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Production of FAMEs from several microalgal lipidic extracts and direct transesterification of the *Chlorella pyrenoidosa*

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ABSTRACT

In this study different methods were applied for lipids extraction from the dry biomass of *Chlorella pyrenoidosa*. The survey was carried under different conditions seeking comparative assessment of extraction methods. The method using chloroform:methanol (2:1 v/v) showed the highest lipid extraction followed by methanol, chloroform, ethanol, and hexane. Afterward, we also assessed the relative influence of the solvent extractor selectivity on the overall FAMEs (Fatty Acids Methyl Esters) yield. The application of the transesterification process on the several lipidic extracts was compared with direct transesterification process from dry biomass. In the extraction using chloroform:methanol system a larger amount of lipids was obtained but the conversion to FAMEs using transesterification process was the lowest from lipids. However, despite the amount of extracted lipids with methanol being smaller, its conversion to FAMEs was higher from lipids. In addition, the extraction with methanol followed by transesterification process also resulted in a higher FAMEs yield from biomass than direct transesterification process using methanol.

1. Introduction

The use of lipids from microalgae biomass for biodiesel production (fatty acids methyl esters, FAMEs) has been described as one of the most promising biomass feedstock with the potential to meet petro-diesel. The production of biodiesel from microalgae oil has previously been demonstrated in the literature using conventional routes, which involves the extraction of the lipids from the microalgae biomass followed by its conversion to FAME [1,2]. Extraction of lipids from the microalgal biomass is an important step in the overall process of biodiesel production.

The genus *Chlorella* contains several species that produce different amounts of lipids, e.g., *Chlorella vulgaris* 14–22%, *Chlorella ellipsoidea* 4.49% and *Chlorella pyrenoidosa* 2–11.9% [3,4]. Varying growth conditions can yield 18.9% (with limitation of N) until 22.2% of lipids (under optimum conditions). However, under this same culture conditions 25.2% and 38.0% of lipids, were obtained, from mutant strains of this species [5].

The cell wall of microalgae is composed of a wide variety of substances such as cellulose, chitin, murein, protein, silica and $CaCO_3$ [6]. Takeda classifies microalgae according to the composition of saccharides in the cell wall [7,8]. According to Okuda divisions of microalgae like Chlorophyta, Rhodophyta and Ochrophyta have cellulosic cell wall [6]. This implies that depending on the nature of the cell wall, several methods must be applied to break the wall such as milling, high-

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pressure homogenization, ultrasonication, and microwave before the solvent extraction.

Due to the wide range of polarity of the lipids found in microalgae different types of solvents can be used in the extraction. There are different types of solvents in the literature. For instance, ethanol, ethanol:hexane, chloroform:methanol:-water mixtures, are applied in the extraction of lipids from *Isochrysis galbana* [9], ethanol:hexane:water for *Phaeodactylum tricornutum* [10] and methylene chloride:methanol for *Chaetoceros gracilis* [11].

The lipid fraction extracted from microalgae may change its content and composition according to the solvent polarity [12]. The methods and appropriate solvents for the disruption of the cell wall are key pieces to increase the extraction efficiency [13].

This work aimed to study the extraction of lipids from microalgae *C. pyrenoidosa*, using Soxhlet extraction, magnetic stirring, and ultrasonic bath with five solvents: mixture chloroform:methanol (2:1 v/v), methanol, chloroform, ethanol, and hexane. Afterward, the application of the extraction followed by transesterification process to several algal lipidic extracts was compared with direct transesterification process and the biodiesel yield was expressed as its weight relative to the biomass.

2. Experimental section

2.1. General

The microalgae *C. pyrenoidosa* dry biomass was obtained commercially by Galena Company. It was 120-mesh particle size, containing 61.3% protein, 21.0% carbohydrate and 2.3% chlorophyll. The algal biomass was previously dried in an oven at 60 °C until constant weight. Before extractions, the samples were maintained in a freezer at −5 °C into a container to be protected from the light. The solvents used in the extractions were obtained from commercial sources (Synth, Brazil and Merck, Germany). The fatty acid profile analysis was performed in a Shimadzu GC-2010, equipped with split/splitless injector and Flame Ionization Detector (FID).

2.2. Lipid extraction using magnetic stirrer or ultrasonication

In a test tube, 1 g of dry biomass with 6 mL of solvent (chloroform:methanol mixture, methanol, chloroform, ethanol, and hexane) was added at room temperature (20 °C) under magnetic stirring (700 rpm) or ultrasonic bath during different time intervals. Later the sample was centrifuged at 2000 rpm for 5 min. The organic phase was carefully collected and the solvent evaporated under reduced pressure. The lipid fraction was dried to constant weight in an oven at 60 °C [14]. This method was subdivided according time extraction (Table 1). All procedures applying these methods were performed in triplicate.

2.3. Lipid extraction using Soxhlet

Sub-samples of 1.5 g of dry biomass were settled in a cellulose cartridge. The extraction was performed during 120 min in a Soxhlet apparatus with 150 mL of solvent (mixture chloroform:methanol, methanol, chloroform, ethanol, and hexane). During this period, about 8–10 solvent evaporation/condensation/percolation cycles were observed in the extraction chamber. After extraction the solvent was evaporated under reduced pressure and the lipid fraction was dried to constant weight in an oven at 60 °C. All procedures with these methods were performed in triplicate.

Table 1 – Lipid extraction methods.

Entry	Method		
1	Magnetic stirring	A1	3 × 20 min.
2		A2	20 min.
3		A3	120 min.
4	Ultrasonication	B1	3 × 20 min.
5		B2	20 min.
6		B3	120 min.

2.4. Fatty acid profiles

The derivatization of the lipid fraction of microalgae was carried out according to the methodology proposed by Metcalfe and Schmitz [15] in which the sample containing lipid fraction (300 mg) was placed in a test tube where a mixture 3 mL of boron trifluoride/methanol was added. The mixture was heated in a water bath at 70 °C for 20 min. For recovery of the fatty acid methyl esters the derivatized mixture was washed into a separatory funnel with 15 mL of hexane and 20 mL of distilled water. The organic and aqueous phases were then separated. The organic phase containing the fatty esters was dried, and the solvent was evaporated at 50 °C. Afterward, the fatty acid profiles was determined by GC-FID as follows: Injector: SPL, 250 °C, split injection 1:50, linear velocity mode, flow rate 1 mL/min, injection volume 2 μL; Column: RTX-wax (30 m length × 0.25 mm i.d. × 0.25 μm film thickness), oven temperature of 200 °C, with total analysis time of 30 min and 260 °C of the detector temperature. The identification of fatty acids was performed by comparison with the retention time of standards. Individual patterns of saturated fatty acids were used from C6:0 to C24:0, in the form of methyl esters. The standard mixture of methyl esters of PUFA N°. 1 from marine source and N°. 3, Menhaden oil were also used, all from Supelco.

2.5. The extraction using A3 method and transesterification process

100 g (+/−1) of dry biomass were mixed to 300 mL of solvent system (chloroform:methanol, methanol or ethanol) in a 600 mL round bottom flask and then the extraction was performed during 120 min. The mixture was filtered and the organic phase was evaporated under reduced pressure and dried in an oven at 60 °C to constant weight.

After lipid extraction, the reaction was carried out using H_2SO_4 acid as catalyst (3 or 10% in relation to the mass of lipids) under constant stirring for 4 h at 60 or 100 °C. The molar ratio of alcohol/lipids was 30:1. Aiming to adjust this stoichiometric ratio for catalytically conversion of algal lipids onto methyl esters, the fatty acids were considered as simple

lipids i.e. mainly triacylglycerols (C_{3n+3} $H_{6n-6l+2}$ O_6) whose molecular weight (g/mol) is easily obtained by the expression:

$$M = 42.078n - 6.048l + 134.042$$

By the rule of mixtures proposed by Kay [16] the average molecular mass is estimated by the individual molecular masses of triacylglycerols weighted by their respective mole (x) or mass (z) fractions in the mixture.

$$\overline{M} = x_1M_1 + x_2M_2 + x_3M_3 + \cdots = \frac{1}{\frac{z_1}{M_1} + \frac{z_2}{M_2} + \frac{z_3}{M_3} + \cdots}$$

Substituting appropriately the mass percentage from chromatogram of each extract (Table 3), we get the average molecular weight. As an example, on extracts by A3 method using solvent systems $CHCl_3$:CH_3OH, $CHCl_3$, CH_3OH and CH_3CH_2OH, was obtained 844.74, 846.48, 877.43 and 844.73 g/mol respectively.

The reaction was monitored by TLC, and performed on glass plates coated with silica gel. The eluent was hexane:diethyl ether (80:20 v/v), and iodine vapor was used for visualization. When the reaction was complete, the excess alcohol was removed under reduced pressure and hexane was added. The mixture was vacuum filtered through a Büchner funnel. The filtrate was separated, and then dried over anhydrous $MgSO_4$. The hexane was removed under reduced pressure to obtain the crude product. The fatty acid methyl esters were purified by column on silica gel/Al_2O_3 to give the pure FAMEs.

2.6. *The direct transesterification or in situ process*

In this procedure 100.89 g of dry biomass and 300 mL of methanol were mixed in a 600 mL round bottom flask to 10.86 mL (20 g) of H_2SO_4 acid catalyst. The reaction was performed at 60 °C for 4 h under constant stirring. The reaction was also monitored by TLC, and performed on glass plates coated with silica gel. The eluent was hexane:diethyl ether (80:20 v/v), and visualized through iodine vapor. After complete reaction, was removed the alcohol excess under reduced pressure and then added hexane. The mixture was vacuum filtered in Büchner funnel. The filtrate was separated, and then dried over anhydrous $MgSO_4$. The hexane was removed under reduced pressure to obtain the crude product. The fatty esters were purified on silica gel/Al_2O_3 column to give the pure FAMEs.

3. Results and discussion

Different methods for lipid extraction from the *C. pyrenoidosa* biomass seeking to determine the lipid content and also the fatty acid profile of each extract were investigated. Afterward, the application of the extraction and transesterification process in several algal lipid extracts was compared with direct transesterification or in situ process (Fig. 1).

3.1. *Extraction of lipids from the* C. pyrenoidosa

The best yields on extracted lipids were shown when chloroform:methanol 2:1 v/v using both magnetic stirring or ultrasonication as has widely been cited in literature on polar and neutral lipid extractions [14]. The methods A1 and B1 were able to extract similar amounts of lipids, i.e., 19.74 and 19.43% of total lipids, respectively (Fig. 2). The compositions of

Table 2 – Profile of fatty lipids of *C. pyrenoidosa* obtained from different extraction methods.

Entry	Method	Solvent	Fatty acids[a] (%)									
			C14:0	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3	18:4
1	A1	$CHCl_3$:CH_3OH (2:1)	0.7	17.3	0.8	7.0	9.3	1.2	3.3	18.5	41.8	ND[b]
2	B1	$CHCl_3$:CH_3OH (2:1)	0.6	16.4	2.0	7.1	9.9	1.2	3.5	18.1	41.2	ND
3	A3	$CHCl_3$:CH_3OH (2:1)	1.5	19.7	0.8	6.9	8.5	1.2	3.6	18.3	39.3	ND
4	B3	$CHCl_3$:CH_3OH (2:1)	0.5	15.9	1.9	5.8	7.8	1.1	3.2	15.2	36.9	2.4
5	A1	$CHCl_3$	0.7	15.8	2.0	6.9	9.1	1.3	3.7	18.8	41.6	ND
6	B1	$CHCl_3$	1.1	24.3	2.6	5.9	5.0	1.7	4.4	19.3	35.7	ND
7	A3	$CHCl_3$	0.9	15.0	1.6	7.2	9.9	0.9	3.4	18.2	42.5	0.2
8	B3	$CHCl_3$	0.6	20.2	2.3	7.0	8.3	1.8	3.4	18.3	38.0	ND
9	A1	CH_3OH	1.2	17.5	2.3	6.9	9.1	1.0	3.3	18.0	40.6	ND
10	B1	CH_3OH	1.0	17.0	2.3	7.2	9.9	1.0	3.5	18.0	39.8	0.2
11	A3	CH_3OH	1.1	16.2	2.1	6.5	9.4	1.3	3.4	17.2	38.7	0.2
12	B3	CH_3OH	1.0	18.9	2.1	6.2	8.0	1.4	3.6	17.5	41.1	ND
13	A1	C_2H_5OH	0.7	17.3	2.4	6.9	9.1	1.0	3.6	18.0	40.9	ND
14	B1	C_2H_5OH	1.1	18.7	2.0	6.3	7.5	1.6	4.6	19.1	36.0	ND
15	A3	C_2H_5OH	1.1	18.7	2.3	6.9	9.0	1.1	3.6	18.5	39.1	ND
16	B3	C_2H_5OH	1.0	18.4	2.2	6.4	8.2	1.2	3.4	18.8	40.3	ND
17	A1	C_6H_{14}	0.7	17.6	1.6	6.9	7.4	1.3	4.2	19.8	37.3	3.0
18	B1	C_6H_{14}	1.2	16.8	1.7	6.9	8.0	1.5	4.5	20.0	39.2	ND
19	A3	C_6H_{14}	0.4	16.9	1.7	5.6	6.1	1.6	4.8	17.1	34.7	3.5
20	B3	C_6H_{14}	0.6	24.9	1.8	4.9	4.5	1.4	5.8	18.8	29.0	ND

a Tetradecanoic (myristic, C14:0), hexadecanoic (palmitic, C16:0), 9-hexadecenoic (palmitoleic, C16:1), 9,12-hexadecadienoic (C16:2), 7,10,13-hexadecatrienoic (hiragonic, C16:3), octadecanoic (stearic, C18:0), 9-octadecenoic (oleic, C18:1), 9,12-octadecadienoic (linoleic, C18:2), 9,12,15-octadecenoic (linolenic, C8:3), 6,9,12,15-octadecatetraenoic (stearidonic, C18:4).
b Not detected.

Table 3 – Experimental conditions for fatty acid methyl esters (FAMEs) production.

Entry	Extraction (23 °C, 120 min)	Transesterification (240 min)	T (°C)
1	$CHCl_3$:CH_3OH (2:1 v/v)	CH_3OH, 3% H_2SO_4	60
2	$CHCl_3$:CH_3OH (2:1 v/v)	CH_3OH, 10% H_2SO_4	100
3	CH_3OH	CH_3OH, 3% H_2SO_4	60
4	CH_3OH	CH_3OH, 10% H_2SO_4	100
5	C_2H_5OH	CH_3OH, 3% H_2SO_4	60
6	–	CH_3OH, 20% H_2SO_4[a]	60
7	–	CH_3OH, 20% H_2SO_4[a]	100

a Direct transesterification or in situ process.

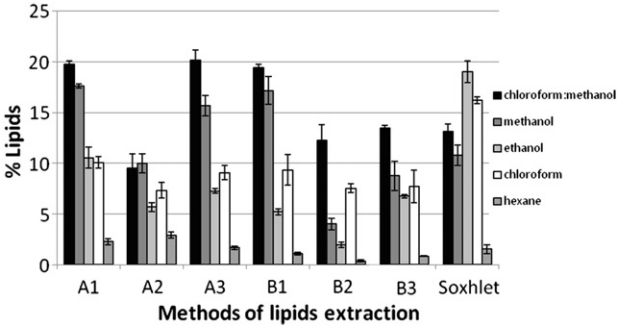

Fig. 2 – Total lipids extracted with different solvents and methods.

these lipid extracts also were similar, suggesting that both methods were equally effective in extracting the various lipid classes present in algal biomass. The solvent system chloroform:methanol (2:1 v/v) applying A2 method barely 50% of total lipids were extracted compared to A1. The B2 method proved to be less effective in extraction, so the B1 method was the most effective. These results show that three successive extraction stages are essential for removal of lipids from algal biomass [17].

Extractions with methanol were effective for both A1 and B1 methods, obtaining 17.17 and 17.59% lipids, respectively. Under Soxhlet extraction, the best performance was with ethanol (19.01%), followed by chloroform (16.20%). Lipids yield in B1 method with ethanol were below than those obtained in A1 method (10.54%) (Fig. 2).

In general, there was an increase of extracted lipids as the solvent polarity was increased. The worst results found using hexane indicate that the amount of nonpolar lipid components such as triacylglycerols in the sample is lower, showing that the vast majority of lipids in *chlorella* biomass are polar in nature, which can also be observed in other microalgae species [18]. Even changing extraction time with hexane the obtained amount did not exceed 3% of the total lipids. The amount extracted by hexane applying Soxhlet and A3 method was similar 1.55 and 1.66%, respectively (Fig. 2).

The main fatty acids identified in the lipid fractions were C16:0, 18:2 and 18:3, with lower percentages of 14:0, 16:1, 16:2,

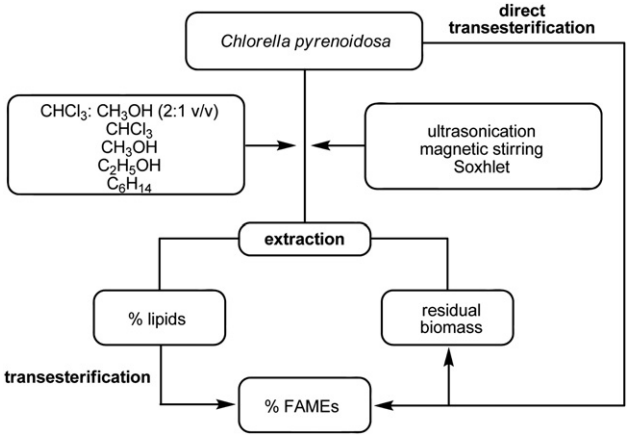

Fig. 1 – Extraction method of lipids and FAMEs production from *C. pyrenoidosa* biomass.

16:3, 18:0, 18:1 (Table 2). Regarding the quantity and quality of fatty acids, there was no variation in the lipid profile when extracted by magnetic stirring, ultrasonication and Soxhlet in the presence of different solvent systems. In all cases was not observed uncommon fatty acids. Table 2 (entries 1, 7, 10, 11, 19 and 20) shows that stearidonic acid (18:4), common in mixed culture of green algae, was detected [19]. The fatty acid present in largest amount was linolenic acid (18:3) ranging between 29.04 and 42.47%. The presence of linoleic acid (18:2) was detected between 15.20 and 20.04% in the samples. This result was consistent with that obtained by Petkov & Garcia [19], 18:2 acid was observed in 18–27% on *C. pyrenoidosa* biomass. In this work were also identified polyunsaturated acids 16:2 and 16:3. Similar amounts of polyunsaturated fatty acids on *C. pyrenoidosa* were also observed by other authors [20,21]. The acid 14:0 was present in small amounts in all samples, ranging from 0.45 to 1.55%. According to literature the percentage of 14:0 in freshwater microalgae does not exceed 1% [19].

The B3 method using hexane showed a higher percentage of 16:0 and 18:0 and lower percentage of 18:3 in comparison with other extraction methods. The amount of 18:0 was 9.45%, while in other fractions this value did not exceed 4.58%.

3.2. *Extraction and transesterification of lipids from the C.* pyrenoidosa

The application of the transesterification process using homogenous acid catalysts for biodiesel production from microalgal lipids is not a novel one. Microalgal biodiesel production is typically performed by oil extraction followed by the transesterification of algal oil [22]. Direct transesterification or in situ process of the raw biomass has also been reported for some algae. Because of the inherent nature of a single–stage reaction, direct transesterification was much less time-consuming than extraction and transesterification. It also avoided the potential lipid loss during the extraction stage; as a result, the direct transesterification led to a higher yield of crude biodiesel and FAMEs. However, extra care must be taken in the design of a direct transesterification method [23–25].

According to literature the biodiesel yield from algal biomass through extraction-transesterification and direct

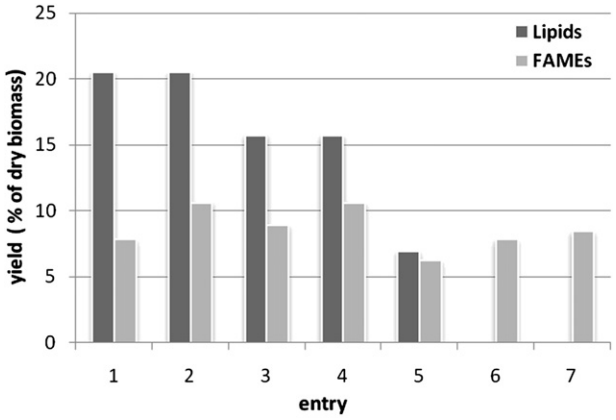

Fig. 3 – Total lipids and fatty acid methyl esters (FAMEs) yields from *C. pyrenoidosa*: extraction followed by transesterification process (entries 1–4) and in situ process (entries 6 and 7).

transesterification were compared [24]. The direct transesterification (with the three solvents, chloroform, hexane, petroleum ether) resulted in a high biodiesel yield. When no solvent was used in the direct transesterification, biodiesel yield was very low (12.7%), indicating that solvent was essential for the reaction. In this case, the biodiesel yield was expressed as its weight relative to the algal oil present in the freeze-dried algal biomass (1 g). The extraction-transesterification also resulted in a high biodiesel yield (98.4%) from algal oil with a fatty acid methyl ester (FAMEs) content of 66.37%.

In this work, the application of the extraction followed by transesterification process to several algal lipidic extracts from *C. pyrenoidosa* was compared within situ process and the biodiesel yield was expressed as its weight relative to the dry biomass (100 g). The FAMEs production was investigated in different conditions (Table 3).

From the lipids extracted with the A3 method (23 °C, 120 min) using chloroform:methanol, methanol, and ethanol, the transesterification reactions were carried in the presence of methanol. The total lipids using different solvents and yields of esters obtained from extraction followed by transesterification process or in situ process are in Fig. 3. In the extraction with Bligh and Dyer method [26] (chloroform:methanol, 2:1 v/v) a larger amount of lipids was obtained (20.5%) but the methyl ester yield performed at 60 and 100 °C were 7.8 and 10.6% of the FAMEs based on dry biomass, respectively (Fig. 3, entries 1 and 2). These results show that the oil in lipid extract is low and it is also constituted by non transesterificable lipids. Despite the amount of lipids extracted with methanol be smaller (15.7%) than chloroform:methanol (2:1 v/v), the transesterification process resulted in a much-higher biodiesel yield, 8.9 and 10.6% from dry biomass at 60 and 100 °C, respectively (Fig. 3, entries 4 and 5). The amount of lipids extracted with ethanol was lower (6.9%) than other extractions, yielding 6.2% of the FAMEs (based on dry biomass weight).

The direct alcoholysis applied on algal dry biomass (in situ process) performed at 60 and 100 °C with methanol not exceeds 7.8 and 8.4% of the FAMEs, respectively (Fig. 3, entries 6 and 7).

According to Fig. 3, the extraction at room temperature using methanol followed by transesterification at 60 or 100 °C (a two-stage method) resulted in high biodiesel yield (entries 3 and 4) than direct transesterification of the algal biomass (entries 6 and 7). In addition, within the experimental conditions, an increase in the reacting alcohol amount and of the catalyst in direct transesterification (a one-stage method) did not show significant an increase in the yield of the FAMEs products obtained when compared with the extraction followed by transesterification process. The direct transesterification to prepare biodiesel from microalgae biomass is also limited due to contact of the biomass with acid catalyst, this is one of the main reasons which would favors the use of methanol in the extraction at room temperature followed by

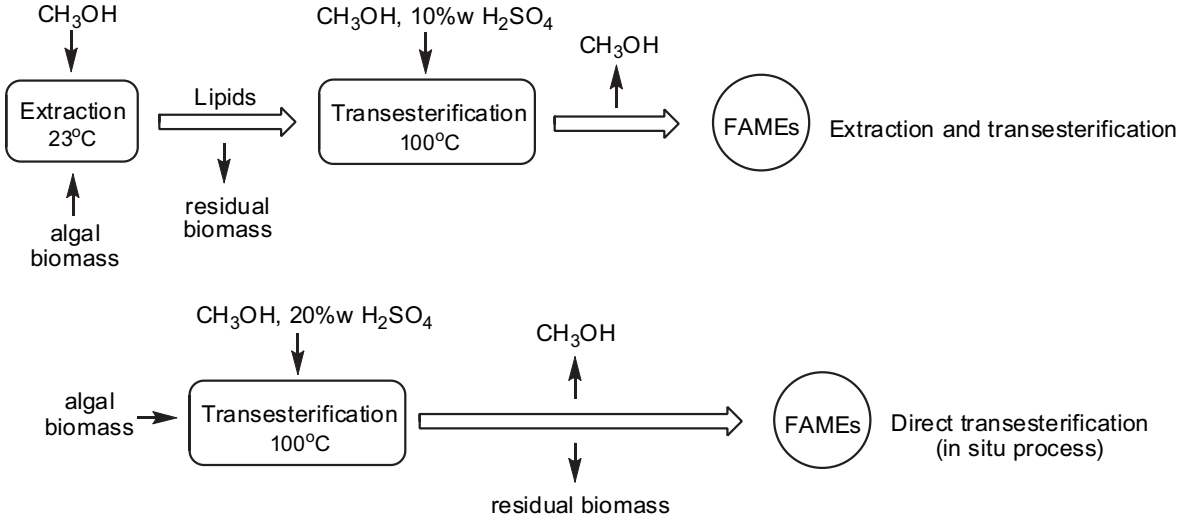

Fig. 4 – The extraction and transesterification and direct transesterification process using methanol.

transesterification process (a two-stage method) of the lipids as shown in Fig. 4.

4. Conclusions

In this study different solvent systems, chloroform:methanol (2:1 v/v), methanol, chloroform, ethanol, and hexane were applied for lipids extraction from *C. pyrenoidosa*. It was observed that chloroform:methanol (2:1 v/v) mixture presented the best result on lipid extraction from microalgae biomass, while hexane showed the lowest extraction yields. These results show that the oil in microalgae biomass is low and it is constituted mainly by polar lipids. There was no significant variation in the fatty acid profile of tested samples. Regardless the method, most abundant fatty acid was 18:3 and the minimum was the saturated 18:0.

Afterward, the advantage of producing FAMEs from different lipidic extracts by acid transesterification was compared with direct transesterification. The extraction and transesterification process, both with methanol, resulted in a higher ester yield than direct transesterification process using methanol.

In summary, the extraction at room temperature using methanol followed by transesterification process (a two-step process) may be especially advantageous for microalgae biomass in relation to direct transesterification (a one-step process). The direct transesterification is also limited by addition of the excess sulfuric acid and alcohol to microalgal biomass, in this case, the energetic cost of the recovery of the excess alcohol and catalyst might, limit the achievable cost reduction. In addition, the use of methanol in the two-step process also eliminates the solvent recovery step required to obtain the oil feedstock as in the conventional method with hexane.

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