Supercritical CO_2 Extraction of Triglycerides from Enriched *Nannochloropsis* Microalgae for Biofuel Applications

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ABSTRACT

This study has been conducted within the French Research Project called Shamash whose aim was to study the production of biofuels from microalgal oil. This work deals with supercritical extraction of triglycerides from dried and crushed *Nannochloropsis occulata* microalga which has been grown under nitrogen limitation to increase the oil content. Experiments have been performed at laboratory scale (with charge of 10 g per batch) and at larger scale (with charges of 1, 2, 6, 7.5 and 15 kg per batch). More, the influence of some operating parameters variations upon extraction yields and kinetics has been evaluated. The influence of pre-treatment (drying mode and grinding), at laboratory scale, and the influence of grinding and solvent/charge mass ratio, at pilot scale, have been studied.

INTRODUCTION

Microalgae are photosynthetic organisms present in multitudes of species and able to develop in many environments, from marine environment to fresh or brackish water, from polar ices to deserts or other extreme milieu. Microalgae are particularly able to accumulate fatty acids up to 80 % of their dry weight and particularly when submitted to nitrogen defaults [1]. During the last two decades, such property has been explored in the energy field for the production of triglycerides leading to biofuels after trans-esterification [2-17]. Extraction of triglycerides from dried seaweeds or microalgae can be performed using large amounts of organic solvents such as *n*-hexane but such commonly-used solvents are toxic, sometimes flammable and generally low selective solvents.

One alternative proposal to avoid the use of *toxic solvents* to extract bio oil is to carry out extraction with supercritical CO_2 as solvent. This technology is well-known today and is considered as a green process thanks to the safety and non-flammability of CO_2. There are numerous advantages in using supercritical CO_2 in order to extract triglycerides: supercritical CO_2 can solubilize non polar compounds as triglycerides; CO_2 is spontaneously and completely removed from the extracts after depressurization; during an operation of extraction, the main part of CO_2 is recycled and therefore used several times, decreasing the consumption per extracted mass; lastly, phospholipids are not solubilised. This last property is particularly interesting for biofuel application as the degumming operation is avoided.

In this work, supercritical CO_2 extractions were performed from lipid enriched *Nannochloropsis occulata*; at laboratory scale (charge of 10 g per batch) and at larger scales (charge of 1, 2, 6, 7.5 and 15 kg per batch). The purpose of this change of scale is to assess

whether a way of producing microalgal biodiesel from oil extracted using supercritical CO_2 would be possible.
At laboratory scale, the influence of pretreatment, drying mode and grinding, on extraction yields and kinetics has been studied. Four drying modes have been compared: drying under low temperature, freeze-drying, atomization and freezing followed by drying. Two particle sizes have been tested: less than 0.160 mm or between 0.315 and 1 mm. Larger scale experiments have been performed and the influence of particle size and solvent/charge mass ratio have been studied again.

MATERIALS AND METHODS

Chemicals and microalgae

Instrument grade carbon dioxide (purity of 99.7%) was supplied by Air Liquide Méditerranée (France).
The studied microalgae was *Nannochloropsis occulata* purchased by Alpha Biotech (Asserac, France). Its culture has been performed under nitrogen limitation to increase lipid content. After the harvest and centrifugation, microalgae have been subjected to various types of drying: drying under low temperature, freeze-drying, atomization and freezing followed by drying. Depending on different samples of microalgae studied, triglycerides content is understood between 12 and 35%. Before extractions, microalgae were grounded and sieved.

Experimental set-ups.

Several set-ups have been used to perform extraction experiments at laboratory scale and at larger scales.

Extractions at laboratory scale.

The laboratory set-up was supplied by Separex (Champigneulles, France). Figure 1 and 2 show a scheme and a photograph of the laboratory set-up, respectively. Experiments were performed in extraction cells of 5, 10 and 20 cm^3 corresponding to approximately 2, 6 and 10 grams of dried microalgae, respectively.

One extraction experiment is performed as follows:
Liquid CO_2 (1) is cooled in a cryogenic bath (2), filtered and pumped (3) towards the extraction cell (6). Before extraction autoclave, CO_2 is heated (4) until the chosen temperature. The extraction autoclave which contains a fixed bed of the dried powder is also heated. Pressure is controlled by a pressure gauge. After the extraction cell, CO_2 is released to gas state through an expansion valve (7). The extracted molecules are collected in a collector (8). The CO_2 flow rate is determined by a flow meter (9) placed at the end of the extraction line.

Regarding the low mass of raw materials and the fact that the extracts contain both neutral lipids and pigments, the extraction yields were obtained through the mass loss (Eq. 1) of the vessel.

$$\text{Mass loss}\,(\%) = \frac{\text{initial mass (g) - mass after extraction (g)}}{\text{initial mass}} \times 100 \qquad \text{Eq. 1}$$

The reproducibility of the experiment has been preliminary determined and estimated at less than 0.3 %, whatever the operating conditions.

At laboratory scale, all experiments were performed using the 20 mL autoclave and the chosen operating parameters were: pressure of 40 MPa, temperature of 333 K, and a CO_2 flow rate of 0.5 $kg.h^{-1}$.

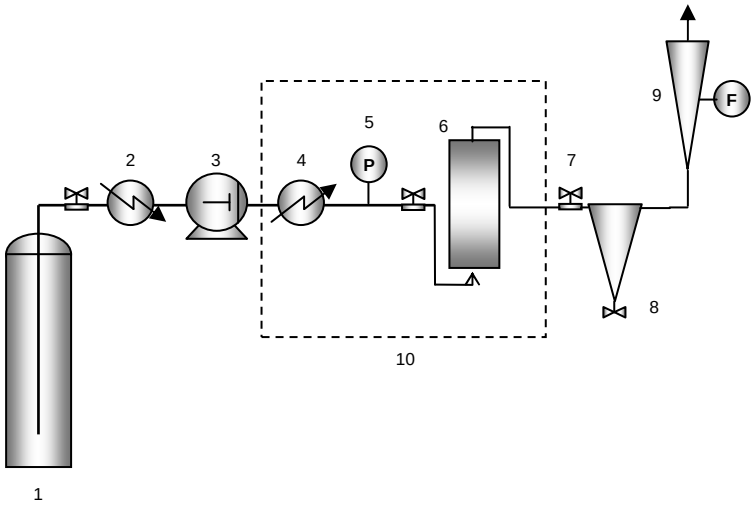

Figure 1: Experimental set-up. 1 – CO_2 cylinder; 2 – Cryogenic bath; 3 – High pressure volumetric pump ; 4 - Heat exchanger; 5 – Manometer ; 6 – Extraction cell; 7 – Expansion valve; 8 – Collector; 9 – Flow meter ; 10 – Thermoregulated area

Figure 2: Laboratory set-up.

Extractions at larger scales.

Experiments at larger scales were also performed.
First, extraction experiments with 2 kg per batch were performed by an equipment (figure 3) supplied by Separex (Champigneulles, France).

Figure 3: 2 kg per batch set-up.

The extractions at larger scales were performed as at laboratory scale except that at larger scales the CO_2 is recycled and pressure can't exceed 35 MPa.
The chosen extraction parameters were: pressure of 33 MPa, temperature of 333 K and a CO_2 flow rate of 15 $kg.h^{-1}$.

Other experiments have been conducted by Separex (Champigneulles, France) with 6, 7.5 and 15 kg per batch and by Hitex (Vannes, France) with 1 kg per batch.
The experiments conducted at larger scales correspond to scale factors of 100, 200, 600, 750 and 1500.

RESULTS

Laboratory scale. Influence of the type of drying on extraction yields and kinetics.

After the harvest and before proceeding to the supercritical fluid extraction, microalgae must be concentrated and dried to eliminate water. In order to obtain good extraction yields using supercritical CO_2, the humidity rate should be less than 12 % [18]. Two pre-treatments are mentioned in literature: freeze-drying [19-29] and drying under low temperature [30-33] but none data are given concerning the influence of such pre-treatments on oil recovery. Extraction experiments were first performed with microalgae submitted to drying under low temperature (35 °C) and freeze-drying. The microalgae are supposed to contain about 30% of neutral lipids. The pressure was fixed at 40 MPa, temperature at 333 K, and the flow rate at 0.5 kg/h. To increase mass transfer, microalgae were grounded and sieved. For these experiments, particle sizes were understood between 0.160 and 0.315 mm. Figure 4 shows the obtained extraction curves.

Extraction curves show that with drying under low temperature the extraction is more rapid. Drying under low temperature seems to be the best pre-treatment. This could be explained by the fact that freeze-drying preserves the integrity of microalgal cells. The mass transfer may then be more limited.

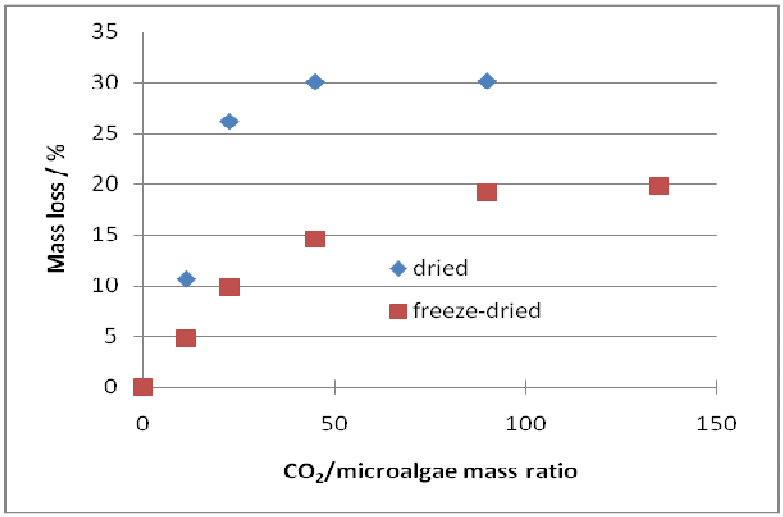

Figure 4: Influence of pre-treatment on mass losses for *Nannochloropsis occulata*

When comparing the mass losses obtained after a 90 minute extraction (P = 40 MPa ; T = 333 K; CO_2 flow rate = 0.5 $kg.h^{-1}$ corresponding to a solvent/charge mass ratio of 60) with *Nannochloropsis occulata* microalgae which has undergone other pre-treatments than freeze-drying and drying under low temperature, the same observations can be made (table 1): drying under low temperature provides the best extraction yields.

Table 1: Mass losses obtained for different pre-treatments

Pre-treatment	**Mass loss (%)**
Atomization	21.78
Freeze-drying	12.55
Drying under low temperature	35.36
Freezing followed by drying	28.12

Extracts from *Nannochloropsis occulata* (figure 4) have been analyzed using Iatroscan technique. The composition of the oil and its evolution versus the extraction duration are given in tables 2 and 3.

Table 2: Composition of extracted phases for dried nitrogen limited *Nannochloropsis*

Duration	Free Fatty Acids (%wt)	Triglycerides (%wt)	Stérol (%wt)	Pigments/polar lipids (%wt)
15 min	2.00	90.17	4.37	3.46
30 min	1.08	93.71	3.70	1.51
60 min	1.14	91.36	3.41	4.09
120 min	2.62	93.82	1.80	1.76
Raw dried *Nannochloropsis*	**0.71**	**72.13**	**4.58**	**22.58** (Pigments + polar lipids)

Table 3: Composition of extracted phases for freeze-dried nitrogen limited *Nannochloropsis*

Duration	Free Fatty Acids (%wt)	Triglycerides (%wt)	Stérol (%wt)	Pigments/polar lipids (%wt)
15 min	0.74	91.31	4.35	3.60
30 min	0.31	91.86	4.57	3.25
60 min	0.43	91.45	5.40	2.72
Raw freeze-dried *Nannochloropsis*	**1.10**	**53.78**	**4.42**	**40.71** (Pigments + polar lipids)

It appears that whatever the pre-treatment, the extracted phases are mainly composed of triglycerides (90 %), and also contain free fatty acids and pigments (carotenes and/or chlorophylls). As expected, phospholipids were not extracted. Pigments remain in the biodiesel phase after the trans-esterification. Lastly, the composition of extracted phases is independent of the extraction duration.

Laboratory scale. Influence of particle size on extraction kinetics.

Dried *Nannochloropsis occulata* (12 wt % of neutral lipids) were manually crushed and sieved. Two particle sizes have been tested: diameters less than 0.160 mm and diameters understood between 0.315 and 1 mm. Each experiment was performed under 40 MPa, 333 K and a CO_2 flow rate of 0.5 kg/h. Figure 5 shows the extraction curves obtained with *Nannochloropsis occulata* for the two studied particle sizes.

Extraction curves show that extraction is rapidly limited by the diffusion phenomenon. The extraction curves illustrate the important role played by crushing. At fixed CO_2/microalgae mass ratio it appears as expected that mass losses are higher with a smaller particle size.

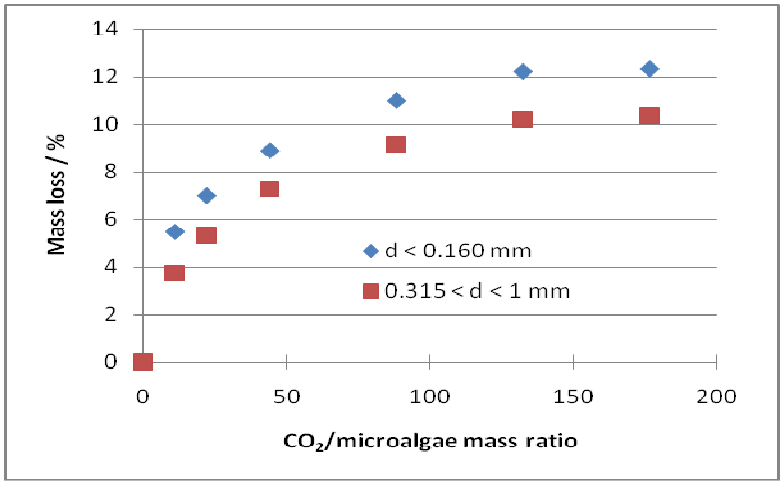

Figure 5: Influence of particle size on extraction curves.

Larger scale (× 200) and (×100). Preliminary experiments and reproducibility on extraction yields and kinetics.

Experiments were performed with an other sample of *Nannochloropsis occulata* whose neutral lipid content was estimated of about 20 %.
First experiments were performed to find the best operating conditions. Table 4 shows the mass losses obtained for each try. All experiments were conducted at 333 K and a CO_2 flow rate of 15 kg/h in an autoclave of 4 L.

Table 4: Mass losses obtained for preliminary experiments.

Pressure / MPa	Duration /h	Particle size /mm	Mass loss /%
31	2	2	11
31	2	2	11
33	3	2	17
33	3	0.5	19

At this scale, the influence of granulometry has also been studied. Figure 6 shows two extraction curves obtained with an average particle diameter of 0.5 mm and 2 mm, respectively.

These results show that large-scale extraction can lead to maximum yields as long as the raw material is grinded and the extraction conducted under high pressure. What is encouraging in these results is that these high yields were obtained for the operating conditions very applicable on an industrial scale, i.e. Pressure close to 30 MPa, solvent mass/biomass ratio less than 30 and particle diameters of 0.5 mm.

Other experiments were conducted by Hitex (Vannes, France) with 1 kg per batch in an autoclave of 2 L. The operating conditions were: pressure of 29 MPa, temperature of 328 K and a CO_2 flow rate of 6 $kg.h^{-1}$. Two particle sizes were tested: less than 0.3 mm and understood between 0.3 and 0.5 mm. The mass losses obtained were 11 % and 10 %, respectively after an extraction time of 6 hours. These results show again the positive influence of granulometry on mass losses.

Larger scales (× 600 and × 750).

These extractions have been performed with one batch of 6 kg and two batches of 7.5 kg under 31.5 MPa, 333 K and a CO_2 flow rate of 100 kg/h. Table 5 shows the yields obtained.

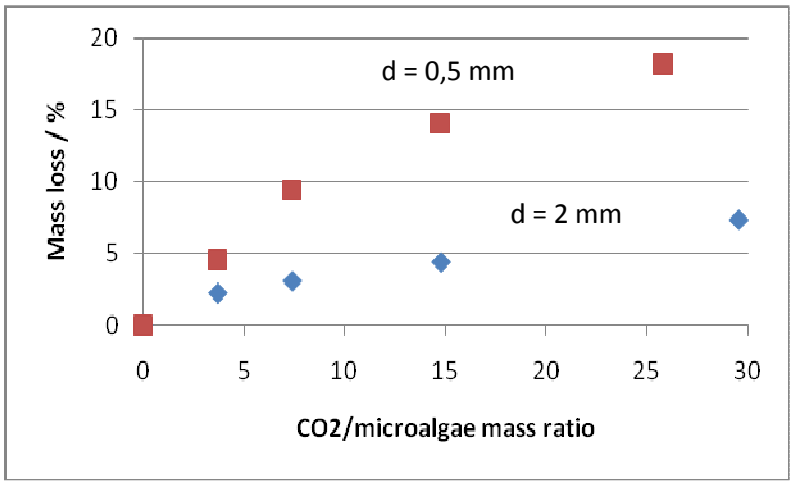

Figure 6: Influence of particle size on extraction curves at larger scale.

Table 5: Mass losses obtained for preliminary experiments.

Batches /kg	Solvent/biomass mass ratio	Recovered mass /g	Mass loss /%
6	30	250	4
2 × 7.5	20	1300	8

The mass losses obtained here are disappointing. Such bad results are explained by the high average particle size understood between 2 and 3 mm, and a too fast CO_2 flow rate.

Larger scale (× 1500).

Finally an extraction on a batch of 15 kg have been performed. The chosen operating conditions were: 30 MPa, 333 K and a CO_2 flow rate of 50 $kg.h^{-1}$. The particle diameter was understood between 0.3 and 0.5 mm. The extraction has been conducted during 4 hours corresponding to a solvent/biomass mass ratio of 13.3 and leaded to a mass loss of 6 %. The disappointing mass loss is explained by a still too large average particle size and a too fast CO_2 flow rate.

CONCLUSION

The aim of this work was to perform supercritical CO_2 extraction from lipid enriched *Nannochloropsis occulata* at several scales. The results obtained show that, at laboratory scale, the extraction of triglycerides using such technology can lead to high yields as long as the microalgae are dried under low temperature, sufficiently grinded and the extraction conducted under high pressure. Unfortunately, the results obtained at larger scales were not encouraging because of an inadequate choice in operating conditions. Further experiments are necessary to optimize larger scale extractions, by decreasing both CO_2 flow rate and particle diameter. However, reducing more the diameter of the particles may cause experimental inconveniences, such as clogging of frits or caking phenomenon leading to preferential paths and thus to poor mass transfer.

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REFERENCES

[1] CHISTI, Y., Biotechnol. Adv., Vol. 25, **2007**, p. 294.
[2] BOROWITZKA, M., BOROWITZKA, L., Micro-algal Biotechnology, Cambridge, UK: Cambridge University Press, **1988**; p 96.
[3] BOROWITZKA, M., J. Biotechnol., Vol. 136, n° 1, **2008**, p. S7.
[4] ARESTA, M., DIBENEDETTO, A., BERBERIO, G., Fuel Process. Technol., Vol. 86, **2005**, p. 1679.
[5] ARESTA, M., DIBENEDETTO, A., CARONE, M., COLONNA, T., FRAGALE, C., Environ. Chem. Lett., Vol. 3, **2005**, p. 136.
[6] BUNGAY, H., Trends Biotechnol., Vol. 22, n° 2, **2004**, p. 67.
[7] MIAO, X., WU, Q., Bioresour. Technol. Vol. 97, **2006**, p. 841.
[8] XU, H., MIAO, X., WU, Q., J. Biotechnol., Vol. 126, **2006**, p. 499.
[9] MIAO, X.; WU, Q., J. Biotechnol., Vol. 110, **2004**, p. 85.
[10] SAWAYAMA, S., INOUE, S., DOTE, Y., YOKOYAMA, S., Energy Convers. Manage**.,** Vol. 36, n° 6-9, **1995**, p. 729.
[11] MINOWA, T., YOKOYAMA, S., KISHIMOTO, M., OKAKURA, T., Fuel, Vol. 74, n° 12, **1995**, p. 1735.
[12] DOTE, Y., SAWAYAMA, S., INOUE, S., MINOWA, T., YOKOYAMA, S., Fuel, Vol. 73, n° 12, **1994**, p. 1855.
[13] GINZBURG, B., Renew. Energy**,** Vol. 3, **1993**, p. 249.
[14] MIAO, X., WU, Q., YANG, C., J. Anal. Appl. Pyrolysis, Vol. 71, n° 2, **2004**, p. 855.
[15] MILNE, T., EVANS, R., NAGLE, N., Biomass, Vol. 21, n° 3, **1990**, p. 219.
[16] DEMIRBAS, A., Energy Convers. Manage., Vol. 42, **2001**, p. 1357.
[17] WIJFFELS, R., BARBOSA, M., EPPINK, M., Biofuels, Bioprod. Biorefin., Vol. 4, n°3, **2010**, p. 287.
[18] EGGERS, R., Supercritical Fluid Extraction of Oilseeds/Lipids in Natural Products. In Supercritical Fluid Technology in Oil and Lipid Chemistry; King, J. W., List, G. R., Eds.; AOCS Press, Champaign, IL, **1996**; p. 35-64.
[19] CHEUNG, P., Food Chem., Vol. 65, **1999**, p. 399.
[20] CHEUNG, P., LEUNG, A., ANG, P., J. Agric. Food Chem., Vol. 46, **1998**, p. 4228.
[21] CHOI, K., NAKHOST, Z., KRUKONIS, V., KAREL, M., Food Biotechnol., Vol. 1, n° 2, **1987**, p. 263.
[22] ANDRICH, G., ZINNAI, A., NESTI, U., VENTURI, F., FIORENTINI, R., Acta Alim., Vol. 35, n°2, **2006**, p. 195.
[23] GOUVEIA, L., NOBRE, B., MARCELO, F., MREJEN, S., CARDOSO, M., PALAVRA, A., MENDES, R., Food Chem.**,** Vol. 101, **2007**, p. 717.
[24] MENDES, R., REIS, A., PALAVRA, A., Food Chem., Vol. 99, **2006**, p. 57.
[25] SAJILATA, M., SINGHAL, R., KAMAT, M., J. Food Eng., Vol. 84, **2008**, p. 321.
[26] MENDES, R., FERNANDES, H., COELHO, J., REIS, E., CABRAL, J., NOVAIS, J., PALAVRA, A., Food Chem., Vol. 53, **1995**, p. 99.
[27] ANDRICH, G., NESTI, U., VENTURI, F., ZINNAI, A., FIORENTINI, R., Eur. J. Lipid Sci. Technol., Vol. 107, **2005**, p. 381.

[28] CRESPO, M., YUSTY, M., Ecotoxicol. Environ. Saf., Vol. 64, **2006**, p. 400.
[29] POLAK, J., BALABAN, M., PEPLOW, A., PHILIPS, A., Supercritical Carbon Dioxide Extraction of Lipids from Algae. In *ACS Symposium Series n°406*; Johnston, K.; Penninger, J., Eds. American Chemical Society: Washington, DC, **1989**, p. 449.
[30] ARESTA, M., DIBENEDETTO, A., BERBERIO, G., Fuel Process. Technol.**,** Vol. 86, **2005**, p. 1679.
[31] ARESTA, M., DIBENEDETTO, A., CARONE, M., COLONNA, T., FRAGALE, C., Environ. Chem. Lett., Vol. 3, **2005**, p. 136.
[32] SUBRA, P.; BOISSINOT, P., J. Chromatogr., A., Vol. 543, **1991**, p. 413.
[33] SUBRA, P., TUFEU, R., GARRABOS, Y., BOISSINOT, P., J. Supercrit. Fluids., Vol. 4, **1991**, p. 244.