophylls which can reach a percentage of 1-2 % of the dry weight, also a good amount of secondary pigments is present, especially β-carotene, astaxanthin, castaxanthin and lutein (Safi et al., 2014). In the last decades, researchers focused on the development of bio-refining processes of biomass extracts. In particular, the utilization of microalgae as animal and fish feed, manures, pharmaceutical, cosmeceutical, environmental and other biotechnological applications are altogether assessed (Bhattacharya and Goswani, 2020). High value-added biomolecules are generally extracted from microalgal biomass by solvent extraction (Lee et al., 2021). However, the fractionation of the extract into different streams, each rich in

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a particular class of biomolecules is a crucial matter which is still an object of investigation (Alavijeh et al., 2020). Recently, researchers attempted at developing innovative system for biorefining, such as aqueous two-phase systems (ATPS). ATPS consist in two immiscible aqueous solutions of hydrophilic non-volatile components. The steric hindrance between the two solutions and the ionic strength leads to the formation of the separate phases in equilibrium with each other. The mechanism of partitioning of the phases is not yet clear, but the gravitational, buoyancy and friction forces certainly intervene. When introduced in the ATPS, the biomolecules contained in the microalgal extract are distributed in the two different phases based on their affinity (Khoo et al., 2020). The factors that regulate the partition of the molecules are: molecular weight of molecules and ATPS components, pH and temperature and hydrophobicity. Commonly, ATPS consist in two polymers (such as polyethylene glycol and dextran), a polymer and a salt (such as a phosphate, sulfate or citrate or organic salts, such as choline chloride) or ionic liquids and alcohols (Iqbal et al., 2016; Dos Santos et al., 2018). The objective of the present study was to evaluate the possibility to develop a sustainable alternative to the classic methods of separation and purification of microalgal extracts. To this end, the possibility of fractionating microalgal extracts through two-phase aqueous biphasic systems (ATPS) was investigated and the sizing of a pilot plant for separation was proposed. An ATPS multistage system allows obtaining high recovery yields of the main biomolecules (lipids, proteins, carbohydrates) (Suarez Ruiz et al., 2020; Liu et al., 2018). The lipidic and carbohydrate fraction could be subsequently separated by heating and gelification of the polymeric phase. Proteins were hypothesized to migrate in the saline phase (Suarez Ruiz et al., 2018). Furthermore, the choice of a natural extraction solvent, such as NaDES, and two-phase aqueous systems for fractionation allows developing a low environmental impact process and to reduce the biomolecules purification costs.

2. Materials and methods

2.1 Extraction of Bioactive compounds

The biomass was obtained from a liquid culture of *Chlorella vulgaris* grown in cylindrical photobioreactors. A 8 h photoperiod was ensured by eight with cold white light fluorescent lamps. In Figure 1, *Chlorella vulgaris* growth curve was derived spectrophotometrically (Shanghai Mapada Spectrophotometer UV- 1800 PC) by measuring the absorbance (OD690) values of the culture for about 20 days. After about seven days, the culture reaches the stationary state and the specific growth rate remains more or less constant and approximately equal to zero. Total protein and carbohydrate contents were evaluated by the Lowry and the Dubois assays, respectively (Dubois et al., 1956; Lowry et al., 1951). It was determined that total protein content was 10.2 %, total carbohydrates were 43.4 % and neutral lipids content was 29.4 % of the biomass.

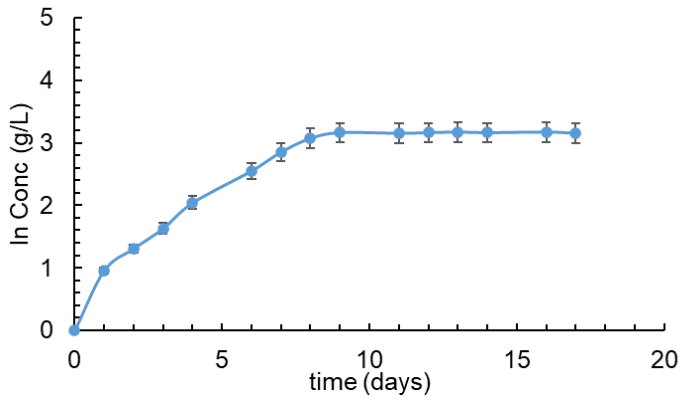

Figure 1: Chlorella vulgaris growth curve

To obtain NaDES-Y, lauric acid ($C_{12}H_{24}O_2$) and caprylic acid ($C_8H_{16}O_2$) were mixed in the molar ratio of 0.33 mol/mol and heated up to 50 °C for 20 minutes until a homogeneous and colorless liquid was formed (Buldo et al., 2019). The solid-liquid extraction of bioactive compounds was performed by adding 1.5 mg of wet biomass to 30 mL of solvent under orbital shaking conditions at 25 °C for 24 hours. To facilitate the mechanical breakdown of the cell wall and improve the contact between the solvent and the intracellular components, 2 mm glass beads were added to the reaction vessel. Subsequently, the solid-liquid separation was carried out by centrifugation at 4000 RPM x g for 20 minutes. The amount of pigments was assessed spectrophotometrically and proteins and carbohydrates contents were evaluated by the Lowry and the Dubois assays, respectively.

2.2 Process flow diagram for the fractionation of the bioactive compounds

The design of the process flow diagram for the fractionation of the bioactive compounds was carried out by hypothesizing the partitions of bioactive compounds based on literature results obtained with similar sistems. The PFD was designed hypothesizing a biomass of 3.5 Kg and 35 Kg of solvent. The PFD is composed of a multistage ATPS system, a gelification unit and a membrane separation unit.

2.3 ATPS fractionation

In order to design the three-stage ATPS separation system, preliminary experiments to assess the components distribution in the ATPS system were carried out. The PEG-choline chloride aqueous two-phase system was prepared in a graduate tube by mixing an aqueous solution of PEG (90% w/w) with an aqueous solution of Choline Chloride (72% w/w). The two phases were separated by setting for 20 minutes at room temperature. The qualitative observations on the components distribution and the densities measured gravimetrically for each phase are reported in Table 1. The fractionation of the main bioactive compounds (lipids, proteins, carbohydrates) contained in the extract was carried out by designing a three-stage separation unit of ATPS. The extract is introduced in a system composed of saturated aqueous solutions of PEG 600 and choline chloride. It was hypothesized that, in the first stage, according to the density (ρ), three phases are formed: a top hydrophobic phase composed of NaDES components, lipids and carbohydrates, an intermediate hydrophilic phase containing choline chloride and proteins a hydrophobic PEG-rich a bottom phase, predominantly containing pigments. To enhance the separation of the main bioactive compounds, the top phase is treated in other two analogous ATPS stages. The separation efficiencies of each ATPS stage were determined according to the study of Suarez Ruiz et al., (2020). Despite the study refers to a system in laboratory scale, different studies reported the possible scalability ATPS systems (Suarez Garcia et al., 2018; Ruiz Ruiz et al., 2013).

Table 1: Component distribution in the ATPS

	Composition
Top Phase (ρ = 900 Kg/m3)	NaDES components; Lipids; Carbohydrates
Intermediate Phase (ρ = 1090 Kg/m3)	Proteins Residual culture salts;
Bottom Phase (ρ = 1120 Kg/m3)	Pigments

2.4 Gelification

The lipid-rich PEG phase was subsequently treated in a gelification unit. According to Mohsen Nia et al. (2006), when an aqueous solution of PEG is heated to is cloud point, the solubility of the polymer decreases up to the formation of a separated phase. The first phase is mainly composed of water and hydrophilic, the other hydrophobic and rich in PEG. In the proposed PFD, the gelification temperature is set to 40 °C, also to avoid the degradation of the bioactive compounds. Therefore, it is hypothesized that the aqueous phase is rich in proteins and the polymeric hydrophobic phase contains pigments, carbohydrates and lipids (Gao et al., 2020). The stratification of the phases is driven by the density difference.

2.5 Ultrafiltration

To recover proteins from the hydrophilic salt-rich phase, a batch ultrafiltration unit is proposed. A spiral wound membrane was hypothesized for this application (Cheryan et al., 1998). The protein- rich phase is collected in a feed tank at atmospheric pressure and sent to the membrane. The retentate current is recirculated to the tank. At the end of the filtration process, the feed tank contains a protein concentrated aqueous solution.

3. Results and discussion

The main results in terms of extraction efficiency (w/w %) are reported in Table 2 and the hypothesized separation efficiencies (%) in each stage are reported in Table 3.
From Table 4, it can be observed that about 71% of the neutral lipids entering with the extract to the multi-stage system are recovered in the top phase. In addition, the amount of proteins and pigments in the top phase is negligible. The majority of the proteins originally in the extract, namely 83%, are located in the intermediate phase and most of the pigments, (91%), remain in the bottom phase. In the light of the hypothesis made and the results obtained, it can be observed that the multi-stage fractionation system proposed a does not allow to complete the purification of triglycerides. As a result, a further downstream treatment is required also for the recovery of carbohydrates and of the two NaDES components which could be recycled and employed to formulate fresh NaDES for the extraction. Furthermore, the three-stage system allows obtaining high proteins and pigments separation efficiencies in the intermediate phase and bottom phase, respectively.
The process flow diagram of the three stage ATPS separation system is displayed in Figure 2. The extract (current 1) enters the first ATPS stirred tank (V-1) and the top, intermediate and bottom phases are formed. Currents 4, 8 and 12 are the top phases exiting the three ATPS stages (V-1, V-2 and V-3). Currents 3, 7 and 11 are the intermediate phases exiting the three ATPS and are fed to the ultrafiltration unit, which consists in the UF membrane, the retentate recirculation tank and the tank that collects the permeate (F-1, V-5 and V-6). Bottom phases are represented by the currents 5, 9 and 13 are sent to the gelification unit (V-4). The gelification unit is a jacketed stirred tank heating the solution from 25 to 40 °C. Single ultrafiltration and gelification units convey all the top and bottom phases, respectively. The top current formed in the gelification unit (current 14) is sent to the UF unit to enhance protein recovery. The UF permeate (current 21) is prevalently composed of water and choline chloride.

Table 2: Extraction efficiencies obtained from the NaDES assisted extraction from Chlorella Vulgaris

Lipids (%)	Proteins (%)	Carbohydrates (%)
86	27	85

For all the ATPS stages, stirred cylindrical vessels with torospheric bottoms operating at atmospheric pressure and 25 °C could be suitable. For the further design of the vessel, a liquid filling degree of 75% would be assumed. Gelification could be carried out in a stirred and jacketed vessel. In this unit, initially, the entire volume of liquid should undergo a heating operation up to 40 °C to allow the formation of the biphasic system. The unit design, therefore, should include the sizing of the vessel, the stirring system and the heat exchange system. The heat exchange could be achieved with a spirally baffled jacket in austenitic stainless steel 316.

Table 3: hypothesized separation efficiencies (w/w %) in each stage

	Proteins (%)	Carbohydrates (%)	Pigments (%)	Lipids (%)	C_8 (%)	C_{12} (%)
First Stage						
Top Phase	65	85	35	89	89	89
Int. Phase	29	7	8	-	-	-
Bottom Phase	6	8	57	11	11	11
Second Stage						
Top Phase	45	92	20	82	82	82
Int. Phase	45	4	2	-	-	-
Bottom Phase	10	4	78	18	18	18
Third Stage						
Top Phase	6	92	-	97	97	97
Int. Phase	83	4	-	-	-	-
Bottom Phase	11	4	100	3	3	3

Table 4: Overall separation (w/w %) efficiency for each component

	Proteins (%)	Carbohydrates (%)	Pigments (%)	Lipids (%)	C_8 (%)	C_{12} (%)
Top Phase	2	72	-	71	71	71
Int. Phase	83	14	-	-	-	-
Bottom Phase	15	14	91	29	29	29

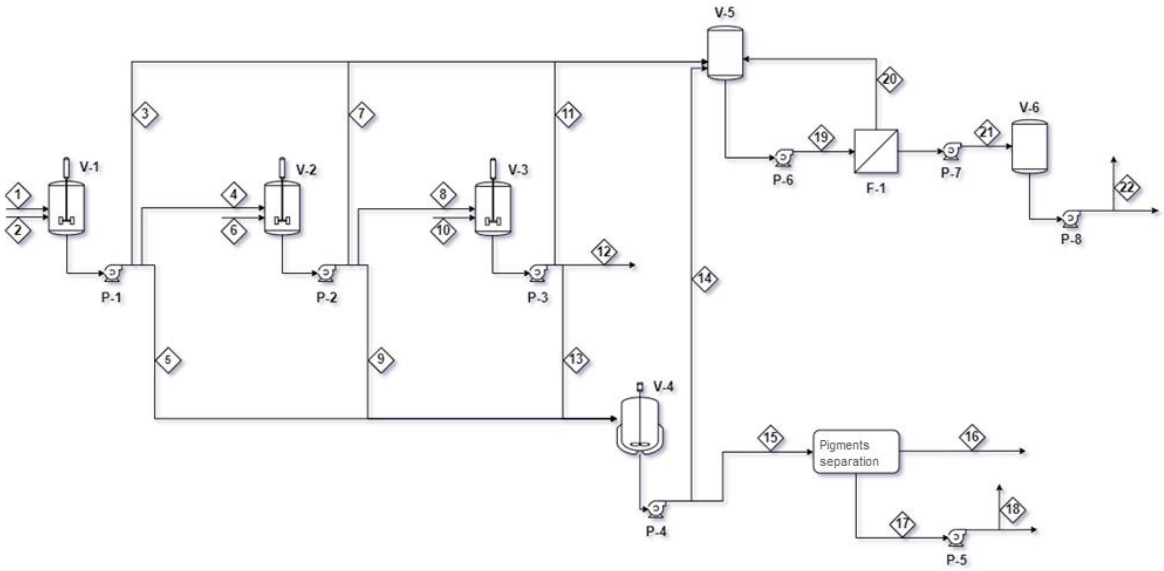

Figure 2: Process flow diagram of the three stage ATPS separation system

4. Conclusions

In this study, a multi-stage ATPS system for the fractionation of the main bioactive compounds contained a microalgal extracts from *Chlorella vulgaris* was configured. This technique potentially allows obtaining high separation efficiencies, requires mild operating conditions and can be carried out with no use of hazardous compounds. A PFD on a pilot scale was configured for the separation process, which allowed recovering about 71 % of the neutral lipids contained in the extract, most of the pigments, (91 %) and 83 % of proteins. These results encourage an experimental validation of the results, both in laboratory and pilot scales.

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References

Alavijeh R.S., Karimi K., Wijffels R.H., van den Berg Eppink M., 2020, Combined bead milling and enzymatic hydrolysis for efficient fractionation of lipids, proteins, and carbohydrates of *Chlorella vulgaris* microalgae, Bioresource Technology, 309, 123321.

Bhattacharya M., Goswami S., 2020, Microalgae – A green multi–product biorefinery for future industrial prospects, Biocatalysis and Agricultural Biotechnology, 25, 101580.

Buldo M., Cicci A., Sed G., Sapone V., Bravi M., 2019, Detoxification of Olive Oil Mill Wastewaters by Liquid-liquid Extraction with Natural Deep Eutectic Solvents, Chemical Engineering Transactions, 74, 1495-1500 DOI:10.3303/CET1974250

Cheryan M., 1998, Ultrafiltration and microfiltration handbook, CRC Press, Boca Raton.

Choix F. J., de–Bashan L. E., Bashan Y., 2012, Enhanced accumulation of starch and total carbohydrates in alginate–immobilized *Chlorella* spp, induced by *Azospirillum brasilense*: II, Heterotrophic conditions, Enzyme and microbial technology, 51(5), 300–309.

dos Santos N.V., Martins M., Santos–Ebinuma, V.D., Ventura, S.P., Coutinho, J.A., Valentini, S.R., Pereira, J.F, 2018, Aqueous Biphasic Systems Composed of Cholinium Chloride and Polymers as Effective Platforms for the Purification of Recombinant Green Fluorescent Protein, ACS Sustainable Chemistry & Engineering, 6, 9383–9393.

Dubois M., Gilles K. A., Hamilton J. K., Rebers P. T., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical chemistry, 28(3), 350–356.

Gao C., Cai C., Liu J., Wang Y., Chen Y., Wang L., Tan Z., 2020, Extraction and preliminary purification of polysaccharides from *Camellia oleifera* Abel. seed cake using a thermoseparating aqueous two–phase system based on EOPO copolymer and deep eutectic solvents. Food Chemistry, 313, 1–7.

Iqbal M., Tao Y., Xie S., Zhu Y., Chen D., Wang X., Huang L., Peng D., Sattar A., Shabbir M.A., Hussain HI., Ahmed S., Yuan Z., 2016, Aqueous two–phase system (ATPS): an overview and advances in its applications, Biol Proced Online, 18, 18.

Lee S.Y., Khoiroh I., Vo D.N., Senthil Kumar P., Show P.L., 2020, Techniques of lipid extraction from microalgae for biofuel production: a review, Environ Chem Lett ,19, 231–251.

Khoo K.S., Leong H.Y., Chew K.W., Lim J.–W., Ling T.C., Show P.L., Yen H.–W., 2020, Liquid Biphasic System: A Recent Bioseparation Technology, Processes, 8, 149.

Liu Y., Chen L., Zhou J., Yan Z. 2018, Extraction of tetracycline using the ionic liquid–based aqueous two-phase systems: single–stage versus multi–stage, Chemical Industry & Chemical Engineering Quarterly, 24, 387–397.

Lordan S., Ross R.P., Stanton C., 2011, Marine bioactives as functional food ingredients: potential to reduce the incidence of chronic diseases. Marine drugs, 9(6), 1056–1100.

Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. 1951, Protein measurement with the Folin phenol reagent, Journal of biological chemistry, 193, 265–275.

Mata T.M., Martins A.A., Caetano N.S., 2010, Microalgae for biodiesel production and other applications: a review. Renewable and sustainable energy reviews, 14(1), 217–232.

Mohsen Nia M., Rasa H., Modarress H. 2006, Cloud–point measure ments for (water + poly(ethylene glycol) + salt) ternary mixtures by refractometry method, Journal of Chemical and Engineering Data, 51, 1316–1320.

Ruiz Ruiz F., Benavides J., Rito Palomares M., 2013, Scaling–up of a B–phycoerythrin production and purification bioprocess involving aqueous two–phase systems: practical experiences, Process Biochemistry, 48, 738–745.

Safi C., Zebib B., Merah O., Pontalier P. Y. Vaca–Garcia C., 2014, Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renewable and Sustainable Energy Reviews, 35, 265–278.

Suarez Garcia E., Suarez Ruiz C.A., Tilaye T.J., Eppink M.H., Wijffels R.H., van den Berg C., 2018, Fractionation of proteins and carbohydrates from crude microalgae extracts using an ionic liquid based–aqueous two–phase system, Separation and Purification Technology, 204, 56–65.

Suarez Ruiz C.A., Emmery D.P., Wijffels R.H., Eppink M.H., van den Berg C., 2018., Selective and mild fractionation of microalgal proteins and pigments using aqueous two–phase systems, J Chem Technol Biotechnol.

Yeh K. L., Chang J.S., 2012, Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga *Chlorella vulgaris* ESP–31, Bioresource technology, 105, 120–127.

Zheng H., Yin J., Gao Z., Huang H., Ji X., Dou C., 2011, Disruption of Chlorella vulgaris cells for the release of biodiesel–producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis, and microwaves. Applied biochemistry and biotechnology, 164(7), 1215–1224.