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Effect of Mechanical Pretreatment on *Nannochloropsis Gaditana* on the Extraction of Omega-3 by Using Accelerated Solvent Extraction Technology

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The omega-3 group includes substances as eicosapentaenoic acid and docosapentaenoic acid that can be partially synthesized by human body and substances as alpha-linolenic acid that have to be necessarily introduced into the body from dietary intake. Omega-3 are important nutrients thanks to their anti-inflammatory properties and healthy properties in the reduction of cardiovascular diseases. The growth forecasts of omega-3 market will lead to an increase in the demand for EPA and DHA and therefore finding new potential sources, such as microalgae which are the first EPA and DHA producers in the marine environment, is very important.
The aim of this work is to evaluate the feasibility of mechanical pretreatment on *Nannochloropsis gaditana* as source of EPA and at the same time evaluating the effect on the extraction yield of two solvents: a mixture of chloroform/methanol/water (Bligh & Dyer methods) and hexane that is GRAS (generally recognized as safe solvent) by using accelerated solvent extraction technology.

1. Introduction

Omega-3 polyunsaturated fatty acids are known for their beneficial effects on human health thanks to their anti-inflammatory properties and healthy properties in the reduction of cardiovascular diseases. Omega-3 PUFAs include substances that have to be necessarily introduced into the body as eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA), useful to keep a correct cardiac activity and exert a control on the level of triglycerides in the blood (van der Voort et al., 2017). According to the World Health Organization (WHO), 250 mg/day of EPA and DHA is the recommended dietary intake for men, while the daily dose of DHA for women and children is in the range of 100-200 mg/day (FAO, 2010).
In this study the focus was on the production of EPA. The main commercial sources of EPA are fish oil and fish meal, but issues related to the contamination and depleting fish supplies due to an intensive fishing, push towards the search for alternative sources that can cope with the growing demand for EPA. Microalgae are the first EPA producers in the marine environment, in particular Nannochloropsis species, belonging to the class Eustigmatophycea, is suitable for EPA production. According to the Nannochloropsis strain, EPA content varies from 1.1% to 5.1% of dry biomass (DW): for N. gaditana EPA content is in the range 2.0-4.3% DW, for N. oceanica 4.4-5.1% DW, for N. salina 1.1-3.5% DW and for N.oculata 2.0-3.0% DW (Ma et al., 2016, Camacho-Rodríguez et al., 2014). Among these strains, *N. gaditana*, that is one of the candidates for EPA production, was chosen for this study. EPA can be used for different applications: pharmaceutical and nutraceutical products, dietary supplements, infant formulas, animal foods, food and beverage (van der Voort et al., 2017). Relatively to the EPA economic value, in Molino et al. (2018) it was reported that the oil, rich in EPA, that can be derived from *Nannochloropsis sp.* has an high price ranging from US$80/kg to US$160/kg. This price, if compared with the fish oil price (US$1.4-1.7/kg (GLOBEFISH Highlights Gennaio 2018)), is not competitive, so it is necessary to increase EPA extraction efficiency in order to lower it, which is feasible by subjecting microalgal biomass to pretreatment methods.

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Pretreatment methods are used to destruct the cell wall of microalgae; in this way intracellular released components can get in touch more easily with solvent molecules during the extraction process, operating cost decreases and lipids quantity and quality increase (Abirami et al., 2016). Pretreatment methods can be classified in biological, chemical and physical methods (Klinthong et al., 2015). Enzymatic pretreatment is a biological method that can improve lipid recovery from microalgae. Castejón et al. (2019) carried out a study on *N. gaditana* in which the effectiveness of enzymatic pretreatment on different extraction techniques was evaluated. The results have shown that using hexane as solvent for pressurized liquid extraction, lipid recoveries with and without enzymatic pretreatment were 17.6% e 9.5%, respectively. Lee et al. (2015) reported the hydrothermal-acid pretreatment (chemical method) to extract EPA from *N. salina*. When the sample was not subjected to the pretreatment, lipid yield was less than 5%, while, using acid pretreatment with sulfuric acid (2% (v/v)), EPA yield was equal to 58.1 mg/g cell. Microwave pretreatment (physical method) was examined in a study on *N. gaditana* of Cancela et al. 2019. Results showed that when the ultrasound extraction was preceded by microwave pretreatment, the percentage of oil extracted was 22.60% of total dry biomass, higher than value without pretreatment (6.22%). A study on *N. gaditana* where different pretreatment methods were tested, was conducted by Safi et al. (2017). Bead milling, a physical method, got the best results with a release of total proteins equal to ±50% (w/w), higher than enzymatic pretreatment (±35% (w/w)). In Abirami et al. (2016) was shown a comparison between acid, enzymatic, thermal, microwave and ultrasonic pretreatment on *N. gaditana*. Results highlighted that EPA yield was high in ultrasonic disruption (EPA content equal to 3.25 % dry wt), while was low in acid treatment (1.89 % dry wt).
The aim of this work was to evaluate the feasibility of mechanical pretreatment on *N.gaditana* as source of EPA. Furthermore, with the use of the accelerated solvent extraction (ASE) technology, the effect of solvents on the extraction yield was evaluated. A mixture of chloroform/methanol/water (C/M/W) (1:2:0.75v/v) used in Bligh & Dyer methods and hexane that is GRAS (generally recognized as safe solvent) were used.

2. Materials and Methods

The biomass of *N.gaditana* was supplied by the company Algalimento (Santa Lucía de Tirajana, Gran Canaria) in the form of freeze-dried powder and was stored at -20 °C.The biomass was subjected to a mechanical pretreatment using the Planetary ball mill Retsch PM200 and to accelerated solvent extraction by Dionex ASE 200.
Experimental design was planned to evaluate the effects on extraction yield when pretreatment conditions changed, in particular the influence of rotation speed (200 - 600 rpm) fixing 5 min as pretreatment time for two different biomass/diatomaceous earth ratio (B/DE) (0.5 and 1.0) and the influence of time (5 - 25 min) fixing 600 rpm as rotation speed for B/DE equal to 1.0. Solvents used for accelerated solvent extraction were hexane (tests 01-15) and a mixture of C/M/W (tests 16-30). All solvents were purchased by Sigma-Aldrich (Saint Louis, MO, USA) with a chromatographic grade. Extraction temperature was 50 °C and pressure was 100 bar. Each extraction was performed with two cycles of 10 min, and four extraction stages were carried out for each sample (80 min as total extraction time). In Table 1 experimental design is reported.

Table 1: Experimental design. Pretreatment conditions (rotation speed, time and B/DE ratio). Tests 01-15 were done using hexane, while chloroform/methanol/water was used for tests 16-30

N. test	Rotation speed (rpm)	Time (min)	Biomass/diatomaceous earth ratio
01/16	no pretreatment		
02/17	200	5	1
03/18	300	5	1
04/19	400	5	1
05/20	500	5	1
06/21	600	5	1
07/22	600	10	1
08/23	600	15	1
09/24	600	20	1
10/25	600	25	1
11/26	200	5	0.5
12/27	300	5	0.5
13/28	400	5	0.5
14/29	500	5	0.5
15/30	600	5	0.5

The liquid extract obtained by ASE 200, was dried at room temperature through a ZymarkTurboVap evaporator (Zymark, Hopkinton, MA, USA) for dry residue weight. The extracts were subjected to a transmethylation according to the standard method UNI ISO 12966-2, using NaOH solution in methanol (0.5 M) and BF3 in methanol (14%) (Sigma-Aldrich Ltd., St. Louis, MO, USA). At the end of this stage, isooctane was added and finally the upper phase was taken and transferred into a GC glass vial for the analysis. A 7820A GC-FID equipped with an HP-88 100 mt x 0.25 mm x 0.2 µm column was used to perform analysis, and the chromatographic conditions were taken from the standard method UNI ISO 12966-4. The temperature of the oven was from 150 °C to 240 °C with a ramp of 4 °C/min and those of the injector and detector were 250 °C. Nitrogen (purity > 99.999%) with a spatial velocity of 30 cm/s was used as a gas carrier. For the quantitative analysis a mixture of 37 fatty acid ethyl esters (C4–C24) (Supelco FAME 37, CRM47885, Sigma-Aldrich Ltd., St. Louis, MO, USA) was used.

3. Results and Discussion

The amount of fatty acids extracted using hexane as solvent increased considerably when the sample was pretreated, as observed in Figure 1. This effect was evident considering the difference between FAs in the no-pretreated sample equal to 31.63 mg FAs/g respect to FAs in the pretreated sample at 600 rpm for 5 min (B/DE= 1.0) (74.31 mg FAs/g). The increase of extraction yield for *Nannochloropsis sp.* under mechanical pretreatment using a high pressure disrupter was also observed by Angles et al. (2017). In fact, the cell disruption rate increased with the increasing of operating pressure of disrupter and TFAs extraction yields using heptane increased with increasing of cell disruption rates. As observed in figure 1, the increase in the extraction yield of FAs using hexane was dependent of the increase of rotation speed for both B/DE ratio. However, a greater quantity of fatty acids was obtained under B/DE equal to 1.0. At a pretreatment of 600 rpm for 5 min, 61.99 mg FAs/g were obtained for B/DE equal to 0.5 and 74.31 mg FAs/g for B/DE equal to 1.0, which meant that the increase of the percentage of diatomaceous earth decreased the yields.

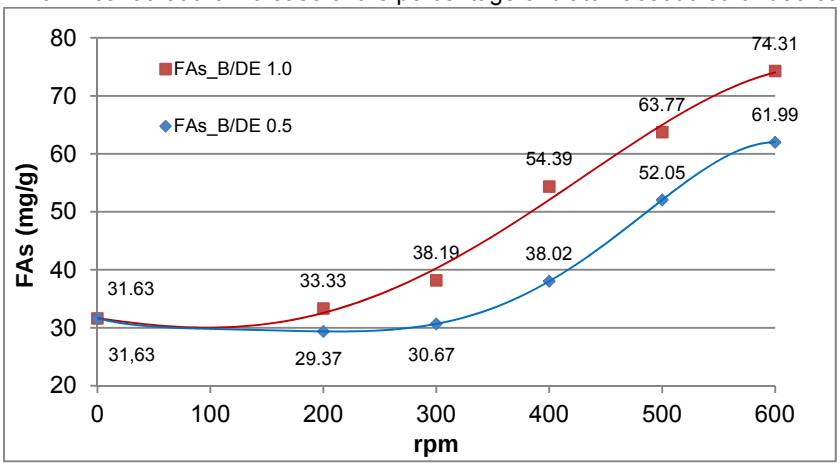

Figure 1: Fatty acids extracted with hexane after a mechanical pretreatment (rotation speed from 200 to 600 rpm, time 5 min and B/DE equal to 0.5 and 1.0)

Figure 2 shows the trend of fatty acids obtained using hexane as a function of the increase of pretreatment time, with rotation speed and B/DE equal to 600 rpm and 1.0, respectively. It is evident that when the time increased there was a drastic drop in the amount of fatty acids extracted. This was already observable starting from 10 min, passing from 74.31 mg FAs/g (time 5 min) to 16.55 mg FAs/g (time 10 min). This result could be explained taking into account that when the pretreatment time increases, temperature increases too, and this would lead to the degradation of fatty acids. On the other hand the fact that there was not an improvement in the amount of extracted FAs when the pretreatment time increases could be partially explained by a work of Postma et al. (2015) about the disintegration of the *Chlorella vulgaris* microalgae using bead milling. At fixed biomass concentration and agitator speed, it was observed that after 200 s of pretreatment about 90% of the cells were already disintegrated. Therefore, since the cell disintegration was already almost complete after 200 s, a greater quantity of fatty acids could not be extracted even if the pretreatment time increased.

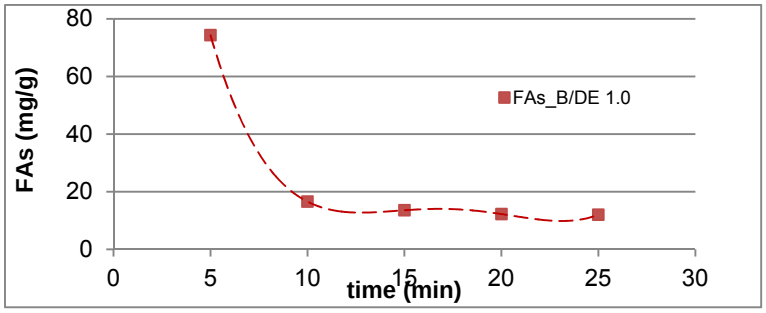

Figure 2: Fatty acids extracted with hexane after a mechanical pretreatment (rotation speed 600 rpm, time from 5 to 25 min and B/DE equal to 1.0)

The increasing of the amount of extracted fatty acids after the pretreatment step, was also observed using the mixture of C/M/W as solvent (Figure 3), maintaining fixed the operational conditions adopted in the case of hexane as extraction solvent. The FAs were 41.19 mg FAs/g in the no-pretreated sample and 116.49 mg FAs/g in the pretreated sample at 600 rpm for 5 min (B/DE= 1.0).The increase of extraction yield of FAs using the mixture was dependent of the increase of rotation speed for both B/DE ratio, but a greater quantity of fatty acids was obtained under B/DE equal to 1.0. At a pretreatment of 600 rpm for 5 min, 94.51 mg FAs/g were obtained for B/DE equal to 0.5 and 116.49 mg FAs/g for B/DE equal to 1.0.When pretreatment was carried out at 600 rpm and B/DE equal to 1.0 changing the time, the trend was characterized by a drop in the amount of fatty acids extracted when the time increased (Figure 4).

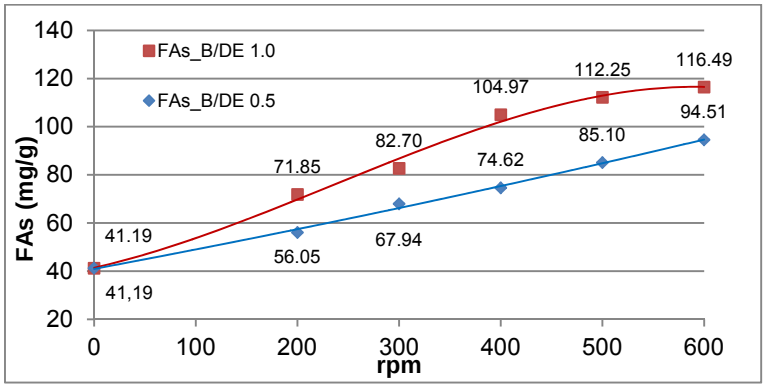

Figure 3: Fatty acids extracted with the mixture of chloroform/methanol/water after a mechanical pretreatment (rotation speed from 200 to 600 rpm, time 5 min and B/DE equal to 0.5 and 1.0)

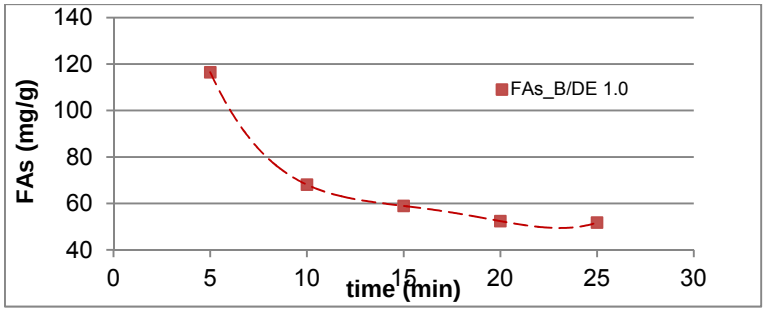

Figure 4: Fatty acids extracted with the mixture of chloroform/methanol/water after a mechanical pretreatment (rotation speed 600 rpm, time from 5 to 25 min and B/DE equal to 1.0)

Observing the results obtained, it was noted that the mixture of C/M/W allowed to extract a greater quantity of fatty acids than that obtained with hexane.Fatty acids obtained without pretreatment were 31.63 mg FAs/g using hexane and 41.19 mg FAs/g using the mixture of C/M/W. This gap became more evident when the sample was subjected to the pretreatment. In particular, with a pretreatment at 600 rpm for 5 min and B/DE

equal to 1.0, the amount of fatty acids was 2.35 times higher than that obtained without pretreatment using hexane, and 2.83 times higher than fatty acids obtained without pretreatment using the mixture of C/M/W. In fact, as shown in Taleb et al. (2016), *N.gaditana* and Parachlorellakessleri, among the numerous strains chosen (including several Nannochloropsis strains) in order to be subjected to mechanical pretreatment using high pressure bead milling, were the best candidates to be pretreated. For them the highest percentages of cells disruption, above 80%, were recorded, which made these microalgae particularly suitable for lipids extraction. By employing hexane and pretreating the sample at 600 rpm for time over 5 min the contents of fatty acids were lower than those without pretreatment, while under the mixture of C/M/W, fatty acids obtained pretreating the sample in the aforementioned conditions were greater than those without pretreatment. This meant that under time over 5 min and using hexane, the pretreatment phase worse the extraction yields.The condition in which the amount of fatty acids was the highest was achieved when pretreatment was carried out at 600 rpm for 5 min with B/DE equal to 1.0 and the extraction process was performed with the mixture of C/M/W. In particular, under these best conditions, since that *N. gaditana* is rich in the EPA content (Chua et al., 2017), in Table 2 it is also shown the amount of EPA extracted in this case.

Table 2: EPA content in the sample pretreated and extracted under the best conditions

N. extract	EPA (mg/g)
1st	20.65
2nd	13.82
3rd	8.83
4th	4.86
Total	48.16

In addition to the EPA, *N.gaditana* contains others fatty acids (Sukenik et al., 1993). To highlight the difference in fatty acids content between a no-pretreated sample and one that was subjected to pretreatment, in Table 3 are reported the characterization of a no-pretreated sample and that of the sample pretreated and extracted under the best conditions.

Table 3: Characterization of a no-pretreated sample and of the sample pretreated and extracted under the best conditions

	No-pretreated sample (mg/g)	Sample pretreated under the best conditions (mg/g)
Myristic acid	0,09	0,22
Pentadecanoic acid	0,25	0,62
Heptadecanoic acid	11,02	32,04
Stearic acid	0,11	0,10
Heneicosanoic acid	0,00	0,02
Docosanoic acid (acid Beenico)	0,17	0,49
Palmitoleic acid	10,21	30,24
cis-10-Heptadecenoic acid	0,13	0,36
Elaidic acid	0,29	0,87
Linoelaidic acid - ω-6	0,95	2,99
GLA γ-Linolenic acid - ω-6	0,13	0,38
EPA (cis-5,8,11,14,17-Eicosapentaenoic acid) - ω-3	17,83	48,16
Total	41,19	116,49

From the registered data, it is possible to observe that fatty acids extracted after pretreatment were greater than those without pretreatment, confirming the effectiveness of this phase. Moreover, it can be observed that fatty acids of which *N.gaditana* is richer are heptadecanoic acid, palmitoleic acid and first of all eicosapentaenoic acid.

4. Conclusions

Recently many studies have been done to increase lipid extraction yields from microalgae pretreating biomass before the extraction process. In this work, the feasibility of mechanical pretreatment on the N.gaditana as source of EPA was evaluated. Tests were carried out using two different solvents in order to study their effect on the extraction yield. When the sample was pretreated the amount of fatty acids extracted increased

considerably: 2.35 times higher than that obtained without pretreatment using hexane and 2.83 times higher than fatty acids obtained without pretreatment using the mixture of C/M/W. FAs extracted increased with the increase of rotation speed and the highest extraction yields were obtained under B/DE equal to 1.0.
The mixture of C/M/W allowed to extract a greater quantity of fatty acids than that obtained using hexane. The condition in which the amount of fatty acids was the highest was achieved when pretreatment was carried out at 600 rpm for 5 min under B/DE equal to 1.0 and the extraction process was performed with the mixture of C/M/W. Under these conditions EPA content in the sample was 48.16 mg EPA/g, an amount greater than that obtained by characterizing the no-pretreated sample.

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